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Efficacy and environmental fate of *Chondrostereum purpureum* used as a biological control for red alder (*Alnus rubra*)

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Abstract

A field trial to assess the ability of *Chondrostereum purpureum* to limit the resprouting of cut red alder (*Alnus rubra*) was established in British Columbia, Canada. Overall, 92% of stumps inoculated with *C. purpureum* died in the first year and 100% were dead by the second year. *C. purpureum* was thus as effective as the herbicide treatments Carbopaste and liquid glyphosate (12% Vision) and reduced resprouts to zero. Naturally occurring *C. purpureum* spores colonized about 40% of untreated cut stumps and about 20% of the stumps treated with chemical herbicides. The peak production of *C. purpureum* basidiocarps occurred about 22 months after the trial was established, with *C. purpureum* on 66% of treated stumps. The following year, the basidiocarps persisted on 23% of treated stumps. About 600 young red alder trees were cut to act as spore traps 2 years after trial establishment, when the most *C. purpureum* basidiocarps were present on treated stumps in the trial. These spore-trap trees were subsequently sampled, and PCR markers were used to verify and characterize 43 dikaryotic *C. purpureum* individuals, none of which were identified as the released isolate 2139. There was little difference in average band-sharing with the applied isolate among pre-trial (37% average) and spore-trap (38% average) populations. There was therefore no evidence that the *C. purpureum* strain applied inundatively to stumps in this trial was detectable on nontarget trees in the local area or had any measurable impact on the local *C. purpureum* population.

Keywords: Biological control; Biological impact; Environmental fate; Genetic marker; Vegetation management

1. Introduction

Environmental concerns have called for the reduction of chemical herbicide use in forests and given support to the concept of integrated vegetation management. The use of chemical herbicides, however, is at present the preferred approach for vegetation management in reforestation sites and in utility company rights-of-way. Mechanical treatment alone is ineffective for many target tree species as they can resprout vigorously without further treatment. There is therefore a need for effective alternative treatments such as indigenous plant pathogenic fungi formulated for use as biocontrol agents for weeds, known as mycoherbicides (Templeton et al., 1979). While biocontrol of forest weeds using indigenous mycoherbicides is designed to minimize risk, it is a new approach that should be carefully evaluated.

The basidiomycete *Chondrostereum purpureum* (Pers.) Pouzar is found worldwide in temperate zones. It is a facultative saprophyte with broad-spectrum pathogenicity towards many hardwood species. A primary invader of woody angiosperms, *C. purpureum* usually enters its host through a fresh wound, cut stump, or stem lesion (Brooks and Moore, 1926; Rayner and Boddy, 1988; Spiers and Hopcroft, 1988). The fungus grows through xylem tissues of the host plant, causing cambial necrosis, decay, sapwood staining, and sometimes death of the host (Rayner, 1977; Wall, 1986, 1991). Infection by

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C. purpureum can also cause foliar discoloration or silvering, known as silver-leaf of orchard fruit trees (Bishop, 1979; Grosclaude et al., 1973; Setliff and Wade, 1973).

In Canada, it has been demonstrated that infection of hardwood stems by *C. purpureum* reduces the number and viability of resprouts (Wall, 1990). The *C. purpureum* biocontrol strategy involves covering the entire surface of freshly cut target stumps with mycelial fragments in a protective nutrient formulation. The effectiveness of *C. purpureum* as a mycoherbicide has been evaluated for the control of *Acer rubrum* L. (red maple), *A. saccharum* Marsh. (sugar maple), *A. macrophyllum* Pursh (bigleaf maple), *Alnus rubra* Bong. (red alder), *Betula alleghaniensis* Britton (yellow birch), *B. papyrifera* Marsh. (paper birch), *Fagus grandifolia* Ehrb. (beech), *Populus tremuloides* Michx. (trembling aspen), *P. grandidentata* Michx. (bigtooth aspen), *Prunus pensylvanica* Linn. f. (pin cherry), and *P. serotina* Ehrh. (black cherry) (de Jong et al., 1990; Dumas et al., 1997; Harper et al., 1999; Jobidon, 1998; Pitt et al., 1999; Wall, 1986, 1990, 1991, 1994, 1997; Wall et al., 1992).

Current understanding of the ecology and pathogenicity of *C. purpureum* is steadily growing. The fungus exists in all biogeoclimatic zones in North America, which precludes the risks associated with the introduction of a foreign species for biological control. A number of studies have examined the population structure of *C. purpureum* at regional to intercontinental scales, using different methods for differentiation of subgroups, including mating interactions (Rayner and Boddy, 1986), SDS–protein profiles (Ekramoddoullah et al., 1993), isozyme analyses (Shamoun and Wall, 1996), ribosomal DNA and mitochondrial DNA profiles (Ramsfield et al., 1996, 1999), random amplification of polymorphic DNA (RAPD; Gosselin et al., 1996, 1999), and morphology, pathogenicity, and RAPD (Spiers et al., 2000). The associated risk of moving isolates over large geographic areas and across biogeoclimatic zones was assessed with the conclusion that the *C. purpureum* population is panmictic and has no geographic or host specialization (Gosselin et al., 1999; Ramsfield et al., 1996, 1999).

