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Indigenous plant pathogens in evaluations of foreign biological control candidates in the United States of America

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Abstract. Risk assessment of biological control candidates relates to their potential for damage to non-target species in the field following release (application). Most evaluations, conducted in laboratories and greenhouses, rely on host specificity as a primary argument for 'low-risk' conclusions about field introductions. However, it becomes difficult to convey a conclusion of low-risk when there is limited (no damage) non-target infection under controlled test conditions. Closely-related plant pathogens indigenous to the United States of America have been used to clarify elements of risk for at least three candidate fungi, *Puccinia carduorum*, *Puccinia jaceae*, and *Myrothecium verrucaria*. The conclusion that *P. carduorum* from Turkey is of low risk for management of *Carduus thoermeri* in the USA was based, in part, on comparative greenhouse data on specificity of *P. carduorum* from *C. tenuiflorus* in California. Results from current research on *P. jaceae* for biological control of *Centaurea* spp. indicate that it is a very weak pathogen of *Carthamus tinctorius* (safflower), when compared with North American isolates of *P. carthami*, the cause of safflower rust. Comparison of *M. verrucaria* from China with isolates from the USA indicates that the Chinese isolate is very similar to those from USA soils and plants. Thus, comparison of the foreign isolate of *M. verrucaria* with indigenous isolates resulted in selection of the USA strains for further development for biological control of North American weeds.

Introduction

The main objective in biological control of weeds (and of other pests) is to create controlled imbalances known as epiphytotics and epizootics. Efficacy is a prerequisite for a potential biological control agent and safety is the central issue in the decision to develop and utilize the agent.

The safety of a biological control candidate must relate to its potential, or lack thereof, for damage to non-target species in the field following release (application). However, most evaluations of safety are conducted in controlled laboratory and greenhouse studies. Making the leap from greenhouse and laboratory data to predictions of events in the field creates a challenge for scientists evaluating candidates for biological pest control (Watson 1986b; Cullen 1990; Weidemann and TeBeest 1990). Even field evaluations require the interpretation of data and judgements about risk (Cullen 1990). Furthermore, evaluations of candidate agents conducted under optimal conditions often result in symptoms without disease on non-target species. These have been described as "equivocal results" by Cullen (1990) and often have been reported as an expansion of host range (Watson 1986b). Cullen (1990) states that, "The interpretation of equivocal results on plant species closely-related to the target weed has become the most important issue in host-specificity studies, particularly with increased concern over possible effects on native species."

A risk assessment may be used to identify potential hazards (Alexander 1990; DeJong *et al.* 1990; Cairns and Orvos 1992). Exposure of non-target organisms is assumed to be inevitable, especially for introductions of foreign biological control agents, because, given enough time, many introduced agents, although not all, would be expected to move through the ranges of non-target species.

The process of evaluating hazard consists of identification and characterization. Hazard identification, involves tests of the agent on non-target species under optimal environmental conditions. If no hazard is identified from such close scrutiny and rigorous testing, then the assumption is that there will be no risk in nature, even if there is exposure. Hazard characterization includes experiments and other procedures made under conditions that are more realistic for the candidate organism. This is followed, if possible, by studies of the host, the agent and other non-target species in the field.

In this paper, we explore approaches to risk assessment when "equivocal results" have been encountered. First, we describe approaches reported in plant pathology that have been used to resolve questions raised where limited non-target effects have been noted. Second, we describe a process used during evaluations of pathogens at the USDA-ARS-FDWSRU as an additional tool to resolve these issues. The latter process is based on comparisons between foreign pathogens of unknown risk potential with closely related indigenous pathogens that have a known potential for damage in nature. This approach has provided important insights and information for risk assessments. Discussion of these aspects is supported by selected examples from the development of plant pathogens for biological weed control, which has general application in risk assessments of insects and genetically engineered microorganisms.

