

Development of genetic markers for risk assessment of biological control agents

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Abstract: Prior to the release of any organism for use as a biological control agent, it is necessary to assess the possible effects of deploying a single isolate over a wide geographic area. There is a distinct possibility that a rare virulence gene or allele could be introduced into a local population of the pathogen following release. Factors such as the isolation of populations by distance or natural barriers to dispersal can allow evolutionary forces such as selection, mutation, and genetic drift to affect gene frequencies in local populations. Analysis of the genetic structure of a species can reveal information about its population structure and permit risk analysis. We have developed a series of molecular markers to assess the impact of releasing selected isolates of the biological control fungus *Chondrostereum purpureum*. These markers were used to analyze gene flow between populations and to assess the risk of the deployment of *C. purpureum* as an agent for the biological control of forest weeds in Canada.

Key words: *Chondrostereum*, biological control, fungi, forest weeds.

Résumé : Avant d'autoriser un nouvel organisme comme agent de lutte biologique, il est nécessaire d'évaluer les conséquences éventuelles du relâchement d'un seul isolat sur une grande région géographique. La possibilité existe qu'un gène ou un allèle de virulence peu fréquent puisse être introduit dans une population locale de l'agent pathogène à la suite d'un relâchement. Des facteurs tels que l'isolement des populations par la distance ou par des barrières naturelles qui gênent la dispersion peuvent permettre aux forces de l'évolution telles que la sélection, la mutation et la dérive génétique d'affecter la fréquence des gènes dans les populations locales. L'analyse de la structure génétique d'une espèce peut informer sur la structure de sa population et permettre l'analyse du risque. Nous avons développé une série de marqueurs moléculaires afin d'évaluer l'impact de la dissémination d'isolats sélectionnés du champignon de lutte biologique *Chondrostereum purpureum*. Ces marqueurs furent utilisés pour analyser le flux de gènes entre populations et pour évaluer le risque du relâchement du *C. purpureum* comme agent de lutte biologique contre les mauvaises herbes forestières au Canada.

Mots clés : *Chondrostereum*, lutte biologique, champignons microscopiques, mauvaises herbes forestières.

Introduction

Biological control is the deliberate use of one or more organisms to suppress the growth or reduce the population of another organism to a level at which it is no longer an economic problem (Hawksworth et al. 1995). Two basic biological control methods include "classical" biological control (CBC) and the "inundative" strategy. The classical strategy is also termed the "inoculative" method, as it usually involves a small "dose" of an exotic biological control organism, applied once or only occasionally, to a host population of weeds that is usually of foreign origin. The "inundative" method involves single or multiple applications of a biological control agent to a pest at much higher concentrations than ordinarily encountered (Daniel et al. 1973). The term mycoherbicide usually refers to an indige-

nous fungus applied in an inundative manner to control native weeds (TeBeest and Templeton 1985). The inundative method involves application of sufficiently high levels of inoculum to the weed population, under conditions that favour disease onset, to quickly create a disease epidemic among plants (Daniel et al. 1973; Templeton et al. 1979; Charudattan 1991).

Virtually all plant species are affected by one or more indigenous pathogens that may kill individual plants, especially when weak or stressed. For effective weed biological control the emphasis has traditionally been on the induction or enhancement of disease epidemics in plants (epiphytotics) by manipulation of epidemic constraints. Epidemics occur when the requirements of a pathogen are fulfilled and are characterized by a rapid rate of disease onset (Anderson and May 1986). Facultative parasites and facultative saprophytes are typically more damaging to their host than obligate parasites, so they are generally more suitable for use as mycoherbicides (Charudattan 1991). The inundative approach has an advantage over the classical strategy in that it is not dependent on the agents being self-sustaining. Since mycoherbicides are generally reapplied to new weeds, the long-term survival of the pathogen is not as important as it would be for the CBC strategy. Following application, mycoherbicides will generally persist locally at an elevated

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level, then return to endemic levels when the targeted weed population is depressed (Charudattan 1988). It is crucial for the success of any biological control program to monitor the progress of the epidemic following the release of any potential agent.

