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Evaluating the Environmental Safety of Broad-host-range Bioherbicides

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ABSTRACT

Broad-host-range pathogens, indigenous in their areas of intended use as bioherbicides and endemic in populations of the weeds of interest, are more appealing commercially than host-specific pathogens because of their wider market potential. However, these pathogens may spread in space and/or in time following their application, thereby potentially increasing the risk of disease to non-target host plants. The ratio of the density of inoculum added to the non-target host plant's environment by the bioherbicide to that occurring naturally, can be used to assess the 'relative risk' of the bioherbicide and determine its acceptability and/or best management practice. Empirical and modelling methodologies have been used for quantifying the additional and natural background inoculum levels of an indigenous plant pathogen being considered for development as a bioherbicide, enabling the ratio of 'added to natural' inoculum density to be determined. We first review how this ratio has been used to define a minimum isolation distance, or safety zone, between areas of application and locations of non-target host plants, using the example of *Sclerotinia sclerotiorum* applied to *Cirsium arvense* in permanent pasture in New Zealand. Secondly, we consider how the ratio has been applied for the same purpose but at a much larger geographic scale using as examples *Chondrostereum purpureum* deployed for weed control in forests in the Netherlands and on Vancouver Island. Lastly we review how the ratio has been used to determine the duration of a withholding period using, again, the *S. sclerotiorum* - *C. arvense* system. Determining an acceptable value for the ratio requires knowledge of the relationship between the disease that a bioherbicide pathogen causes in non-target host plant populations, and the pathogen's inoculum density.

Keywords: *Chondrostereum purpureum*, mycoherbicide, relative risk, risk analysis, *Sclerotinia sclerotiorum*, weed

CONTENTS

INTRODUCTION

Bioherbicide candidate pathogens have generally been selected because they are host specific to the target weed (Watson and Wymore 1990; Auld and Morin 1995), precluding any disease risk to non-target plant species and thereby increasing the likelihood of regulatory approval. However, strict host specificity is a barrier to commercialisation because it limits the market potential of the bioherbicide and thus the ability of companies to invest in the development of such a product (Templeton and Heiny 1990). By contrast, broad-host-range (plurivorous) plant pathogens are generally more appealing because of the wider market potential of a product effective against several weed species. Selecting broad-host-range pathogens indigenous (naturally occurring) in the region of intended use, and endemic in populations of the target weeds and non-target host species, reduces risks that prospective bioherbicides will cause novel diseases in non-target plant species (Templeton and Heiny 1990). In support of the idea of using broad-host-range pathogens as bioherbicides, Auld and Morin (1995) stated that "where sufficient spatial and temporal separation occurs between target weeds and other susceptible plants, strict host specialisation should not be regarded as a requirement for a bioherbicide, just as it is not required of chemical herbicides".

Attempts have been made to broaden the host range of host-specific bioherbicide pathogens by combining them with other control strategies and other pathogens (Watson and Wymore 1990). It has also been suggested that hybridisation could be explored as a way of broadening the host range of a host-specific bioherbicide pathogen (Auld and Morin 1995).

The deployment of an indigenous broad-host-range plant pathogen as a bioherbicide may conceivably increase the level of disease in non-target plant populations by adding to the natural background of the pathogen's inoculum. One solution to this problem is to use a strain of the bioherbicide pathogen that cannot spread in space or time. Templeton and Heiny (1990) commented .... "Ideal fungal pa-

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thogens (as bioherbicides) are those whose poor capacity to disseminate is their single most important epidemiological constraint". To this end, the auxotrophic mutants developed in *Sclerotinia sclerotiorum*, for which chemical-dependencies may be overcome only in the applied formulation, so restricting the pathogen to one generation at the site of application (Miller *et al.* 1989a; Sands and Miller 1993; Harvey *et al.* 1998), may prove effective in diminishing risks to non-target plants. Alternatively, the non-sclerotial mutants of *S. sclerotiorum* (Miller *et al.* 1989b) and *S. minor* (Abu-Dieyeh and Watson 2006), that cannot produce the ascospores that enable aerial dispersion, may prove effective.