Risk assessment of plant pathogens for weed control

The ideal biological control agent is one that causes severe damage to the target species only. Among plant pathogens are several fungi that meet this standard based on host-range determinations in greenhouse tests. Strains of *Puccinia chondrillina* are known for their specificity within *Chondrilla juncea* (rush skeletonweed) (Hasan *et al.* 1995). Fungi in the genus *Septoria* also have very restricted host-ranges and many have been proposed for biological control, including *S. passiflorae* Syd. against *Passiflora tripartita* var. *tripartita* (banana poka vine) (Trujillo *et al.* 1994), *S. cirsii* Niessl against *Cirsium arvense* (Canada thistle) (Leth 1986; Hershenhorn *et al.* 1993), and a *Septoria* sp. against *Lantana camara* (bush lantana) (Trujillo and Norman 1995).

Strains of *Puccinia canaliculata* have been identified that are specific to a biotype or variety of *Cyperus esculentus* (yellow nutsedge) and strains from *C. rotundus* do not infect *C. esculentus* (Phatak *et al.* 1983; Bruckart *et al.* 1988; Scheepens and Hoogerbrugge 1991). In the case of *Uromyces rumicis*, a heteroecious rust fungus, host specificity was demonstrated for both the uredinial and the telial stages of the fungus (Inman 1971; Schubiger *et al.* 1986a, b). The smut fungus *Entyloma compositarum*, used successfully for biological control of *Ageratina riparia* (Hamakua pa-makani), has been reported to be very host specific (Morris 1991; Trujillo 1986; Trujillo *et al.* 1988).

For many other fungal pathogens, however, "equivocal results" have led either to their abandonment as biological control candidates or to further research that clarifies both their risk and potential in biological control. Several approaches have been used to develop the needed information for proper risk assessments. The most common approach has been to quantify the relative susceptibility of the target (= the standard) and non-target plant species (Politis *et al.* 1984; Mortensen 1986; Bruckart *et al.* 1986; Bruckart 1989; Morris 1989; Bruckart and Peterson 1991; Morris 1991; Mortensen *et al.* 1991; Baudoin *et al.* 1993; Yang and Jong 1995a, b; Bruckart *et al.* 1996). Relative susceptibility is determined on the basis of disease severity (i.e. disease ratings, leaf area infected, or pustules per unit leaf area) or disease incidence (i.e. the proportion of individual plants infected compared to controls).

Microscopic and histological examinations of infection events have been used to clarify issues arising from host-range testing. The nematode, *Subanguina picridis*, attacked non-target species closely related to *Acroptilon repens* (Russian knapweed), including *Cynara scolymus* (artichoke). Histological study of plant reactions to nematode colonization indicated that only *A. repens* (Russian thistle) supports the nematode through its life cycle; the nematode failed to mature in other plant species (Watson 1986a). Evaluations of the relative susceptibility of *Rubus* spp. (blackberries) to infection by *Phragmidium violaceum* incorporated whole leaf clearing and examination of infection events as part of the determination of a rating for the amount of disease (Bruzzese and Hasan 1986). Histological studies also provided data that were used to gain approval for introduction of a species of *Phaeoramularia* into South Africa for the biological control of *Ageratina adenophora* (Morris 1989).

Another approach involves measuring relative plant damage, such as effects on plant biomass, height, or yield. Isolates of *Puccinea jaceae* from four species of *Centaurea* were compared for effects on biomass reduction of the source *Centaurea* species. A significant reduction in biomass occurred only on *C. solstitialis* (yellow starthistle) inoculated with yellow starthistle strains of *P. jaceae* and not on the other host-*P. jaceae* combinations. None of the isolates caused reductions in the biomass of cornflower, *C. cyanus* (Shishkoff and Bruckart 1993). The effects of *P. jaceae* on biomass of these *Centaurea* species seemed related to production, maintenance, and loss of foliage in rosettes; yellow starthistle was the only species to produce one set of leaves which, when infected, were not replaced with new foliage. From this we concluded that, although *C. cyanus* can be infected by several strains of *P. jaceae,* it is not damaged by these infections even under optimal conditions for inoculation.

Biochemical and molecular comparisons between foreign and indigenous organisms have been used to clarify taxonomy and to characterize candidate biological control agents for rapid identification. Protein patterns for *P. jaceae* from *Centaurea diffusa* (diffuse knapweed) later served to clarify differences between evaluated isolates and those that were discovered in nature (and therefore indigenous?) in British Columbia (Kim and Mortenson 1986; Mortensen *et al.* 1989). Isolates of foreign and indigenous *Puccinia* species have been characterized both by isozyme banding patterns (Bruckart and Peterson 1991) and by amplification of ITS region DNA (Berthier *et al.* 1995).