Mycoherbicide impact assessment

Biological control is often promoted as an effective and environmentally sound method for the management of pests that can include insects, plant pathogens, and weeds. There are, of course, risks associated with perturbing the natural environment through the biological control approach. What happens following inundative biological control of weeds with indigenous plant pathogens? This question can be approached from a "biological" perspective in which the application or release of a biological control agent constitutes an ecological experiment (Anderson and May 1986). Applied biological control research is usually concerned with a more utilitarian perspective. Practical concerns usually focus on efficacy and utility, whereas the biological perspective demands that we closely examine the potential risks to the environment.

Biological impact assessment is a systematic examination of the likelihood and magnitude of establishment of any released organism and a determination of the potential positive and negative effects of establishment of the organism within the ecosystem (Teng and Yang 1993). The objective of this type of analysis is to maximize the benefits and minimize the risks involved. For biological control, the objective is to impact the pest population to some threshold level, while minimizing harm to nontarget organisms. Decisions on the use of biological control agents therefore require knowledge and assessment of biological impact over time. Effects on an ecosystem from the inundative application of mycoherbicide agents are not easily identified and are even less easily predicted. Impact assessment includes fate assessment; the measurement of the growth and survival of introduced organisms; and consequence assessment, which includes the identification of potential side effects towards nontarget organisms. Examination of biological impact has two components: risk assessment and benefit assessment.

Risks and benefits

Risk is defined as the probability of the occurrence of a hazard or potentially harmful effect. Included in risk are three potential hazards: (i) the displacement of nontarget organisms; (ii) allergenicity to humans; (iii) toxigenicity or pathogenicity to nontargets. The probability of the occurrence of a hazard increases in proportion to scale of use (Cook et al. 1996). Displacement of nontarget organisms by the use of a mycoherbicide is not generally considered to be a significant risk, as the effects of local population inundation by indigenous fungi are temporary and limited in scale compared with potential displacement of nontarget organisms by exotic CBC pathogen releases (Charudattan 1991). Allergenicity and toxicity of spores or other preparations of a candidate fungal agent to mammals and other organisms is routinely tested during mycoherbicide development prior to any field release (Templeton 1992). Candidate agents are

also tested for nontarget pathogenicity against a group of plants related to the target host, plus important crop plants considered at risk (Wapshere 1974). Gene transfer may potentially allow traits to be transferred from biological control agents to other organisms in the environment and is increasingly being considered as a part of risk assessment (TeBeest et al. 1992; Cook et al. 1996). Benefits usually include factors such as increase in yield of the crop that is normally impacted by the weed. Benefits may also include secondary features such as reduction in the usage of chemical herbicides, which account for 85% of pesticide use in the United States (Templeton 1992).

Population dynamics following release

Biological control theory has been primarily concerned with the effects of the release of a new organism to an area for control of a pest (Huffaker et al. 1976; Murdoch and Briggs 1996). Perturbation of a population by inundating with one indigenous individual may have much more cryptic effects. It is essential to monitor the effects of a candidate mycoherbicide after it has been applied to assess efficacy and fate. Quantitative epidemic data may be collected over the course of field observation of the pathosystem and as a result of monitoring laboratory, greenhouse, and field experiments. As a precursor to actual field releases, a simulation model can provide a simplified description of population dynamics and weed-pathogen interactions of a biological control strategy and allow the assessment of potential effects over time and space. Models can also provide an estimation of both the probability and the magnitude of an effect. Yang and TeBeest (1993) proposed a model to describe efficacy of mycoherbicide use. This model takes into account the level of primary and secondary weed host infection and rate of disease development to predict effectiveness of mycoherbicide use over time. Indigenous pathogens chosen for mycoherbicide use have co-evolved with their hosts and are likely to be adapted to prevailing environmental conditions. There may be specific conditions or a preferred time period during which the host plants are maximally susceptible to infection. This is often referred to as the "infection window." Adjuvants may be incorporated into the mycoherbicide formulations to provide protection of the pathogen from environmental conditions such as desiccation or UV damage and to maximize contact with the host (van Drieshe and Bellows 1996). This serves to "open the infection window" and render target weeds more susceptible to disease following mycoherbicide application. Optimal infection of the host requires application of a sufficient amount of fungal inoculum at a critical time in the hosts' life cycle to provide specific control of the targeted weed plants. This is followed by the pathogen's return to endemic levels (Yang and Tebeest 1993). This theoretical prediction has been corroborated in several cases by observations over time of mycoherbicide populations following application (Wall 1991; Yang and Tebeest 1993). Models describing the progression of disease caused by mycoherbicide fungi are useful tools for assessing the dynamics of candidate mycoherbicide agents. As a hypothetical framework for a pathosystem model expands, there is an in-