The wide host range of *C. purpureum*, recorded on over 100 hosts, is an advantage when considering a candidate biocontrol agent, but infection of nontarget plants due to biocontrol application of *C. purpureum* would be undesirable. Studies have indicated a low risk of direct nontarget infection associated with the application of *C. purpureum* mycelium (Becker et al., 1999a,b), but secondary infection from spores originating from the biocontrol-treated stumps is of concern (de Jong, 1992; de Jong et al., 1990; Gosselin et al., 1999). Basidiocarps are usually produced by the fungus on infected stumps and trees and may persist for up to 2 years (Wall, 1997). When environmental conditions are favorable, basidiospores are released that may infect freshly wounded deciduous trees. In Canada and in other countries, *C. purpureum* is commonly found on windblown trees, logged areas, woodpiles, and pruned areas, and these may be important inoculum sources for the phytopathogen. A similar increase in spore load with a similar environmental impact is hypothesized to be associated with the use of *C. purpureum* as a stump treatment. An extensive study was performed in 1993 and 1994 of the natural occurrence of *C. purpureum* on Vancouver Island in relation to its use as a biocontrol agent, which concluded that the added fructification due to biocontrol use was the same order of magnitude or less than naturally occurring levels (de Jong et al., 1996). A fate assessment of *C. purpureum*, concerned with the release and dispersal of spores, was performed by modeling the dispersal part of the epidemiological process to simulate the dissemination of the disease (de Jong, 1992; de Jong et al., 1990, 1991).

Many factors influence or limit the spread of *C. purpureum* spores into the environment: weather conditions that influence the release, dispersal, deposition, germination, and survival of spores as well as the stress level and susceptibility of the hosts (Grosclaude et al., 1973; Wall, 1991). The requirement of a fresh wound for infection allows *C. purpureum* to be applied specifically to host individuals. If the *C. purpureum* formulation is applied to only part of a stump, resprouting often occurs in the unaffected region of the stump. This dependence on a freshly wounded host is hypothesized to be the most important constraint limiting infection by *C. purpureum*. The application of *C. purpureum* mycelium to freshly cut stumps largely removes this constraint and hence efficacy of biocontrol is then dependent upon other factors affecting colonization and competitive ability such as lateral rate of growth and production of phytotoxins.

The following experimental trial was established to gain information on the biological impact, in terms of efficacy and environmental fate, of *C. purpureum*, applied to *A. rubra* under nearly operational conditions, as a formulation that could be developed as a commercial product. Spore traps were established to assess the impact of the field trial on nontarget host plants and the local *C. purpureum* population. It was hypothesized that the amount of *C. purpureum* infection due to spores from the biocontrol trial would increase on nontarget hosts around the treated area for a relatively short period of time, then return to background levels. This field trial was established to examine the dynamics of this mycoherbicide pathosystem and to evaluate (1) the potential of an application of *C. purpureum* mycelium to inhibit stump sprouting in red alder compared to manual brushing and chemical herbicide treatments, (2) the persistence of *C. purpureum* sporocarps, (3) the succession of other fungi on stumps, and (4) the incidence of infection of nontarget red alder stumps by spores from treated stumps.

2. Materials and methods

2.1. Pre-trial population sampling

Prior to the establishment of the field trial, samples of wood were collected from stumps that were adjacent to the trial site, that had been cut because of their location in the right-of-way (ROW) under the power lines. Many of these stumps had become naturally infected by *C. purpureum*, and had produced *C. purpureum* basidiocarps. Wood chips containing cambium tissue were taken from immediately beneath *C. purpureum* basidiocarps and were surface sterilized, cultured, identified, and stored as previously described (Becker et al., 1999b).

2.2. Site description and experimental design

The field-trial site was established at latitude 48°49′N and longitude 123°50′W, in a B.C. Hydro power line ROW in the Cowichan lake district of Vancouver Island, part of the Coastal Western Hemlock biogeoclimatic ecosystem zone. The experimental area straddled a shallow stream and varied in slope and drainage. An even-aged stand of red alder was selected having a tree height of about 10 m and a stem diameter of between 5 and 7 cm. Buffer strips (3 m wide) were cleared between plots during the early summer of 1994 and plots were established in September, a time of year considered suitable according to previous studies. On the day the trial was established, Sept. 19, 1994, the maximum temperature was 27.5 °C, the minimum temperature 11.5 °C, and the mean temperature 19.5 °C (Environment Canada, 2004). It had not rained for 4 days. A total of 25 treatment plots were established, consisting of five replicates of each treatment in a randomized block design. Each block consisted of five treatment plots. Within each treatment plot, 20 healthy red alder trees with stem diam of 5–10 cm at base height were chosen and tagged (each consisted of up to 30 stems in a coppice). Plots were 3 m wide and as long as needed to contain the 20 experimental trees. Trees were cut by chainsaw and all stems within plots were immediately treated with one of the following: *C. purpureum* isolate 2139, Vision glyphosate spray (12%), Carbopaste (12% Vision glyphosate in a paste base material, developed by B.C. Hydro), formulation alone, and control stumps that were cut but left untreated. Slash was removed from trial plots and buffer zones to promote maximum resprouting of stumps.

2.3. Preparation of C. purpureum inoculum

Chondrostereum purpureum isolate 2139 was collected in southern Vancouver Island in June, 1994 from *A. rubra*. Inoculum was prepared from actively growing petri-dish cultures growing on 1.25% malt agar. Plugs, 5–8 mm in diam, were removed aseptically from the advancing zone of the culture, macerated, and suspended in 100-ml aliquots of 1% malt extract broth in Erlenmeyer flasks. Flasks were placed on 100 rpm rotary shakers at room temperature for 2–5 days and the contents then aseptically added to the dry mixture.