Epidemiology was used in one instance to clarify issues concerning risk. *Chodrostereum purpureum* is a pathogen of weedy, ornamental, and commercial *Prunus* species in the Netherlands. Recently, it was considered for use in biological control of weedy *Prunus serotina* (DeJong *et al.* 1990). At issue was the potential for a significant disease increase in beneficial *Prunus* species from the artificial inoculation of weedy trees. An epidemiological model based on spore counts in the field was related to locations of target and non-target *Prunus* species. Increases in aerial spore concentrations from artificially inoculated trees remained within the same order of magnitude as natural levels of inoculum. This was considered an insignificant elevation in inoculum and a proposal was made to use *C. purpureum* against *P. serotina* in the Netherlands.

In Australia, the introduction of *Uromyces heliotropii* for biological control of *Heliotropium europaeum* was proposed and permitted despite the fact that a native species, *H. crispatum* was found to be susceptible. Even though a hazard was identified, the authors argued that damage would be a very remote possibility since the native *H. crispatum* sustained minor infections in controlled tests and was geographically, climatologically, and phenologically separated from the target species (Delfosse *et al.* 1989).

In plant pathology, there also is an option to manipulate certain biological control agents and use them as bioherbicides. Usually, these are evaluated in the same way as foreign candidates, with host specificity being desired in order to eliminate issues associated with hazard. However, a different tactic with these pathogens promotes the use of known broad-spectrum pathogens for weed control. The use of these broad-spectrum pathogens is justified by developing ways to control their activity or spread in the field (i.e. to minimize or eliminate exposure). At least two broad-spectrum bioherbicidal products have been proposed using this approach (Sands *et al.* 1990; Yang and Jong 1995a, b).

Use of related indigenous fungi for comparisons in risk assessments

One advantage of a containment greenhouse and laboratory facility is the option for side-by-side comparative studies of organisms from many different parts of the world. This approach has been used in risk assessments of both *P. carduorum* and *P. jaceae* in the determination of the relative merit of a foreign isolate of *Myrothecium verrucaria* compared with isolates from the United States of America, for biological control.

Puccinia carduorum *on* Carduus thoermeri *(musk thistle)*

A few, small pustules developed on *Cynara scolymus* (artichoke) and a few *Cirsium* species became infected with a Turkish strain of *P. carduorum* from musk thistle (Politis *et al.* 1984). Infections were minor, individual plants were not damaged, and the pathogen could not be maintained on these species under optimal greenhouse conditions, but the question remained concerning the risk under field conditions.

A strain of *P. carduorum* is known to infect *Carduus tenuiflorus* (slenderflower thistle) in California (Watson and Brunetti 1984). However, there are some facts about *P. carduorum* and the slenderflower thistle relatives in California that help to assess the potential field performance of a related strain of the pathogen. First, infected *C. tenuiflorus* is sympatric with both artichoke and the closely-related *Carduus pycnocephalus* (Italian thistle), and yet these plant species do not have rust diseases in California and no rust disease has been observed on artichoke anywhere in the world. Also occurring in California is safflower rust, caused by *P. carthami*, a morphologically similar fungus to *P. carduorum* (Savile 1970). Although safflower was not susceptible to the musk thistle strain of *P. carduorum*, isolates of both species from California, *P. carduorum* from slenderflower thistle and *P. carthami* from safflower, were used in side-by-side comparisons with the musk thistle strain of *P. carduorum*.

In host range determinations, isolates of *P. carduorum* were clearly most aggressive on their source species of *Carduus* (Politis *et al.* 1984). This was verified for the *C. tenuiflorus* isolate (Bruckart and Peterson 1991). All isolates of *P. carduorum*, including the isolate from *C. tenuiflorus* in California, also caused a few, very small pustules on artichoke, but much less than on musk thistle or the other *Carduus* species. The lack of infection by the *C. tenuiflorus* isolate on other *Carduus* species, along with the few pustules on artichoke that were similar to those from other strains of *P. carduorum*, strengthened conclusions that the musk thistle strain has a very strong preference for *C. thoermeri* and that it is very unlikely to damage, if it infects, artichoke in the field. These conclusions were later supported in a field study in Virginia (Baudoin *et al.* 1993).