creased demand for testing mechanisms that potentially affect the system.

The community can be viewed as an open dynamic system in which interactions between human disturbances and the biology of resident species determine patterns of succession or directional change in the composition, abundance, and spatial pattern of these species. The factors driving population change are unique for each community ecosystem and will vary in influence over time. In natural systems, persistence of diseases at endemic levels ensures succession and aids evolutionary adaptation by removing weak and stressed individuals. Plants vary in disease susceptibility or tolerance, and their pathogens may be weakly to highly virulent. Genetic diversity is established and maintained through evolutionary processes, and this variation ensures the coexistence of plant and pathogen.

Assessing population structures for biological control agents

An important consideration during the development of any biological control agent is the possible introduction of rare virulence alleles from the released organism into a local population of the pathogen (Templeton et al. 1979). Gene flow refers to changes in gene frequency due to movement of genes from one population to another (Slatkin 1987). Evolutionary forces such as selection, mutation, and genetic drift are balanced by the effects of migration or gene flow, which tend to increase genetic homogeneity between populations (Wright 1951). Fixation of particular alleles in a local population will be prevented by sufficient migration from another population (Wright 1951). Genetic variation of the population must be examined to assess the impact of the release of a single isolate on the natural population. Ideally, strains chosen for development as mycoherbicides should be genetically similar to local populations to reduce the risk of introducing novel alleles into a local population. For the use of a single isolate across ecozones it should be established that there has been sufficient gene flow between the regions such that the risk of introduction is minimized.

Our research group has been working towards the development of the fungus *Chondrostereum purpureum* (Pers.:Fr.) Pouzar as a biological control agent of hardwoods in reforestation sites and utility rights-of-way. Because little was known about the population structure of this pathogen, an assessment of the genetic variation in *C. purpureum* was considered a prerequisite to field testing. Initial studies indicated that there was no host or geographic specialization in this fungus (Shamoun et al. 1991; Shamoun and Wall 1996). Genetic variation was assessed using markers for ribosomal DNA (rDNA), mitochondrial DNA, and randomly amplified polymorphic DNA (RAPDs) (Ramsfield et al. 1996; Ramsfield et al. 1999; Gosselin et al. 1999). Analysis of the distribution of these markers indicated that there were indeed no barriers to gene flow and that the introduction of rare pathogenicity alleles from isolates used as biological control agents would occur at a low probability.

In the first genetic survey of *C. purpureum*, variation in the rDNA repeat region was analyzed in 107 isolates collected from Europe, New Zealand, and North America (Ramsfield et al. 1996). Variation in the large non-

transcribed spacer region of the rDNA was determined for the entire collection of isolates following amplification by the polymerase chain reaction (PCR) and analysis of restriction fragment length polymorphisms (RFLPs). Three distinct nuclear type patterns were identified using the restriction endonuclease *HaeIII*. Nuclear type I was found in North American, European, and New Zealand isolates. Nuclear type II was only detected in isolates collected from North America, and nuclear type III was observed in isolates collected from both Europe and New Zealand. Therefore, the North American population of *C. purpureum* could be distinguished from the European and New Zealand populations based on rDNA differences. Within North America, the type I pattern was predominantly found in eastern regions, indicated by a frequency of 0.78, while the type II occurred with a relative frequency of 0.89 in western regions. Gene flow across the continent was indicated by near equal nuclear type distributions (nuclear type I: 0.41, nuclear type II: 0.59) in central North America, but geographic separation has led to unequal nuclear type distributions across North America (Ramsfield et al. 1996). These results suggested that gene flow had occurred throughout the Canadian *C. purpureum* population; however, the actual rate of gene flow could not be determined by analysis of this genetic marker alone.