The dry formulation consisted of 375 g talc, 100 g kaolin, 12.5 g corn starch, 5 g pectin, 5 g monosodium glutamate, 1 g monopotassium phosphate, and 1.5 g yeast extract. Ingredients were thoroughly mixed, placed in autoclavable plastic bags, and autoclaved at 15 psi for 20 min. After cooling, the bags were inoculated with the malt broth culture, controls (formulation only) receiving sterile malt broth only. After inoculation, the contents of each bag were agitated to distribute the mycelial fragments and the bags were incubated at room temperature for 3–8 weeks. During incubation, bags were agitated at weekly intervals to maintain uniform growth. Immediately before inoculation in the field, the contents of each bag were mixed with 600 ml of sterile 1% sucrose, 200 ml canola oil, 60 g finely powdered cellulose (Polyphila, LePages's, Brampton, Ont.) and the yolks from two Grade A large eggs. The mixture was stirred vigorously to form a smooth paste, and applied to stumps in a plastic squeeze bottle.

2.4. Field treatments

Trees were felled with a chainsaw to leave 15 cm high stumps with horizontal surfaces. The diameter of each stump was recorded and inoculum applied within 30 min of felling. Inoculum was applied to the cambial region of each stump as a continuous band, at the rate of about 0.2 ml of liquid formulation per cm of stump circumference (e.g., a 10-cm-diameter stump would have received $0.2 \times 10\pi = 6.3$ ml). Sterile control stumps (formulation only) were treated in the same manner using uninoculated formulation. Stumps treated with glyphosate were either sprayed to saturation with 12% spray or a thin bead of Carbopaste was placed on their perimeter (cambium).

2.5. Field-trial assessment

Cut stumps in all the treatment plots were examined for resprouting during the mid summer of each of the succeeding 2 years. The number of living sprouts was recorded for each of the 100 tagged stumps per treatment. An absence of living sprouts on a stump or coppice indicated stump mortality. The presence of basidiocarps of *C. purpureum* and other basidiomycetes on stumps was monitored and was assessed 18 months after inoculation. Analysis of variance was used to assess the differences among treatments using ANOVA and Duncan multiple range tests with SAS software version 6.12, 1997 (SAS Institute, Cary, NC).

2.6. Spore-trap establishment and sampling

Untreated alder trees cut at a height of about 3 ft were established as spore traps in the fall of 1996 during a period of massive fruiting of *C. purpureum* within the field trial. Approximately 200 trees (2–5 cm diam) were cut in each of three spore-trap sites at distances of 50, 700, and 1000 m from the field site along the same ROW. Trees chosen as spore traps were healthy and vigorous, previously uncut, and were primarily from the edges of the ROW. During the following 12–24 months, the spore traps were monitored and wood showing symptoms or signs of infection (staining, presence of basidiocarps) was cut and taken to the laboratory for culturing of fungi and genotype analysis.

2.7. DNA extraction and amplification

Mycelium for DNA extraction was grown in 5 ml volumes of 1.5% malt extract broth for approximately 2 weeks, collected by filtration, washed with water, frozen, and freeze dried. Dry mycelium was ground with sterile sand with plastic pestles in 1.5-ml tubes, and genomic DNA extracted according to Möller et al. (1992).

For genetic analyses, amplification reactions using the APD13 primers were performed as previously described (Becker et al., 1999b), using an annealing temperature of 60 °C for 30 cycles. Reactions were each independently amplified twice, separated on 1.5% agarose gels, and visualized under ultraviolet light by ethidium bromide staining. Photographed gels were scored at least twice and banding patterns for each isolate were recorded. All DNA samples were also amplified using species-specific primers APN1 (Becker et al., 1999b) for verification of identity as *C. purpureum* and as a positive control for DNA quality (data not shown).

2.8. Analyses

To test the hypothesis that inundative biocontrol application would cause a local effect on the resident population by the applied isolate, the similarity of the released biocontrol strain to *C. purpureum* collected before the trial was compared to its similarity to the *C. purpureum* recovered from spore traps. A similarity index based on the proportion of bands shared by two individuals was computed as twice the number of shared bands divided by the total number of bands scored in the two individuals (Nei and Li, 1979).

This similarity index was computed for each isolate in the pre- and post-trial populations, compared with the released isolate.

The computer program PAUP Version 4.0b4a (Swofford, 2000) was used to compute a distance matrix among all isolates. Isolates were compared using the neighbor joining algorithm (Saitou and Nei, 1987) to test for subgrouping within and among the populations. Data were resampled by bootstrapping to test for reliability of clustering.

3. Results

3.1. Trial assessment

Sprouting of cut stumps began during the spring of 1995 and had reached a maximum height of 50 cm by mid summer, the time of the first assessment. Fewer living sprouts were found on stumps treated with *C. purpureum* or chemical herbicides than were found on the untreated, slash controls (Table 1). Sprouting had occurred on many of the stumps treated with *C. purpureum*, but 92% of stumps were dead by mid-July.

Slash and blank formulation-treated stumps had sprouted about four sprouts per stump by the first year after trial, but mortality levels were high in these control stumps (65–70%) (Table 1). By the second year after treatment, 72% of formulation control stumps and 86% of slash controls had no sprouts (Table 1), and were considered to be dead.

The number of living sprouts on stumps treated with *C. purpureum* (zero per stump) was less than on those in the slash control (1.18 sprouts per stump) but this was not statistically less than the number of sprouts per stump in the formulation control plots (0.37 sprouts per stump) (Table 1).

