

Chapter 58. TRANSGENIC MYCOHERBICIDES – NEEDS AND SAFETY CONSIDERATIONS

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running title: TRANSGENIC MYCOHERBICIDES

1. INTRODUCTION

The biocontrol of weeds was discussed by Boyetchko (previous chapter), at great length in a comprehensive book (TeBeest 1991), and in excellent recent reviews by Auld and Morin (1995) and Weston (1999). This chapter is an updated and highly condensed version of a chapter entitled “Molecular biology in weed biocontrol” (Gressel 2002), by permission of the publisher. Hypervirulence for the purpose of this chapter is defined as obtaining a higher level of virulence than found in the most virulent native strain of a mycoherbicide.

We must face up to the fact that almost all proposed biocontrol agents specific for major weeds in arable row crop agriculture have not lived up to expectations. Most of the successes described in the previous chapter have been in the control of alien weeds by introducing the pathogen that kept it in balance in its center of origin (“classical biocontrol”). In contrast, most of the important weeds of major row crops have become globally distributed, quite evolved from their cohorts at the centers of origin, and cannot be controlled by the classical methods. Pathogens have been isolated that prey on many of these major global weeds and can provide a modicum of control when huge levels of inoculum are applied. Usually $>10^4$ spores per cm^2 are thus “inundatively” applied, which is more than four orders of magnitude than required for 100% efficiency of the initial application. Thus, most proposed organisms have not been sufficiently cost-effective, despite efforts in efficient production of inocula and formulating the inocula (Boyette et al. 1991; Quimby et al. 1999) and in designing application technologies. Biological control should seem to be an easier technology to perfect than herbicides; herbicides are used at relatively high efficiency. Few herbicides could be made to work at 10 times lower rate let alone a ten thousand fold lower rate. The difference in orders of magnitude between practice and theory should be the target for improvement of weed biocontrol agents.

A host-specific hypervirulent pathogen that controls a major row-crop weed to the extent that farmers require, (i.e. similar to control achieved with a chemical herbicide), would have gone extinct soon after evolution, as would the target weed; dead weeds and dead mycoherbicides cannot reproduce. Considering the lack of success attained with potential inundative mycoherbicides, despite years of research (albeit at low budget), it has been suggested that a solution could come from genetically engineering hypervirulence genes into weed-specific pathogens, where the pathogen is kept from becoming extinct by preservation of inoculum in the laboratory (Gressel 2002; Kistler 1991).

1.1. Ecological needs/justification for biotechnologically upgrading mycoherbicides

It is only by conferring some type of hypervirulence that biocontrol agents will have the ability to augment or compete with herbicides in row cropping situations. The ecological balance achieved with classical agents in an extensive pasture or forestry situation cannot suffice in intensive agriculture. Basically, a homeostasis is achieved. Despite fluctuations (Arditi and Berryman 1991), (which in themselves would be unacceptable to the intensive farmer), the homeostasis cannot be broken with the

classical agents that have co-evolved for such a long period with their hosts (Dennill and Hokkanen 1990; Frank 2000). Indeed, it is the long-term co-evolutionary war between plants and their natural enemies that is thought to be the source of evolutionary diversity among plants (Rausher 2001).

Engineering genes for hypervirulence into a classical agent may tip the balance away from the homeostasis, but such an agent may “put itself out of a job”, and re-infestation will be needed when the weeds make a come-back. Thus, the relationship will no longer be classical, with a one-time infection, but closer to inundative.

2. NEEDS FOR ENGINEERING TO GREATER VIRULENCE FOR CONTROLLING WEEDS

Laboratory control of early stages of weed growth may not translate into an effective mycoherbicide. Weed populations in the laboratory are typically synchronous. Weed populations in the field are typically asynchronous with germination in a number of flushes. The farmer wants herbicides (and thus a mycoherbicides) to control a number of flushes, preferably until the crop canopy closes, precluding competition.