Polymorphisms in the mitochondrial DNA restriction patterns were also used to assess genetic variation in the *C. purpureum* population (Ramsfield et al. 1999). The ATPase VI – cytochrome *b* region was amplified from a sample population of 84 isolates of *C. purpureum* collected from North America ($n = 44$), Europe ($n = 22$), and New Zealand ($n = 18$) and digested with *NsiI*. PCR amplification produced a 5.1-kb product in every case, and there were no size polymorphisms that would indicate gene duplication or large insertion or deletion events. Mitochondrial haplotypes were assigned as type I if *NsiI* cleaved the fragment or type II if the fragment was not cleaved. There was a near equal distribution of mitochondrial haplotypes in New Zealand, while Europe and North America had twice the number of mitochondrial type I over type II. In the North American population, mitochondrial type I was the predominant type in the east, while in western isolates, the haplotype distribution was near equal. Comparison of the mitochondrial haplotype distribution with assigned rDNA types indicated that, although the distributions were similar, each marker was independently inherited. When the nuclear and mitochondrial types of each isolate were compared to measure the association between them using the χ^2 test ($\chi^2 = 2.567$), no significant relationship between the nuclear and mitochondrial types was found ($p = 0.633$). This indicated that, although the trend was the same for both marker systems, they were distributed independently of each other. The distribution of mitochondrial DNA types corroborated that gene flow has occurred across the entire continent and that there were no natural barriers to *C. purpureum* dispersal across the North American continent.

In an independent study recently completed by Gosselin et al. (1999), the extent of genetic diversity among 93 isolates of *C. purpureum* was studied in populations corresponding to four of the five major Canadian ecozones. The ecozone concept provides a useful guide to determine

whether an organism is indigenous or nonindigenous to the region of intended use. It is important to evaluate the population structure in relation to the ecozone concept and to use this standard for risk evaluation. If a high similarity between populations in different ecozones can be demonstrated, the perceived risk of deployment would be decreased. It is, therefore, important to determine the population structure with a fairly high degree of resolution for any potential biological control agent. Pseudoallelic frequencies were estimated at each of 22 RAPD loci by scoring for the presence or absence of amplified fragments of DNA in haploid mycelial cultures (Gosselin et al. 1999). The analysis of the population structure revealed that genetic diversity within populations accounted for most of the total genetic diversity, while genetic diversity between populations represented only a small proportion of the total diversity. Differences in allele frequencies were detected among the four populations studied, but no evidence of population structure was revealed. The RAPD analysis indicated that *C. purpureum* is a highly heterogeneous fungus with a continuously distributed population across Canada. It was concluded by Gosselin et al. (1999) that the amount of gene diversity within subpopulations was great enough to negate the risk of introducing new genes into a local population. As genetic exchange occurs naturally throughout the country and because no significant genetic variation has been attributed to the different ecological regions, the use of any native isolate that matches the criteria for effective biological control should not be restricted.