Both the number of live resprouts and the mortality of stumps differed significantly among treatments by 1995, 1 year after establishment of the trial (ANOVA, $P < 0.05$) (Table 2). The overall effects of *C. purpureum* treatment were not different from those due to chemical herbicide treatment, but were different (Planned contrasts, $P < 0.01$) from the overall untreated formulation and slash controls (Table 2).

Field examination made in mid summer of 1996, the second growing season after field-trial establishment, showed near-complete mortality of stumps treated with Carbopaste or *C. purpureum* and >96% mortality of liquid glyphosate-treated stumps (Table 1). In comparison

Table 1
Percent mortality and number of living sprouts on cut and treated *A. rubra* stumps

Treatment	1995		1996	
	% Mortality	Sprouts per stump	% Mortality	Sprouts per stump
Slash control	65.0 b	4.45 a	86.0 ab	1.18 a
Formulation control	70.0 b	3.49 ab	72.0 b	0.37 b
C. purpureum 2139	92.0 a	0.45 c	100.0 a	0.00 b
Glyphosate	97.0 a	0.35 c	99.0 a	0.01 b
Carbopaste	100.0 a	0.01 c	100.0 a	0.00 b

Treatments with the same letter are not significantly different by Duncan's multiple range test at $P \leqslant 0.05$.

Table 2
Analysis of variance and planned contrasts (P values) of stump mortality and resprouting of *A. rubra* treated with *C. purpureum* and chemical herbicides

Source/contrast	1995		1996	
	Mortality	Living sprout #	Mortality	Living sprout #
Overall treatment	0.0010	0.0147	0.0737	0.0031
Cp vs. untreated	0.0022	0.0030	0.0163	0.0013
Cp vs. herbicides	0.0680	0.5965	0.8380	0.9809
Cp vs. glyphosate	0.5422	0.9424	0.9232	0.9461
Cp vs. carbopaste	0.3330	0.7509	1.0000	1.0000
Cp vs. formulation	0.0130	0.0378	0.0128	0.2198
Cp vs. slash	0.0032	0.0084	0.1868	0.0006

Cp, *Chondrostereum purpureum.*

with 1995, all plots exhibited less sprouting and more mortality of stumps (Table 1). By the second year after trial establishment, the *C. purpureum*-treated red alder stumps had no resprouts, which was equivalent to those treated with chemical herbicides, and was significantly less resprouting than on the untreated formulation and slash controls (Table 2).

3.2. Basidiocarp assessment

The fungus *C. purpureum* generally produces basidiocarps about 18 months after infection of red alder stumps. On the experimental site, the peak production of *C. purpureum* basidiocarps occurred in the spring of 1996 on 66% of the stumps treated with *C. purpureum*, about 19% of the stumps treated with chemical herbicides, and 43% of stumps in plots treated with blank formulation or slash control (Table 3). Basidiocarps of *Trametes versicolor* (L.) C.G. Loyd, *Schizophyllum commune* (L.) Fr., and other basidiomycetes were also observed on many of the stumps in all of the treatment plots (Table 3).

In a follow-up assessment of the trial in July 1997, basidiocarps of *C. purpureum* were observed on 23% of the stumps treated with the fungus. On the other treatments, occurrence of *C. purpureum* was <2%. No living sprouts were found on any stump bearing *C. purpureum* basidiocarps. Evidence of secondary colonization and advanced decay were apparent, and basidiocarps of *T. versicolor*, *S. commune*, and other basidiomycetes were recorded on many of the stumps.

Table 3
Percentage of *A. rubra* stumps in each treatment with basidiomycete fruiting bodies

Treatment	*Chondrostereum purpureum*	*Schizophyllum commune*	*Trametes versicolor*	Others
Slash control	42 bc	19 a	28 a	37 a
Formulation control	43 bc	17 a	13 ab	21 b
C. purpureum 2139	66 ab	13 a	18 ab	19 b
Glyphosate	15 c	3 a	6 b	11 b
Carbopaste	23 c	13 a	4 b	8 b

Treatment means with the same letter are not significantly different by Duncan's multiple range test at $P \leqslant 0.05$.

3.3. Pre-trial population vs. spore traps

In the summer of 1997, eight isolates verified to be *C. purpureum* were recovered from 100 tagged alder stumps from the spore-trap site established 50 m from the field trial. The spore traps that were 700 m from the field site yielded 34 verified isolates of *C. purpureum* from 200 spore traps. Sporocarps of *C. purpureum* were also noted on slash, cut from spore traps. At a distance of 1000 m from the field site, only one isolate of *C. purpureum* was recovered from 200 alder stems used as spore traps. Field-collected samples were amplified using SCAR fingerprinting markers (Becker et al., 1999b) and banding patterns (Fig. 1) were compared. None of the 43 *C. purpureum* isolates recovered from spore traps amplified the characteristic banding pattern of isolate 2139.