We explore in this review an alternative approach to using mutants of broad-host-range pathogens to limit spread. A comprehensive understanding of the spatial and temporal dynamics of the pathogen can provide guidance as to the acceptability of using it as a bioherbicide and/or the development of strategies to manage the risk to non-target plants. A metric suitable for evaluating this risk is the ratio of the density of inoculum added by the bioherbicide to a non-target host plant's environment, to that occurring naturally. The rationale for using the pathogen's inoculum density is based on the plant pathology principle that inoculum density (number of infective propagules), together with infection potential (environmental conditions), plays a fundamental role in a disease epidemic, whether it is set at the beginning of a growing season (as for monocylic pathogens), or grows with the epidemic (polycylic pathogens) (Lucas 2002). It is appropriate to consider the ratio of 'added to natural' inoculum density since the pathogen chosen as the bioherbicide is, as a consequence of its broad host range, naturally present at endemic levels in populations of a range of weed and non-target host plant species. The ratio provides a measure of the additional non-target plant disease risk, due to the use of the pathogen as a bioherbicide, relative to the natural background risk of the disease in non-target species.

The addition of inoculum to a non-target host plant population by the bioherbicide may occur spatially through atmospheric dispersion of propagules such as spores formed during or after the disease develops in the weed population (de Jong *et al.* 2002b). Addition of inoculum may also occur temporally through the persistence of propagules, such as sclerotia, at the bioherbicide application site (Bourdôt *et al.* 2000).

Values for this 'added to natural' inoculum density ratio equal to or less than 1.0 (when inoculum density in the non-target plant population is, at most, doubled by the bioherbicide) may be considered acceptable. For example, in a decision tree recently developed in Europe for regulatory guidance on the environmental safety of microbial plant protection products, a bioherbicide is judged an 'acceptable risk' if it adds no more inoculum than an amount equal to the background level of exposure to the pathogen, and if the pathogen neither persists at the application site nor presents any unacceptable hazards such as allergenicity and toxicity (Mensink and Scheepmaker 2007).

The effect of doubling the natural background inoculum density in a non-target host plant population on the disease level in this population will, however, depend on the background inoculum density and the shape of the relationship between inoculum density and disease level in the non-target plant population. Thus a doubling of the background inoculum level may, in some cases, result in a significant and unacceptable increase in disease in a non-target plant population. While background inoculum densities have been estimated for some bioherbicides (de Jong *et al.* 1990b; de Jong 1992; Bourdôt *et al.* 2000, 2006), the shape of the disease-inoculum density relationship in non-target plant populations has not been determined for any bioherbicide pathogen (Bourdôt and Saville 2010). These two parameters are likely to vary between host-pathogen systems and their quantification is necessary before a judgement can be made as to the appropriate value of the 'added to natural' inoculum density ratio as a regulatory decision-making parameter (Bourdôt and Saville 2010).

We consider in this paper how the 'added to natural' inoculum density ratio has, to date, been estimated and used to inform the analysis and management of additional disease risks in non-target plant species. We use, as case studies, two bioherbicide systems based on indigenous broad-host-range fungi, which are currently being used in Europe and Canada, and investigated in New Zealand.

APPLICATIONS OF THE 'ADDED TO NATURAL' INOCULUM DENSITY RATIO

The 'added to natural' inoculum density ratio is unlikely to be a constant. It can be expected to vary spatially with proximity of the bioherbicide application site to a non-target host plant population as the inoculum produced at, and dispersed from the application site, decreases with distance. The ratio can also be expected to vary temporally as the inoculum produced by the bioherbicide pathogen declines in density over time. The ratio at any point in space and time is also likely to be affected by land management activities occurring at the bioherbicide application site that impact upon the density of inoculum produced by the bioherbicide and its dispersal from the site. Other environmental conditions that affect inoculum formation and its dispersal in space and time will also affect the ratio. This complexity is alluded to by Mensink and Scheepmaker (2007) in their discussion of the use of 'exposure data' in a decision tree for regulators of bio-pesticides. They state "Quantification of the natural background level of a micro-organism and the additional exposure due to an MPPP (microbial plant protection product).....has not been dealt with. It is complex and requires considerable knowledge of spatial and temporal processes....e.g. soil, leaf surrounding environment..."

To date, the 'added to natural' inoculum density ratio has been applied to the risk analysis of bioherbicides to (1) establish minimum isolation distances (safety zones), (2) to estimate geographic mean values for the ratio and (3) to estimate a withholding period. We summarise and discuss each of these uses below.