The use mycoherbicides is an orphan science, with little study of weed-pathogen interactions. Conversely, much research focused on understanding the molecular biology of infection of crops (Dangl and Jones 2001), and how to overcome these infections, while we need to know how to successfully cause epidemics. It is very important to understand the natural infection process, and there is much to learn from it. Phytopathologists have been studying the defenses in so-called compatible responses, where the fungus typically infects the crop. Those engaged in biocontrol of weeds have begun to learn that the terms compatible and incompatible are misnomers. Infection is not qualitative, it is quantitative with thresholds and then dose dependency. The molecular defense responses in compatible systems have been a barrier to greater success of biocontrol agents. As each barrier is removed, virulence is enhanced and less inoculum is needed. Thus, the more barriers removed, the greater the enhancement of virulence, often in a synergistic manner.

By default, we may learn much from molecular studies of fungal virulence to crop plants and animals. Many upregulations of enzymes are being recorded during pathogen attack. As the genes are known, the expression of the genes can be transgenically suppressed, and the suppression correlated with a lack of virulence. In this manner it has been shown that the glyoxylate cycle and phospholipase are each required for *Candida* and *Mycobacterium tuberculosis* virulence in mammals (Ghannoum 2000; Lorenz and Fink 2001). Thus, the utility of molecular tools such as gene disruption for finding virulence genes has been demonstrated and genes are being pinpointed (Kwon 1998). For use in enhancing virulence of mycoherbicides it is necessary to demonstrate that: (a) overexpression will enhance virulence. This is not a foregone conclusion. For example, the lack of cAMP has been correlated with a loss of virulence (Kronstad 1997) but it is doubted that overexpression of this signal transducer will enhance virulence; it is more like an on/off switch; (b) that the genes will work in heterologous systems; and eventually (c) that weeds will not quickly evolve mechanisms to overcome the enhanced virulence. The latter is not a moot point when we realize that strain resistance to pathogens is often correlated with resistance to virulence enhancing toxins, e.g. Pedras and Biesenthal (2000), which is analogous to well documented evolution of herbicide resistance (Gressel 2002).

2.1. Weed defenses against biocontrol agents – problems to overcome

The weeds attacked by mycoherbicides initiate various defenses to suppress the spread of invading pathogens including: (a) PR (pathogenicity-related) proteins (chitinases and glucanases as well as other stress-related proteins) can be induced by pathogen attack. The chitinases and glucanases degrade fungal cell walls, preventing mycoherbicide penetration and growth in the tissue; (b) constitutive, or pathogen induced-phytoalexins, non-protein, secondary metabolites of various

chemistries that poison the fungus; (c) physical barriers composed of polymers such as suberin, lignin, callose, or their mixtures are induced. Often papillae of callose rapidly form, blocking the advancing fungal hyphae. Such papillae can contain small amounts of lignin. Traces of lignification can severely suppress degradation of carbohydrate polymers such as callose and cellulose by fungal enzymes (Gressel et al. 1983).

Different species initiate different defenses, and the levels of induced and pre-existing defenses can vary during weed growth and development. The outcome of this is that different quantities of inocula are needed at different stages of growth, a major complication to using biological control agents. Similar age dependencies often exist with some chemical herbicides, and herbicide chemists have strived to develop chemicals with less age dependency, a goal that must be paralleled with mycoherbicides.

There can be a strong genetic load to a pathogen from having a gene or genes introduced. Such a load might reduce growth rate (on rich media, or heterotrophically in the environment) but should be an advantage when attacking a weed. This genetic load could be deemed as positive, not just because it kills the weed. It is positive because from an ecological point of view (as well as the commercial point of view, which is immaterial to this discussion) it is better to have the transgenic organism dissipate from the environment when the weed is not present. This sounds like planned obsolescence, but it is really planned biosafety.

3. LESSONS FROM THE TRANSGENIC BIOCONTROL OF INSECTS AND DISEASES

The paucity of reports on transgenic biocontrol of weeds suggests that it may be worthwhile to take a cue from biocontrol of insect pests and diseases. A wide variety of transgenic viruses, bacteria, and fungi have been successful in controlling insects and diseases (Vurro et al. 2001a). Microorganisms have been doing this to each other throughout evolutionary history using e.g. streptomycin (from Actinomycetes), penicillin (from mycelial fungi), and killer toxins (yeast). Many of the genes controlling their production are inherited on plasmids that are passed around among organisms.