Assessing the environmental fate of an individual genotype

While the analysis of the described markers can yield information on the historical dispersal patterns of an organism, monitoring the fate of a released organism requires a higher degree of resolution. Tracking of specific genotypes following release relies on the development of markers that allow detection and identification of the released agent. For the biological control fungus *C. purpureum*, such markers could be used to monitor introgression of a released isolate into the local population (Hintz and Shamoun 1996). While RAPD markers have been useful for the discrimination of individual genotypes, there is considerable variability in the specificity and reproducibility of amplification with RAPD primers. This inconsistency led to the development of sequence-characterized amplified regions (SCAR) as genetic markers (McDermott et al. 1994). Whereas RAPD primers amplify anonymous fragments of DNA based on annealing of random-sequence primers, SCAR primers are designed according to authentic sequence derived by the sequence characterization of the RAPD amplification products. Genomic DNA isolated from the isolate used for field release in British Columbia was amplified using the primer OPD-13 (5'-GGGGTGACGA-3'). The RAPD amplification products were individually cloned and the sequences determined. For each of the cloned products, oligonucleotide primers were designed that extended beyond the original priming site such that the estimated annealing temperature (T_m) of the primers was between 62 and 64°C. Because of the much longer length of the primers (21–30 nucleotides),

a much higher annealing temperature could be used during amplification using the SCAR primer pairs.

During the routine screening of the RAPD-derived SCAR markers, one PCR primer pair having a rather unusual property was uncovered. Instead of amplifying a single band, as would be expected for a single primer pair, up to 20 bands were simultaneously amplified from target *C. purpureum* genomic DNA (Becker et al. 1999). This pattern was highly reproducible and very specific for each individual genotype tested. The primers designated AP-D13F (GGGGTGACG-AGGACGACGGTG) ($T_m = 63.2^\circ\text{C}$) and AP-D13R (GGG-GTGACGACATTATACTGCAGGTAGTAG) ($T_m = 61.2^\circ\text{C}$) consistently differentiated the isolate PFC2139 from other *C. purpureum* isolates. To better understand which loci were being amplified, several of the amplification products were cloned for sequence comparison. Initial comparisons indicated that the repeated DNA extended beyond the 20- to 30-bp (base pairs) annealing sites and that the primers likely amplified polymorphic versions of a repetitive DNA element. Southern hybridization with individually cloned SCAR bands revealed multiple regions of the genome having sequence homology. This repetitive element therefore appears to be dispersed throughout the genome, and the positions of this element are highly variable from one genetic individual to the next. These features provide a very informative genetic test and allow for the identification of specific isolates following release and recapture.

These markers were used to assess the environmental fate of *C. purpureum* released at our Cowichan Valley, British Columbia, field site. To provide a baseline for genetic diversity of isolates at the proposed release site, a collection of 28 isolates was made prior to the field release of isolate PFC2139 in the summer of 1994. Approximately 18 months following the field release of PFC2139, when nutrients in the dead or dying stumps had become limiting and fruiting body formation had reached its maximum, a series of spore traps was established at various distances from the release site. This was done to map the distance traveled by spores of PFC2139 following release. The spore traps consisted of 100–200 trees (2- to 5-cm diameter) cut at a height of 1 m. The trap sites were located at distances of 50, 700, and 1500 m from the release site. The trap sites were monitored over the next 12–20 months for the presence of new fruiting bodies on the cut stems that would represent infection that had occurred at the time of wounding. There was no correlation between the distance from the release site and the number of isolates recovered. A total of 28 isolates was recovered and confirmed to be *C. purpureum*. There were 8 *C. purpureum* isolates collected at 50 m and 20 isolates collected at 700 m. The site at 1500 m was very dry, and no *C. purpureum* was collected.

The extent of genetic variation within the 28 pretrial isolates collected from Cowichan Valley was compared with the set of isolates recovered postrelease. If there were a significant impact resulting from the release of PFC2139, it would be expressed as an increased similarity to the released isolate of the postrelease population. All of the *C. purpureum* DNAs were amplified using the SCAR AP-D13 primer pair and scored as discrete character states represented by a binary code, with a 0 or 1 at each band position representing the presence or absence of a band at that

Table 1. Similarity of isolate PFC2139 to the *C. purpureum* isolates collected prior to and following field release.