Minimum isolation distance

The 'added to natural' inoculum density ratio has been used to define minimum isolation distances (i.e. safety zones) for the broad-host-range fungus *Chondrostereum purpureum* for use in forest weed control in the Netherlands (de Jong *et al.* 1990a) and *Sclerotinia sclerotiorum* for use against weeds in pastures in New Zealand (Bourdôt *et al.* 2006). Similar methodologies were used for both pathogens and we present only the *S. sclerotiorum* example here.

Large tracts of New Zealand are in permanent pasture that is typically not in close proximity to market garden (vegetable) crops susceptible to the fungus. Nevertheless, a risk analysis was considered necessary to determine how close to such crops a bioherbicide based on *S. sclerotiorum* could be deployed without unacceptable added disease risk. The approach taken was firstly to model the density of naturally-occurring *S. sclerotiorum* ascospores in the air above market garden crops (Bourdôt *et al.* 2006). Secondly, the density of 'added' bioherbicide-derived spores around a biocontrol source in each of a sheep and a dairy pasture was modelled. Thirdly, in two-dimensional space around each of the two virtual biocontrol sources (sheep and dairy), the density contour of 'added' spores that equated to the median density in the market garden area (1:1 ratio of added to natural spores), and to one tenth of the median market garden density (1:10 ratio), was located. These spore density contours were the safety zones for their respective ratios of added to natural spores (levels of relative risk) for a typical sheep and dairy pasture.

Two mechanistic, weather-driven models were used (Bourdôt *et al.* 2006) to quantify the emission of spores from a biocontrol site and their transmission from the site (**Fig. 1**). The emission model (SPORESIM-1D (de Jong *et*

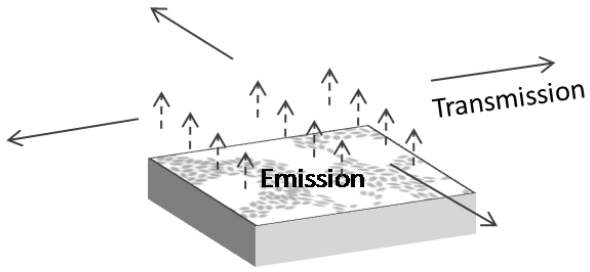

Fig. 1 Schematic of the emission and transmission of the airborne spores from an 'added' or 'natural' source.

al. 2002b)), parameterised using short-term biological data on sporulation, predicted the fraction of the spores released at ground level from apothecia formed by the over-wintered sclerotia (soil-borne resting bodies) of the fungus escaping the vegetation canopy of the 'added' (pasture) or 'natural' (market garden) source, giving an emission rate for a 1 ha *C. arvense*-infested pasture treated with the fungus.

Although the emission of airborne spores from a field site (**Fig. 1**) can be estimated only to within an order of magnitude (Zadoks and Schein 1979), this does not affect the ratio of 'added' to 'natural' immission at various distances beyond a biocontrol source so long as the emissions from the 'added' and 'natural' sources can be assumed to fluctuate in parallel. Thus, the beauty of using the spore density ratio rather than the absolute levels of sporulation, is that this circumvents the need to consider the interacting factors involved in the 'disease triangle' (fungal pathogen, susceptible crop and environmental factors such as temperature, humidity and leaf wetness) (Zadoks and Schein 1979) that lead to the high variability in spore emission estimates.

The transmission model described the atmospheric dispersion of the emitted spores using a Gaussian plume model (PC-STACKS (Erbrink 1995)). The latter provided estimates of the density of the emitted spores in 3-dimensional space (downwind, across-wind and vertical) at immission points around the source. The results for the Broadfield area in Canterbury, one of the six regionally different cases that were modelled (using hourly micro-meteorological data from the ten years 1992-2001) (Bourdôt *et al.* 2006) are given in **Fig. 2**. Firstly, it is evident that the 1:10 spore ratio contour is further away from the pasture source than the 1:1 contour, an expected consequence of the downwind dilution of the spores in the Gaussian plume model.

Secondly, it is evident that the 1:10 and 1:1 contours are closer to the dairy pasture source (**Fig. 2B**) than to the sheep pasture (**Fig. 2A**). This was due to the greater spore trapping ability of the dairy pasture which had a higher leaf area index (LAI), and hence lower spore emission rate in the SPORESIM model. This dramatic effect of pasture LAI on downwind spore density resulted in the 1:1 ratio being within the dairy pasture boundary, and suggests that minimizing the intensity of grazing in treated sheep and dairy pastures during the spore emission period (September – November) would reduce the risk to an adjacent non-target crop and effectively increase the level of safety associated with these 1:1 or 1:10 safety zones for *S. sclerotiorum*.