Organisms can be engineered to higher levels of virulence without recombinant technologies; a strain of *Trichoderma* has been developed that effectively controls various plant pathogens; it is a heterokaryon fusion product between two other strains (Harman and Donzelli 2001). Similarly, mutant mycoherbicidal fungi that overproduce and secrete valine have been selected using amino acid analog antimetabolites such as norvaline or penicillamine. The resistant strains are elicited the same symptomatology as herbicides inhibiting acetolactate synthase (Sands and Pilgeram 2001).

The ideal mycoherbicide envisaged will contain a group of transgenes that will synergistically enhance; (a) mycoparasitism (cutinolytic and cellulolytic genes); (b) antibiosis (yeast killer toxin genes); (c) enhanced competition for nutrients or space (stronger siderophores); and (d) inactivation of the plant pathogens defensive enzymes (proteases). Some of these genes have already been engineered into biocontrol agents of pathogens (e.g. genes encoding chitinases) (Harman and Donzelli 2001), and into fungi that parasitize insect pests (St. Leger and Screen 2001).

Two approaches have been taken to improve the action of viruses on insects. Different nucleocapsid nucleopolyhedroviruses were tested for control of *Helicoverpa* and *Spodoptera* in cotton, but were too slow acting. Their efficacy was improved by using recombinant virus with the ecdysteroid UDP-glucosyltransferase (*egt*) gene deleted (Flipsen et al. 1995). The speed of killing was synergistically further improved by inserting the insect-specific toxin gene *AaIT* in the *egt* locus (Chen et al. 2000).

The lessons to be learnt from the transgenic biocontrol of insects and fungi are the same as being learnt with chemical herbicides: (a) Formulation or engineering for stability is important. BT toxin in its native *Bacillus thuringiensis* is UV unstable. Engineering the toxin gene into a melanized, UV impermeable spore forming organism such as *Pseudomonas*, stabilized it. Conversely, perhaps the Bt

itself could have been engineered to be melanized; (b) Transgene products that initiate cascades are preferable. Engineering a cuticle degrading protease into a hemolymph-attacking fungus activates a pro-phenoloxidase system (St. Leger et al. 1996); (c) Containment is important. The pro-phenoloxidase system described in (b) removes the substrate needed for fungal sporulation, limiting spread (St. Leger et al. 1996); (d) Selectivity is important. Host specificity should not be altered by transgenically enhancing virulence. Thus, potency is elevated while selectivity is retained; and (e) Synergies are important for decreasing inputs as discussed above. Nature typically uses multiple mechanisms both to synergistically protect organisms or to synergistically prey on other organisms. Plants transformed to produce both Bt and chitinase (Regev et al. 1996) or with pea trypsin inhibitor (Fan et al. 1999) kill later larval instars with less transgenic protein than can be achieved by high levels of BT alone.

Resistance to pathogens has been engineered into crops by inserting the chitinase that appears in the *Trichoderma* strain used for biocontrol (Lorito et al. 1998; Lorito et al. 2001). Three way synergies have been shown in anti-pathogenic mycofungicides. cDNAs encoding three barley proteins; a class-II chitinase, a class-II beta-1,3-glucanase and a Type-I ribosome-inactivating protein were expressed in tobacco plants. There was enhanced synergistic protection against *Rhizoctonia solani* infection compared to isogenic lines expressing a single barley transgene to a similar level (Jach et al. 1995).

4. BIOTECHNOLOGICALLY UPGRADING BIOCONTROL AGENTS

Organisms can potentially be modified to increase pathogenicity by transformation with genes for virulence from other species, by increasing the endogenous expression of genes, or by transfer from other organisms by protoplast fusion (Gressel 2002; Harman and Stasz 1991; Harman and Donzelli 2001; Kistler 1991). Increasing virulence, especially by gene transfer, requires extreme care due to environmental impact, i.e. the possibility of increasing the host range to include crops. Thus engineering “failsafe devices” into the pathogens (Section 7) may be a necessity.