On the basis of isozyme analysis, *P. carduorum* isolates from three *Carduus* species were found to be nearly identical (Coefficient of Similarity 90%) and therefore considered to be a single species, distinctly different from *P. carthami* (Bruckart and Peterson 1991). It was learned that with the right set of enzymes, isolates could be distinguished by source *Carduus* species (Bruckart and Peterson 1991). Characterizations based on PCR amplification of ITS region DNA and restriction enzyme digests indicated that *P. carduorum* could be separated by source *Carduus* species and isolates from four species of *Carduus* fell into one of two distinct groups (Berthier *et al.* 1995). Amplifications of other parts of the genome would probably improve the separation of isolates and satisfy the hypothesis that isolates from individual species of *Carduus* could be distinguished. In every case, isolates of *P. carduorum* were much different from those of *P. carthami* and *P. chondrillina*.

Puccinia jaceae *on yellow starthistle,* Centaurea solstitialis

Three species of *Puccinia* were compared with *P. jaceae*. Initial host-range determinations indicated that *C. tinctorius* (safflower) and *C. cyanus* (cornflower) were susceptible to infection by *P. jaceae* from yellow starthistle and other species of *Centaurea* (Mortensen 1986; Bruckart 1989; Mortensen *et al.* 1991). In the USA, safflower is attacked by *P. carthami* and cornflower is attacked by *P. cyani*. Morphologically, these two rust fungi are distinctly different from *P. jaceae* (Savile 1970), which is expected to be a valuable attribute for tracking the pathogen in the field. If non-target infection of related introduced weeds becomes an issue, other isolates of *P. jaceae* on weedy *Centaurea* spp. may be identified on the basis of host plant infection.

Infection of safflower by *P. jaceae* and *P. carthami* was quantified. Disease severity, based on pustule counts, was determined for ten safflower cultivars inoculated with *P. jaceae* and *P. carthami*. The results indicated that most commercial safflower cultivars have some level of resistance to *P. carthami* when compared with a very susceptible cultivar, 'Pacific 1' (Bruckart 1989). The reaction of 'Pacific 1' inoculated with *P. jaceae* resulted in 86% fewer pustules than 'Pacific 1' inoculated with *P. carthami*. The remaining cultivars had proportionately fewer pustules than 'Pacific 1', averaging 4% of the number of pustules from *P. jaceae* compared with the *P. carthami* controls. Also, pustules of *P. jaceae* on safflower were much smaller than those of *P. carthami*. Finally, *P. jaceae* could not be maintained under optimal greenhouse conditions on any of the cultivars except, with difficulty, on 'Pacific 1'. From this, it was concluded that safflower is not a good host for *P. jaceae*, even under optimal greenhouse conditions. This is particularly clear when considering the amount of disease caused by the indigenous safflower pathogen, *P. carthami*, under the same conditions.

In another study, strains of *P. jaceae* from four species of *Centaurea* were compared for the damage each caused on their respective host species. Damage was based on the effects of inoculation on plant dry-biomass compared with uninoculated controls. For comparison, rush skeletonweed was inoculated with *P. chondrillina*, a pathogen of known benefit in the control of skeletonweed both in California (Supkoff *et* *al.* 1988) and in Australia (Cullen 1986). Significant reductions in plant biomass occurred from a single inoculation of yellow starthistle and of skeletonweed by their respective pathogens; the other species of *Centaurea* were not affected (Shishkoff and Bruckart 1993). The amounts of damage seemed to vary according to the production and replacement of leaves by each *Centaurea* species. Yellow starthistle produced one set of leaves in the rosette, without additional new growth until bolting. Leaves of *C. diffusa* and *C. maculosa* (knapweeds) did not live as long as those on yellow starthistle, and eventually they were replaced by new leaves. Leaves of *C. calcitrapa* (purple starthistle), were relatively short-lived compared with the other *Centaurea* species, and they were the most rapidly replaced of the species tested (Shishkoff and Bruckart 1993).