Thirdly, the spore ratio contours are strongly asymmetrical (**Fig. 2A, 2B**), a phenomenon caused by the asymmetry of the wind during the simulation periods (**Fig. 2C**). As a consequence of this asymmetry, taking the maximum distance to this contour as the safety zone, as would be necessary in practice, would build in a significant margin of additional safety for all other directions from the pasture.

Fourthly, the 90th percentile contours were little further away from the sources than the 10-year mean contours (**Fig. 2A, 2B**), suggesting that safety zones derived as the 10-year mean contours are robust. However, by taking the 90th percentile contour rather than the mean contour (a more risk-averse stance in both space and time), the probability over

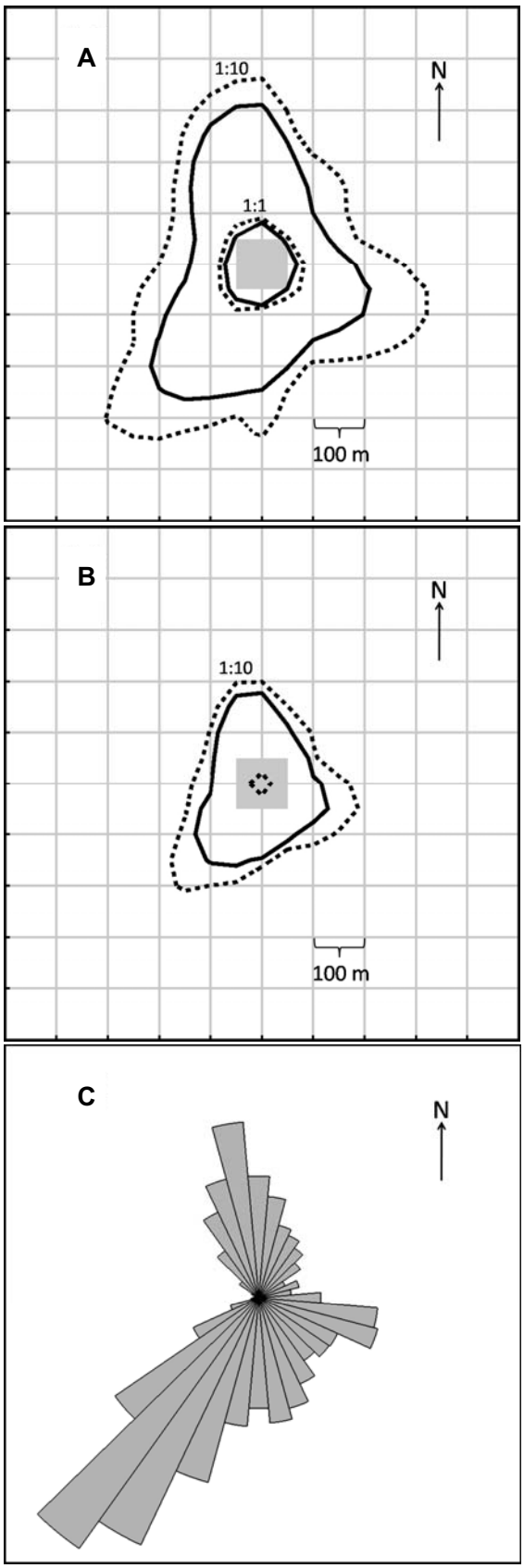

Fig. 2 Simulated safety zones for the application of *Sclerotinia sclerotiorum* as a mycoherbicide to control *Cirsium arvense* in a sheep (A) and dairy (B) pasture in the Broadfield locality of Canterbury, New Zealand. The central grey square represents a 1 ha biocontrol source of spores. The inner and outer pairs of contours represent the 1:1 and 1:10 added to natural (market garden) spore ratio contours respectively. The solid and dotted lines are the 10-year mean and 90th percentile contours respectively. The wind rose (**C**) illustrates the wind run (in the direction the wind is blowing towards) in 10° quadrants, the radius of the sector being the relative wind strength in that direction. Diagrams modified from Bourdôt *et al*. (2006).

time of exceeding the specified added to natural spore density ratio would be reduced to only 10% of years with a relatively small increase (11-41%) in the maximum width of the safety zone.