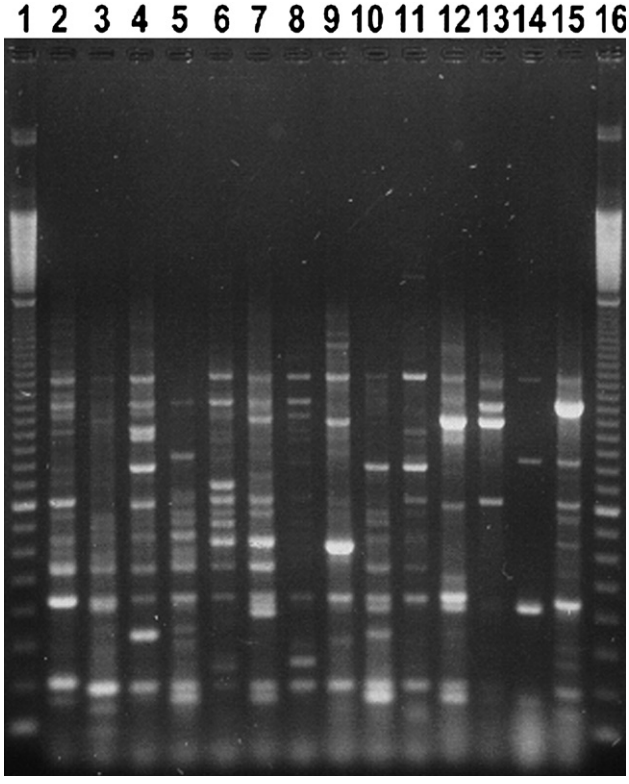

Fig. 1. Banding patterns generated by isolate-specific PCR marker, from pre-trial and spore-trap collections of *C. purpureum*. Results of amplifications using SCAR primer pair APD13F+R with template DNA are shown for a selection of field samples. Biocontrol isolate 2139 is shown in lane 2, along with spore-trap samples ST056 (lane 3), ST062 (lane 4), ST083 (lane 5), ST105 (lane 6), ST114 (lane 7), ST122 (lane 8), ST156 (lane 9), ST157 (lane 10), ST167 (lane 11), and pre-trial samples F0167 (lane 12), F0168 (lane 13), F0170 (lane 14), and F0172 (lane 15). Size marker (100 bp ladder, Pharmacia), which has an intensely staining 800 bp band, is shown in lanes 1 and 16.

Table 4
Similarity of *C. purpureum* isolate 2139 to isolates collected prior to and following field release, expressed as band-sharing coefficients (Nei and Li, 1979)

Pre-trial isolates		Spore-trap isolates		
Isolate	Similarity to 2139	Isolate	Distance from field trial (m)	Similarity to 2139
F0080	0.35	ST001	50	0.38
F0082	0.53	ST003	50	0.43
F0083	0.38	ST013	50	0.40
F0085	0.44	ST021	50	0.18
F0086	0.40	ST022	50	0.30
F0087	0.38	ST025	50	0.25
F0088	0.50	ST053	50	0.24
F0092	0.53	ST056	50	0.33
F0115	0.35	ST062	700	0.42
F0117	0.50	ST081	700	0.32
F0118	0.33	ST082	700	0.25
F0119	0.12	ST083	700	0.42
F0121	0.21	ST103	700	0.48
F0122	0.13	ST104	700	0.38
F0125	0.38	ST105	700	0.64
F0128	0.13	ST114	700	0.56
F0129	0.59	ST116	700	0.57
F0130	0.47	ST122	700	0.60
F0131	0.53	ST156	700	0.22
F0158	0.42	ST157	700	0.40
F0159	0.13	ST167	700	0.48
F0163	0.50	ST194	700	0.29
F0164	0.38	ST196	700	0.33
F0166	0.33	ST402	700	0.32
F0167	0.33	ST406	700	0.12
F0168	0.22	ST407	700	0.50
F0170	0.21	ST415	700	0.42
F0172	0.64	ST422	700	0.33

Relative similarity to the released *C. purpureum* isolate 2139 determined by band-sharing was computed for each member of the pre-trial population and ranged from 12 to 64% (Table 4 and Fig. 2). Members of the post-release population also ranged from 12 to 64% similarity to the released isolate (Table 4 and Fig. 2). There was little difference in average band-sharing among pre-trial (37.18% average) and spore-trap (37.71% average) populations (Fig. 2).

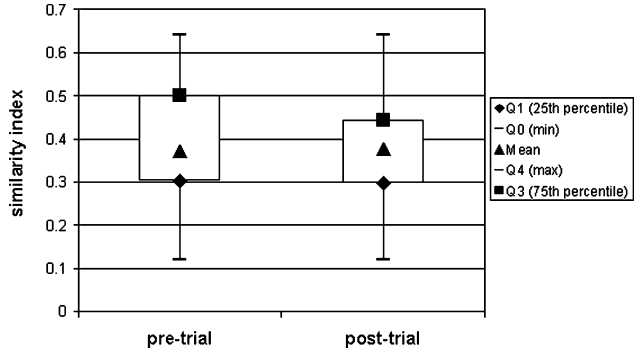

Fig. 2. Box plots providing statistics (mean, 25% quartile, 75% quartile, min and max) for similarity data (given in Table 4) from pre-trial and post-trial (spore traps) *C. purpureum* populations.

A distance matrix was computed that compared all isolates (data not shown). The phylogenetic tree produced using the distance-based neighbor joining algorithm did not cluster taxa of pre- and post-trial population subgroups (data not shown). Bootstrap resampling of data (1000 times) resulted in a polytomy with no grouping of any taxa. Likewise, when data from the released isolate (2139) were added to the analyses, no nodes were supported and there was no clustering among taxa. When the tree was rooted using the released isolate (2139), branch lengths from pre- and post-trial populations to the root were equal (data not shown).