Pretrial isolates				Spore-trap isolates				
Isolate	Total no. of bands	Bands shared with 2139	Similarity to 2139 (Nei and Li 1979)	Isolate	Distance from field trial (m)	Total no. of bands	Bands shared with 2139	Similarity to 2139 (Nei and Li 1979)
F0080	6	3	0.35	ST001	50	10	4	0.38
F0082	8	5	0.53	ST003	50	12	5	0.43
F0083	5	3	0.38	ST013	50	9	4	0.40
F0085	7	4	0.44	ST021	50	11	2	0.18
F0086	9	4	0.40	ST022	50	9	3	0.30
F0087	5	3	0.38	ST025	50	5	2	0.25
F0088	9	5	0.50	ST053	50	6	2	0.24
F0092	8	5	0.53	ST056	50	7	3	0.33
F0115	6	3	0.35	ST062	700	13	5	0.42
F0117	9	5	0.50	ST081	700	8	3	0.32
F0118	7	3	0.33	ST082	700	5	2	0.25
F0119	6	1	0.12	ST083	700	13	5	0.42
F0121	8	2	0.21	ST103	700	10	5	0.48
F0122	4	1	0.13	ST104	700	10	4	0.38
F0125	5	3	0.38	ST105	700	14	8	0.64
F0128	5	1	0.13	ST114	700	14	7	0.56
F0129	6	5	0.59	ST116	700	10	6	0.57
F0130	6	4	0.47	ST122	700	9	6	0.60
F0131	8	5	0.53	ST156	700	7	2	0.22
F0158	8	4	0.42	ST157	700	14	5	0.40
F0159	5	1	0.13	ST167	700	10	5	0.48
F0163	5	4	0.50	ST194	700	10	3	0.29
F0164	5	3	0.38	ST196	700	7	3	0.33
F0166	7	3	0.33	ST402	700	8	3	0.32
F0167	7	3	0.33	ST406	700	6	1	0.12
F0168	7	2	0.22	ST407	700	9	5	0.50
F0170	8	2	0.21	ST415	700	8	4	0.42
F0172	11	7	0.64	ST422	700	7	3	0.33
Mean			0.37					0.38

position for each isolate. Change in the variation within each population was estimated by statistical comparisons of binary data of the two sets of isolates. A similarity matrix was constructed to provide a measure of variability (Table 1). This depended on the number of shared bands and number of total amplified bands for each pair of isolates according to the formula:

$$\text{Distance} = 1 - \frac{2N_{xy}}{N_x + N_y}$$

where N_{xy} is the number of shared bands and $N_x + N_y$ is the total number of amplified bands (Nei and Li 1979).

Examination of the distribution of specific amplified fragments revealed that there were no fragments amplified from PFC2139 that did not also amplify at some low frequency in the pretrial set of isolates. This was not unexpected as isolate PFC2139 was originally collected on Vancouver Island. The index of similarity of the pretrial isolates to PFC2139 ranged from 0.13 to 0.64 and averaged 0.37 for the entire data set. There was no significant change in the average similarity to PFC2139 (0.38) for the postrelease population. Values ranged from 0.12 to 0.64 with an average of 0.38 (Table 1). The frequency of recovering the re-

leased isolate (PFC2139) was so low compared to the trapping of wild type isolates as to be practically undetectable. This would suggest that there is little risk of infection to nontarget trees from release of *C. purpureum*.

Summary

The main concern to both regulatory agencies and the general public in using fungal plant pathogens for control of weeds is their threat to nontarget plants. Risk analysis based on epidemiological modeling systems (de Jong et al. 1990) of indigenous fungal pathogens such as *C. purpureum* have indicated that the risk is extremely low. Empirically based models based on monitoring biological control agents can reveal patterns in the system that may be further tested. To maximize efficacy and limit undesired effects, it is recommended that mycoherbicides be monitored after all field applications. Recent advances in molecular and genetic technologies have permitted the monitoring of pathogen releases and provide a reliable tool for studying plant disease epidemiology. These approaches can be used a complementary tool to existing modeling systems. Therefore, the most effective vegetation-management decisions concerning mycoherbicide use will integrate the knowledge of all fac-

tors and mechanisms influencing the dynamics of an ecosystem to reduce weeds while minimizing potential nontarget effects.

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