In this example, numerical quantification and statistical analyses were restricted to the 1:10 spore ratio contour since the 1:1 ratios (long-term mean and 90th percentile) were within the dairy pasture source in all six regions and no further than ca. 50 m beyond the sheep pasture source. This implied that for the 1:1 level of risk, a safety zone for a *S. sclerotiorum*–based bioherbicide is unnecessary for dairy pastures in New Zealand (illustrated for the Broadfield site in **Fig. 2B**); a conclusion also reached earlier using one year of weather data (de Jong *et al.* 2002a). However, a safety zone is necessary for sheep pasture for the 1:1 level of risk, and a universal distance of *ca*. 50 m would appear to suffice as illustrated for the Broadfield site (**Fig. 2A**). If, alternatively, safety zones were to be based on the 1:10 ratio, an order of magnitude lower level of relative risk as compared to the 1:1 ratio, these zones would extend out to distances of 429 and 260 metres from the centre of a 1 ha bioherbicide site for sheep and dairy pastures respectively in the Broadfield area (90th percentile contours in **Fig. 2A, 2B**).

The 1:1 ratio distances are, however, relatively short distances compared with the sizes of most pastoral farms in New Zealand (e.g. 66% of sheep and 60% of dairy farms in 2007 exceeded 100 ha (1 km^2) in area (Anonymous 2007). This means that the 1:1 safety zone would often be contained within the boundary of the farm deploying the bioherbicide, hence resulting in an extremely low additional risk to non-target crops on neighbouring farms.

Wind is a key driving force in the emission and transmission models used by Bourdôt *et al*. (2006) to locate the 1:1 and 1:10 spore density ratio contours and this is evident for the Broadfield site (**Fig. 2A, 2B** cf. **2C**). Mean hourly wind speed explained 56 and 76% of the variance for sheep and dairy pasture respectively in linear regressions of maximum distance to the 1:10 ratio contour on wind speed across the six locations modelled. Bourdôt *et al*. (2006) suggested that these regressions could be interpolated to determine regionally specific safety zones based on mean wind speed. Their extrapolation to wind speeds beyond those considered in the underpinning emission and transmission models relies upon the assumption of continued linearity between maximum distance to the 1:10 ratio and mean wind speed.

Geographic estimates of the added to natural inoculum density ratio

In contrast to using the 'added to natural' inoculum density ratio to define the width of a safety zone that might be widely applicable or regionally specific, the ratio may also be used in a wider geographic context in the analysis of relative risk. To this end, two different approaches have been taken: (1) a spatially explicit model-based analysis of the 'added to natural' inoculum density ratio within a real landscape in which a bioherbicide might be deployed (de Jong *et al.* 1990a, 1990b), and (2) an empirically-based estimate of the overall geographic mean value for the ratio (de Jong *et al.* 1996).

The spatially explicit real landscape approach was taken in the Netherlands at the time the broad-host-range fungus *Chondrostereum purpureum* was proposed as a bioherbicide for controlling black cherry (*Prunus serotina*) in pine plantations. The safety of fruit trees growing nearby was of concern. *C. purpureum*, like *S. Sclerotiorum*, releases spores that are dispersed on wind currents and these can infect fruit trees through pruning wounds, causing the silver-leaf disease (de Jong *et al.* 1990a). Because of these epidemiological similarities, the underlying method for determining the 'added to natural' inoculum density ratio at distances beyond a site at which *C. purpureum* is used was fundamentally the same as that taken for the proposed *S. Sclerotiorum*–based bioherbicide in New Zealand. Models of