One issue remains in tests of *P. jaceae* from yellow starthistle. All the tests to date have concerned susceptibility of safflower foliage to the urediniospores of *P. jaceae*. However, losses in safflower production are greatest from seedling infections resulting from teliospore contamination of seed. Side-by-side comparisons between *P. jaceae* and *P. carthami* are planned to resolve this question. The *P. carthami* teliospore model has been used to develop a standard greenhouse protocol for manipulation of small, 1 µl quantities of teliospores (Bruckart and Koogle 1995) and to establish a protocol for quantitative inoculation of safflower with *P. carthami* teliospores. These techniques have been applied in processing a shipment of *P. jaceae* teliospores from yellow starthistle collected in Iran. We know from this that the teliospores of *P. jaceae* from Iran were viable, even following surface sterilization with a 1% bleach solution (= 0.05% NaOCl).

Myrothecium verrucaria *on leafy spurge and other broadleaf plants*

An isolate of *M. verrucaria* from leafy spurge collected in China was found to be pathogenic only under the extreme dew conditions of 30°C for 18 h (Yang and Jong 1995b). Damage to leafy spurge and other plants, similarly affected under the dew conditions described, was increased to high levels by applying conidia in an invert emulsion in the absence of dew (Yang and Jong 1995b). Since this effect occurred on broadleaf plants, the concept of using *M. verrucaria* applied in a special invert emulsion carrier as a bioherbicide has been pursued. Eight additional isolates of *M. verrucaria*, including at least one non-pathogen from the USA (S.M. Yang unpublished), were tested on 14 plant species with or without dew following inoculation (Yang and Jong 1995a). The results were similar to those in the first tests, and the USA isolates were chosen for further development, because of potential complications in the regulatory process that can be expected for a foreign pathogen.

Conclusions

The greatest activity in risk assessment involves determination of host range and characterization of non-target effects. Very few, if any, analyses of indirect effects on community function or structure are conducted. The use of modelling or the establishment of expert systems as part of a biological impact assessment (Teng and Yang 1993) may be useful in the future, but these approaches require much detailed data acquired over several years. In certain instances, such data may be available for modelling agricultural pests but it is not readily available for evaluations of candidate biological control agents.

Most risk assessments are based on data originating from artificial tests. These data require interpretation, and conclusions about risk are based on judgments of what the data indicate. All interpretations and judgments are relative; they are compared to a standard. Usually, the standard is the effect of a candidate agent on the target species. Other standards have been used, including the background concentration of *Chondrostereum purpureum* spores from natural infections in the case of *C. purpureum* (DeJong *et al.* 1990).

In the present paper, another standard is described in the process of risk assessments. It involves side-by-side comparisons of a candidate biological control agent and a related organism whose biology is at least partially understood. From this, useful experiments about the potential risk under field conditions can be conducted in controlled laboratory and greenhouse tests. In common with other tools in the risk assessment process, data from these studies need interpretation and judgments about risk still need to be made.

Risk assessments are made on a case-by-case basis, and they require all the information that can be garnered. Different tools must be utilized in each risk assessment, including, host-range tests that favour the candidate agent, and characterization of non-target reactions under more realistic conditions in greenhouse and field evaluations. Cullen (1990) describes the possible need for a certain amount of experimental improvisation and the reliance on careful interpretation of results.

Final outcomes from risk assessments are uncertain. The perception of risk for biological control agents, particularly if they are foreign, is one of low probability and high consequence (Hammitt 1989), a definition used originally to describe risk associated with genetically-engineered organisms. Interpretations and judgments about results from experimental research serve to support regulatory decisions and alleviate public concerns. Cairns and Orvos (1992) note that, "one cannot adequately assess risk unless end points are defined and agreed upon as being pertinent". DeJong *et al.* (1990) also point out that policy decisions remain subjective, regardless of the data. It is certain, however, that the best decisions are based on the best information, and the best information inspires confidence within scientific, regulatory, and public sector communities. We have excellent tools and philosophical perspectives needed to solve these problems, but better communication with regulators and the public is needed.

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