4. Discussion

4.1. Efficacy

Field trials can provide a proof of concept as well as an ecological experiment, which may allow us to disprove hypotheses and address questions posed by theoretical models, such as, "what are the biological impacts of mycoherbicide application?" In this study, control of red alder by *C. purpureum* stump treatment was as effective as that obtained with glyphosate. There was no statistical difference between treatment of red alder with *C. purpureum* and treatment with glyphosate; both resulted in significant reductions in both the number of resprouts and the maximum height of resprouts. In spite of the high mortality in the untreated controls, the field applications resulted in statistically significant reduction in the amount and vigor of sprouting on red alder stumps treated with *C. purpureum* isolate 2139. Suppression of regrowth by *C. purpureum* was as effective as the liquid formulation of glyphosate and almost as effective as Carbopaste. Plots treated with *C. purpureum* or with chemicals had little or no tree growth and would not require retreatment for at least a decade. While mortality levels were high in untreated controls, stumps that did resprout in the untreated control plots had vigorous growth which might require removal within 5 years to prevent encroachment on power lines. This is the first report of an operational-scale field trial of this promising biocontrol agent on the target host species *A. rubra*. Additional field trials of *C. purpureum* on *A. rubra* as well as on other tree species might allow us to more clearly differentiate the effects on target trees due to biocontrol application from effects due to factors such as natural infection of cut stumps during biocontrol application, which may have contributed in this study to high levels of mortality in nontreated stumps.

Generally, mycoherbicides will persist locally at an elevated level then return to endemic levels as the targeted weed population is depressed (Charudattan, 1988). In the case of *C. purpureum*, the fungus is thought to persist as a saprophyte and then return to endemic levels as its

substrate (fresh wood) is depleted. As a primary invader of wounds, *C. purpureum* causes sapwood stain, decay, and eventually host death (Rayner, 1977; Rayner and Boddy, 1986). Upon weakening the host, *C. purpureum* is replaced by other more aggressive saprobic fungi such as *T. versicolor* (L.:Fr.) Pilát and *S. commune* Fr.:Fr. (Rayner and Boddy, 1986; Wall, 1997), which was observed in *C. purpureum*-infected stumps in this trial. These fungi may kill the host and it is believed that the rapid succession ensures *C. purpureum* will not persist at high levels following the inundation of an area for biocontrol treatment.

Several biochemical features of *C. purpureum* have been characterized, including endopolygalacturonase (endoPG) (Miyairi et al., 1985), an enzyme responsible for silvering of infected plant foliage, and a unique group of sesquiterpene metabolites, sterpurenes (Strunz et al., 1997; Xie et al., 1992), but only endoPG has been linked to pathological symptoms. Virulence is dependent on the ability of the fungus to rapidly colonize its host, as well as phytotoxin production and their effects. Hence, the pathogenic mechanism of *C. purpureum* is likely dependent on many factors and has not yet been elucidated. Ekramoddoullah et al. (1993) found that most *C. purpureum* isolates varied slightly in virulence, and Wall et al. (1996) observed few significant differences in virulence of mono- and dikaryotic isolates of *C. purpureum* on thimbleberry (*Rubus parviflorus* Nutt.) tissue cultures and rooted poplar cuttings.

The isolate chosen for biocontrol use (2139) was selected on the basis of its virulence expressed as canker size on red alder (Wall et al., 1996). This selection criterion does not, however, automatically correlate to virulence expressed as silver-leaf symptoms, necrosis in plate disk assays, or resprout suppression (i.e., biocontrol efficacy). Isolates selected for biocontrol use are not necessarily more "fit," but there was concern that these isolates might persist and cause a local increase in the incidence of silver-leaf disease in fruit trees.

This could be detected as an increase in the similarity of the post-trial population to the released isolate, as compared to the similarity of the released isolate to the pre-trial naturally occurring (background) population. Spores were collected by creating an environment for their germination on freshly cut alder trees in the vicinity of the trial to test the hypothesis that inundative biocontrol application would cause a local increase in the resident population of the applied isolate.

4.2. APD13 marker

Molecular genetic markers have provided tools for monitoring populations at different scales, from the fine scale of individual differentiation to across biogeoclimatic zones. The SCAR marker APD13 has shown its utility for highly reproducible characterization of individuals and has been utilized for monitoring the *C. purpureum* infection frequency within infected stumps in field trials (Becker et al., 1999a,b). Polymorphism is detected as band presence versus absence and may be caused either by failure to prime a site in some individuals because of nucleotide sequence differences or by insertions or deletions in the fragment between two conserved primer sites. The presence or absence of shared fragments can be used to estimate the relatedness of the DNA samples. These estimates of divergence do not depend on absolute counts of bands but on the proportion of bands that are shared by samples. Fortunately, the estimation of similarity using a comparison of band-sharing is independent of the efficiency of PCR in amplifying all possible target fragments. If only a fraction of expected fragments are actually detected, providing this fraction is consistent for monomorphic and polymorphic sites, the estimation of the proportion of shared bands will remain valid (Clark and Lanigan, 1993). Preliminary characterization of this marker has revealed its distribution throughout the genome in a repeated motif. No bands are unique to the released isolate 2139 but it has a characteristic pattern that can be easily identified.

4.3. Conclusions from spore-trap studies

We were unable in this experiment to detect the genotype of the released isolate in spore traps following inundative application of this strain, nor did we detect any increase in similarity of the post-trial population to the released isolate that would have suggested its presence in the local population. Although not conclusive, this was the first study in which molecular distance methods were used to compare pre- and post-trial *C. purpureum* collections to test the hypothesis that there would be a temporary local increase in spores from the released strain in the background population of *C. purpureum*.