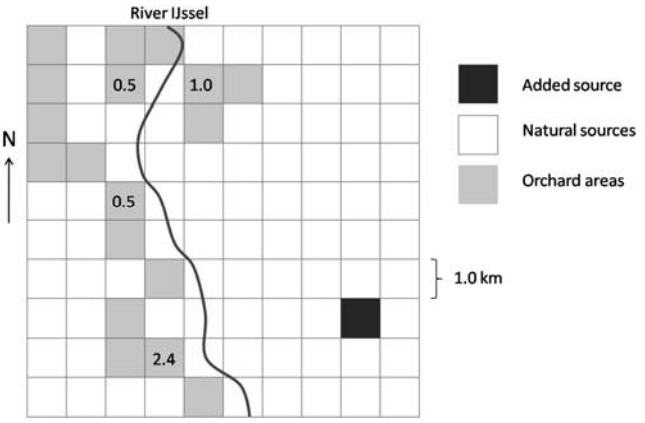

Fig. 3 Map of a 10 × 10 km area near the village of Olst in the Netherlands with the location of natural and added sources of the spores of *Chondrostereum purpureum* and of fruit-growing areas (assumed free of *C. purpureum* basidiocarps due to phytosanitory measures). The numbers in the cells are the long-term average ratios of added to natural spore densities. Diagram modified from de Jong *et al*. (1990a).

emission and transmission of *C. purpureum* spores (using SPORESIM-1D (de Jong *et al.* 2002b) and PC-STACKS (Erbrink 1995) respectively) (**Fig. 1**) were developed to estimate their density at immission points downwind of a source (de Jong *et al.* 1990a; de Jong *et al.* 1990b). As in the New Zealand study, these two models used long-term meteorological data and short-term biological data on sporulation, the latter obtained in experiments over several years (de Jong 1988; de Jong *et al.* 1990b).

However, rather than defining only a safety zone for a typical site treated with the fungus, the two models were also used to simulate the emission and transmission of the spores of the fungus from many contiguous added and natural sources within two different 10 × 10 km areas in rural localities in the Netherlands, each containing orchards with susceptible fruit trees (assumed free of *C. purpureum* due to phytosanitory measures), natural sources (infected deciduous forests, mixed forests, small woods and roadside tree plantings) and added sources (*C. purpureum* applied as a bioherbicide in pine plantations) (de Jong *et al.* 1990b). This enabled the 'added to natural' inoculum density ratio, and hence relative disease risk, to be mapped for each of the modelled areas. An example is shown in **Fig. 3**, where the simulated weed control operations in the pine forest near the village of Olst, applying *C. purpureum* to the cut stumps of *P. serotina*, resulted in an average 'added to natural' inoculum density ratio of about 0.8 (calculated from Table IV in de Jong (1990b) as the ratio of the average over the four modelled orchard areas and over day and night for the *added* spore density to the corresponding average for the *natural* spore density). This overall relative risk in the locality of Olst was in agreement with model results for the other area modelled, in the locality of the village of Oosterbeek (de Jong *et al.* 1990b). These real landscape modelling results predicted that if *C. purpureum* were to be deployed for controlling stump re-growth of *P. serotina* in pine forests in the Netherlands, fruit trees would be at no greater risk from the bioherbicide than they are already from natural sources of the fungus in deciduous forests.

By contrast to the above model-based spatially explicit approach to quantifying the 'added to natural' inoculum density ratio in the Netherlands, a purely empirical approach was taken for assessing the disease risk from a *C. purpureum* bioherbicide on Vancouver Island, Canada (de Jong *et al.* 1996). An empirical approach was necessary here because of the unsuitability of the Gaussian plume model in the mountainous Canadian terrain, which precluded simulations of spore dispersal. Natural fructification on the Island was determined from field measurements of basidiocarp surface area in urban/agriculture areas and in unin-

habited forest-covered wildland areas, providing a mean value of 152 cm^2 basidiocarp surface area (1000 m^2 of land area)$^{-1}$. Estimates of added fructification on the Island, expected as a result of an assumed frequency of hardwood tree control in wildland areas and measurement of fructification on trees treated with *C. purpureum*, were 86, 36, 62 and 5 cm^2 basidiocarp surface area (1000m^2 of land area)$^{-1}$. The respective estimates of the 'added to natural' ratio of basiocarp surface area were 0.57, 0.23, 0.41 and 0.03. It was concluded that since this ratio was consistently < 1.0, a strong case could be made for the registration of this fungus as a bioherbicide for forestry use in Canada (de Jong *et al.* 1996). Furthermore, it was concluded that, because of the pronounced geographical separation between fruit-growing areas and commercial forests in Vancouver Island, the comparison of the added and natural fructification can be considered a conservative estimate of actual risk.

The conclusion that *C. purpureum* could be deployed safely as a bioherbicide for woody weed control in plantation forests is further supported by genetic marker studies. Bioherbicide isolates of *C. purpureum* were found to occur at low frequency compared to natural isolates 50 and 700 m downwind of treatment sites in British Columbia, Canada (Hintz *et al.* 2001), and contributed to less than 15% of infections in the vicinity of treatment sites in the Great Lakes-St. Lawrence forest region, Canada (Gosselin *et al.* 1999a).

Based on the analyses of the relative risk associated with the use of *C. purpureum* in the Netherlands and in Canada, it has been argued that the fungus could also be used for woody weed control in plantation forests in New Zealand (Ramsfield 2006). The fungus occurs on 23 angiosperm hosts in New Zealand and is present in every geographic region in the country resulting in an ubiquitous natural background of inoculum (Ramsfield 2006). Also, considerable genetic diversity and extensive intermixing of isolates occurs throughout New Zealand (Spiers *et al.* 2000), as has been found also in Canada (Ekramoddoullah *et al.* 1993; Ramsfield *et al.* 1996; Shamoun and Wall 1996; Gosselin *et al.* 1999b), precluding any concerns that a locally-sourced bioherbicide strain might introduce rare virulence alleles to natural populations of the pathogen (Hintz *et al.* 2001). The efficacy of this fungus against a wide range of woody weed species is the subject of a research project in New Zealand.

Withholding period

When the pathogen that is intended for use as a bioherbicide produces perennating structures that enable it to survive between years, these will add to any natural risk of the disease occurring in a crop sown in the future at the site where the bioherbicide was applied. The relative magnitude of this added risk can again be quantified as the ratio of the 'added to natural' inoculum density and a withholding period may be defined. This requires knowledge of the density of both the naturally occurring and added perennating structures and also the decay rate of the added structures at the bioherbicide application site.

Such an analysis was conducted for *S. sclerotiorum* intended as a bioherbicide for *C. arvense* in pastures in New Zealand. The perennating bodies of this fungus, sclerotia, are produced at the final stage of disease development and may survive in the soil for several years, initiating disease in future-sown crops (Bourdôt *et al.* 2000). To quantify this disease risk as the 'added to natural' inoculum density ratio, firstly a field survey was conducted to measure the natural background density of the sclerotia in soils under permanent pasture, arable crop/pasture rotations, continuous crops and market gardens in Canterbury. This survey indicated that market garden cropping land in Canterbury supports a natural background density of nine sclerotia m^{-2}. Additional measurements on the control plots of field efficacy experiments with the bioherbicide indicated that pastures containing *C. arvense* in Canterbury have a natural background density of three to four sclerotia m^{-2}.

Secondly, experiments were conducted to measure the density of sclerotia deposited in the soil following application of the bioherbicide to pasture to control *C. arvense*. The number of sclerotia remaining in cohorts of sclerotia artificially buried in two different years at each of four sites, was also measured, and decay rates estimated (Bourdôt *et al.* 2000). These measurements revealed that the density of sclerotia, S_0, deposited after application of *S. sclerotiorum*, varied between 13 and 360 m^{-2}, and that they decayed (in number) exponentially according to $S = S_0 e^{-kt}$, where t is years after deposition and k is the decay rate. The burial study provided eight estimates of the decay rate varying from k = 0.5 to 1.23 with a mean of 0.72, and the estimates from the bioherbicide application study were within this range (Bourdôt *et al.* 2000).