Factors involved in nontarget infection by a released isolate of *C. purpureum* include: the inoculum potential of the infected hosts (i.e., treated trees), spore dispersal ability, the availability of fresh wounds to colonize, and the competitive ability of the fungus to establish itself. The heterothallic tetrapolar mating system (Nakasone, 1990) and presence of many mating types of *C. purpureum* (Wall et al., 1996) promote the outcrossing of haploid basidiospores derived through meiosis. Likewise, the absence of barriers to gene flow among populations of *C. purpureum* (Gosselin et al., 1999; Ramsfield et al., 1996) limits the possibility of fixation of alleles due to selection or stochastic effects (Wright, 1951).

5. Conclusions

Inoculation with *C. purpureum* was as effective as chemical herbicides Carbopaste and liquid glyphosate in causing cut stump mortality and in reducing resprouting.

Overall, more than 90% of stumps treated with *C. purpureum* died in the first year and 100% died in the second year. In comparison with manual cutting or slashing, *C. purpureum* caused 90% reduction in stump sprouting. Naturally occurring *C. purpureum* spores colonized about 40% of untreated cut stumps and about 20% of stumps treated with chemical herbicides. The peak production of *C. purpureum* basidiocarps occurred about 22 months after the trial was established, with *C. purpureum* on 66% of treated stumps, then by the following year, *C. purpureum* basidiocarps persisted on 23% of treated stumps. Amplification of the repeated marker DNA APD13 by the PCR was a rapid and highly reproducible means of detecting polymorphisms for strain identification and analysis of genetic distance. Molecular markers were successfully used to identify and characterize *C. purpureum* genotypes in order to compare populations and monitor effects of mycoherbicide application. Approximately 600 spore traps, established in the vicinity of the trial site during a period of basidiospore production, yielded 43 verified *C. purpureum* isolates, the compatible pairs of 86 spores. The released isolate 2139 was not identified among these recovered genotypes. There was no significant difference in average band-sharing with the applied isolate among pre-trial (37% average) and spore-trap (38% average) populations. There was therefore no evidence that the *C. purpureum* strain applied inundatively to stumps in this trial was detectable on nontarget trees in the local area or had any measurable impact on the local *C. purpureum* population.

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References

Becker, E.M., Ball, L.A., Dumas, M.T., Pitt, D.G., Wall, R.E., Hintz, W.E., 1999a. *Chondrostereum purpureum* as a biological control agent in forest vegetation management. III. Infection survey of a national field trial. Can. J. Forest Res. 29, 859–865.

Becker, E.M., Ball, L.A., Hintz, W.E., 1999b. PCR-based genetic markers for detection and infection frequency analysis of the biocontrol fungus *Chondrostereum purpureum* on Sikta alder and Trembling aspen. Biol. Control 15, 71–80.

Bishop, G.C., 1979. Infection of cherry trees and production of a toxin that causes foliar silvering by different isolates of *Chondrostereum purpureum*. Aust. J. Agric. Res. 30, 659–665.

Brooks, F.T., Moore, W.C., 1926. Silverleaf disease V. J. Pomology 5, 61–97.

Charudattan, R., 1988. Inundative control of weeds with indigenous fungal pathogens. In: Burge, M.N. (Ed.), Fungi in Biological Control Systems. Manchester University Press, New York, pp. 86–110.

Clark, A.G., Lanigan, C.M.S., 1993. Prospects for estimating nucleotide divergence with RAPDs. Mol. Biol. Evol. 10, 1096–1111.

de Jong, M.D., 1992. Risk assessment for the application of biological control of a forest weed by a common plant pathogenic fungus. Risk Anal. 12, 465–466.

de Jong, M.D., Scheepens, P.C., Zadoks, J.C., 1990. Risk analysis for biological control: a Dutch case study in biocontrol of *Prunus serotina* by the fungus *Chondrostereum purpureum*. Plant Dis. 74, 189–194.

de Jong, M.D., Sela, E., Shamoun, S.F., Wall, R.E., 1996. Natural occurrence of *Chondrostereum purpureum* in relation to its use as a biological control agent in Canadian Forests. Biol. Control 6, 347–352.

de Jong, M.D., Wagenmakers, P.S., Goudriaan, J., 1991. Modelling the escape of *Chondrostereum purpureum* spores from a larch forest with biological control of *Prunus serotina*. Neth. J. Plant Pathol. 97, 55–61.

Dumas, M.T., Wood, J.E., Mitchell, E.G., Boyonoski, N.W., 1997. Control of stump sprouting of *Populus tremuloides* and *P. grandidentata* by inoculation with *Chondrostereum purpureum*. Biol. Control 10, 37–41.

Ekramoddoullah, A.K.M., Shamoun, S.F., Wall, R.E., 1993. Comparison of Canadian isolates of *Chondrostereum purpureum* with respect to temperature response, virulence, and protein profiles. Can. J. Plant Pathol. 15, 7–13.

Environment Canada, 2004. Cowichan Lake Forestry Daily Data Report for September 1994. Accessed Feb, 2005, from: <http://www.climate.weatheroffice.ec.gc.ca/climateData/dailydata_e.html>.

Gosselin, L., Jobidon, R., Bernier, L., 1996. Assessment of genetic variation within *Chondrostereum purpureum* from Quebec by random amplified polymorphic DNA analysis. Mycol. Res. 100, 151–158.

Gosselin, L., Jobidon, R., Bernier, L., 1999. Genetic variability and structure of Canadian populations of *Chondrostereum purpureum*, a potential biophytocide. Mol. Ecol. 8, 113–122.

Grosclaude, C., Ricard, J., Dabos, B., 1973. Inoculation of *Trichoderma viride* spores via pruning shears for biological control of *Chondrostereum purpureum* on plum tree wounds. Plant Dis. Rep. 57, 25–27.