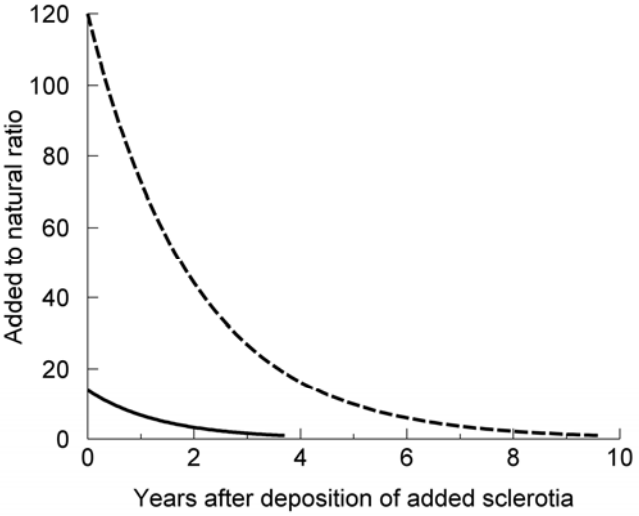

Fig. 4 Decline in the added to natural ratio of soil-borne sclerotia of *Sclerotinia sclerotiorum* in a pasture treated to control *Cirsium arvense*. Two scenarios from Bourdôt *et al.* (2000) are illustrated: an extreme scenario where 360 sclerotia m^{-2} are deposited by the bioherbicide and these decay slowly to a low natural background density of 3 sclerotia m^{-2} (------), and a mean scenario where 125 sclerotia m^{-2} are deposited and these decay at a medium rate to a mean 'market garden' natural density of 9 sclerotia m^{-2} (——). The models for the extreme and mean scenarios are *added*:*natural* = $(360e^{-0.5t})/3$ and *added*:*natural* = $(125e^{-0.72t})/9$ respectively, where t is years after deposition of the added sclerotia. Ratios of 1:1 are reached at 9.6 and 3.7 years respectively.

The withholding period was defined as the point in time, after addition of the sclerotia to the soil by the bioherbicide, when the ratio of the added to naturally occurring sclerotia has declined to 1.0 (Bourdôt *et al.* 2000). This point in time was found, using the exponential decay equation, for all 18 combinations of three deposited densities (13, 125 and 360 m^{-2}), three decay rates (k = 0.5, 0.72 and 1.23) and two natural background densities (9 and 3 sclerotia m^{-2}). This analysis indicated that the withholding period may be less than one year under some circumstances, while under others it may be close to 10 years. However, some of the combinations of the parameter values may be biologically unreasonable. In particular, low decay rates may be unlikely when initial sclerotium densities are high, since high densities may facilitate high rates of hyperparasitism and hence high decay rates.

For a conservative, risk-averse withholding period, a high initial sclerotium density (360 m^{-2}), a low decay rate (k = 0.50) and a low natural sclerotium density (3 m^{-2}) would be appropriate. This combination of parameter values gave a withholding period of 10 years (**Fig. 4**). Alternatively, for a less risk-averse withholding period recommendation, a medium initial sclerotium density (125 m^{-2}), a medium decay rate (k = 0.72) and the natural market garden sclerotium density (9 m^{-2}) could be used. This gives a withholding period of 4 years (**Fig. 4**).

GENERAL DISCUSSION AND CONCLUSION

The case studies we have summarised here illustrate empirical and modelling methodologies for quantifying the additional and natural background inoculum levels of an indigenous plant pathogen proposed as a bioherbicide, enabling the ratio of 'added to natural' inoculum density to be quantified. These methods are relevant to any bioherbicide pathogen that exists endemically in the target weed species, and combined with modern spatial analysis tools such as Geographic Information Systems, could provide very powerful insights into the relative risk of using such pathogens as bioherbicides.

However, as pointed out by de Jong *et al*. (1990a), while these studies can estimate the relative risk to susceptible non-target species, they cannot answer the question "what level of absolute or relative risk is acceptable (to growers of non-target crops)". The notion that a 1:1 ratio is reasonable is based on the idea that a greater increase in the natural inoculum density than a doubling is typically necessary for a measurable increase in plant disease level. This idea that a relatively small change in inoculum density will have little effect on the level of disease is implicit in experiments studying the disease responses of plants to increasing density of spores of pathogenic fungi in which experimental spore densities are typically varied according to a logarithmic series (Makowski 1993; Elmer and Ferrandino 1995; Masangkay *et al.* 1999; Graham *et al.* 2006; Kurt and Tok 2006; Nao 2008).

The 1:1 ratio was considered to be an acceptable level of risk by the Plant Protection Service at Wageningen, the Dutch State Authority charged with making a judgement on the safety of using *C. purpureum* as a bioherbicide against black cherry in forests (de Jong *et al.* 1990b; de Jong *et al.* 1990a). It is also acceptable under the 'risk decision tree' developed by Mensink and Scheepmaker (2007). However, it is impossible to be confident that a doubling of natural background levels of a pathogen's inoculum brings with it little increase in disease risk without knowledge of the shape of the plant disease-inoculum density relationship for the non-target species and pathogen in question. This information is required in addition to knowledge of the natural background density of that inoculum. Only then can the question "what effect does a doubling of the background density of inoculum have on disease incidence or severity?" be answered (Bourdôt and Saville 2010).

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