Harper, G.J., Comeau, P.G., Hintz, W.E., Wall, R.E., Prasad, R., Becker, E.M., 1999. *Chondrostereum purpureum* as a biological control agent in forest vegetation management. II. Efficacy on Sitka alder and aspen in western Canada. Can. J. Forest Res. 29, 852–858.

Jobidon, R., 1998. Comparative efficacy of biological and chemical control of the vegetative reproduction in *Betula papyrifera* and *Prunus pensylvanica*. Biol. Control 11, 22–28.

Miyairi, K., Okuno, T., Sawai, K., 1985. Purification and properties of endopolygalacturonase I from *Stereum purpureum*, a factor inducing silver-leaf symptoms on apple trees. Agric. Biol. Chem. 49, 111–118.

Möller, E.M., Bahnweg, G., Sanderman, H., Geiger, H.H., 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. 20, 6115–6116.

Nakasone, K.K., 1990. Cultural studies and identification of wood-inhabiting *Corticiaceae* and selected *Hyphomycetes* from North America. Mycol. Mem. 15, 1–412.

Nei, M., Li, W., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269–5273.

Pitt, D.G., Dumas, M.T., Wall, R.E., Thompson, D.G., Lanteigne, L., Hintz, W., Sampson, G., Wagner, R.G., 1999. *Chondrostereum purpureum* as a biological control agent in forest vegetation manage-

ment. I. Efficacy on speckled alder, red maple, and aspen in Eastern Canada. Can. J. Forest Res. 29, 841–851.
Ramsfield, T.D., Becker, E.M., Rathlef, S.M., Tang, Y., Vrain, T.C., Shamoun, S.F., Hintz, W.E., 1996. Geographic variation of *Chondrostereum purpureum* detected by polymorphisms in the ribosomal DNA. Can. J. Bot. 74, 1919–1929.
Ramsfield, T.D., Shamoun, S.F., Punja, Z.K., Hintz, W.E., 1999. Variation in the mitochondrial DNA of the potential biological control agent *Chondrostereum purpureum*. Can. J. Bot. 77, 1490–1498.
Rayner, A.D.M., 1977. Fungal colonization of hardwood stumps from natural sources. II. Basidiomycetes. Trans. Br. Mycol. Soc. 69, 303–312.
Rayner, A.D.M., Boddy, L., 1986. Population structure and the infection biology of wood-decay fungi in living trees. Adv. Plant Pathol. 5, 119–160.
Rayner, A.D.M., Boddy, L., 1988. Fungal Decomposition of Wood: Its Biology and Ecology. Wiley, New York.
Saitou, N., Nei, M., 1987. The neighbor joining method, a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Setliff, E.C., Wade, E.K., 1973. *Stereum purpureum* associated with sudden decline and death of apple trees in Wisconsin. Plant Dis. Rep. 57, 473–474.
Shamoun, S.F., Wall, R.E., 1996. Characterization of Canadian isolates of *Chondrostereum purpureum* by protein content, API ZYM and isozyme analyses. Eur. J. Forest Pathol. 26, 333–342.
Spiers, A.G., Hopcroft, D.H., 1988. Ultrastructural studies of basidial and basidiospore development and basidiospore release in *Chondrostereum purpureum*. Eur. J. Forest Pathol. 18, 367–381.
Spiers, A.G., Brewster, D.T., Slade, A., Gardiner, S.E., 2000. Characterization of New Zealand isolates of *Chondrostereum purpureum* with regard to morphology, growth, pathogenicity and RAPD banding patterns. Mycol. Res. 104, 395–402.
Strunz, G.M., Bethell, R., Dumas, M.T., Boyonoski, N., 1997. On a new synthesis of sterpurene and the bioactivity of some related *Chondrostereum purpureum* sesquiterpene metabolites. Can. J. Chem. 75, 742–753.
Swofford, D.L., 2000. PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4.0b4a. Sinauer Associates, Sunderland, MA.
Templeton, G.E., TeBeest, D.O., Smith Jr., R.J., 1979. Biological weed control with mycoherbicides. Annu. Rev. Phytopathol. 17, 301–310.
Wall, R.E., 1986. Pathogenicity of *Chondrostereum purpureum* to yellow birch. Plant Dis. 70, 158–160.
Wall, R.E., 1990. The fungus *Chondrostereum purpureum* as a silvicide to control stump sprouting in hardwoods. North. J. Appl. For. 7, 17–19.
Wall, R.E., 1991. Pathological effects of *Chondrostereum purpureum* in inoculated yellow birch and beech. Can. J. Plant Pathol. 13, 81–87.
Wall, R.E., 1994. Biological control of red alder using stem treatments with the fungus *Chondrostereum purpureum*. Can. J. Forest Res. 24, 1527–1530.
Wall, R.E., 1997. Fructification of *Chondrostereum purpureum* on hardwoods inoculated for biological control. Can. J. Plant Pathol. 19, 181–184.
Wall, R.E., Macey, D.E., Sela, E., 1996. Virulence and interfertility of *Chondrostereum purpureum* isolates. Biol. Control 7, 205–211.
Wall, R.E., Prasad, R., Shamoun, S.F., 1992. The development and potential role of mycoherbicides for forestry. Forest. Chron. 68, 736–741.
Wright, S., 1951. The genetical structure of populations. Ann. Eugenics 15, 323–354.
Xie, J., Liping, L., Ziqin, D., 1992. Isolation and identification of two new metabolites from silver leaf fungus *Stereum purpureum*. J. Org. Chem. 57, 2313–2316.