4.1. The possible needs to upgrade the crops.

One consideration for enhanced biocontrol is to utilize non-specific, general mycoherbicides. For example the use of an asporogenic mutant, amino acid auxotrophic *Sclerotinia* was proposed as a general mycoherbicide, that could not spread or reproduce in the field beyond the target (Sands and Miller 1993). It was never developed beyond the experimental stage, probably languishing because it was not virulent enough. Enhancing its virulence might turn it into the glyphosate of a decade ago; an organism capable of total weed control – but with a limited market. The markets of glyphosate and other herbicides was enhanced manifold by engineering crops to be resistant to the herbicides (Gressel 2002). A corollary could be considered for mycoherbicides, transgenically generate a crop specifically resistant to a broad spectrum mycoherbicide. *Sclerotinia* is a heavy excreter of oxalate, which is an important (Zhou and Boland 1999), but not sole determinant of pathogenicity (Callahan and Rowe 1991). A gene for overexpression of oxalate decarboxylase has been engineered into crops, conferring resistance to *Sclerotinia* (Kesarwani et al. 2000). Plants engineered to overproduce oxalate oxidase were also resistant to fungal attack (Zaghmout et al. 1997). Another case to consider: the broad spectrum mycotoxin deoxynivalenol targets L3 protein in 60S ribosomes. Plants transformed with a modified RPI3 gene were resistant to the mycotoxin (Harris and Gleddie 2001).

5. GENES THAT MAY ENHANCE WEED BIOCONTROL

Biocontrol agents could also be engineered to convey genes that convert pro-herbicides to herbicides. This would then allow the mycoherbicide to be applied together with the pro-herbicides. Such a gain of function mutation has been engineered into the tobacco etch virus (TEV), encoding a P450_{sul} that activates a sulfonylurea pro-herbicide (Whitham et al. 1999). The same gene could also be

put into any other biocontrol agent. Systemic and complete infection may not be needed, if a systemic herbicide is released by the activated gene product. This would have distinct advantages with weeds with underground propagules such as *Cyperus* that regrow after foliar killing. As the biocontrol agent is specific to the weed, it would not cause the conversion of pro-herbicide to herbicide in crops.

Many genes are becoming available that might be appropriate for enhancing the activities of mycoherbicides. Some single genes that may be appropriate are summarized in Table 1. At one time it was thought that it would be problematic to transform organisms to produce complicated molecules such as non-protein toxins because of the large number of genes involved in the pathways to produce such secondary metabolites. Eukaryotic organisms do not have polycistronic gene sequences as in bacteria. Thus, the concept of complex molecule biosynthesis seemed daunting because of the need to hunt genes scattered over the genome. Indeed genes for primary metabolism are scattered over the genome in fungi unlike their polycistronic nature in bacteria. This seems not the case for toxin-biosynthesis genes; they are clustered (Kimura et al. 2001; Walton 2000). One of the concepts supporting the possibility of horizontal gene transfer in fungi is based on the same clustered synteny of secondary metabolite biosynthesis in unrelated organisms (Kimura et al. 2001; Walton 2000). Thus, with luck one may be able to isolate and transform such clustered complex pathways into biocontrol agents.

Genes encoding characterized biochemical functions will be summarized below, but there are also known virulence genes isolated that have no known function. For example, double stranded (ds) RNA similar to and related to cryptic viruses has been correlated with fungal virulence in *Nectria*. Virulence is lost in “cured” strains from which the dsRNA disappeared and was restored through heterokaryon anastomosis (Ahn and Lee 2001). If/how such elements can be moved among organisms, and whether they will induce hypervirulence is yet an open question.

Hypervirulence genes that can possibly be used “off the shelf” to try to enhance biocontrol agents may be classed as “soft”, i.e. those genes whose products dissolve the weed host or detoxify its defense mechanisms, or that produce compounds which already occur human diet, and appear on the official “GRAS” (generally regarded as safe) lists, and are lightly regulated. There is a spectrum of possibilities from these to very “hard” genes producing strong toxins. The regulatory agencies should look with care more than once at organisms with “hard” genes, but with an open mind. Many of these genes may produce compounds that are toxic to mammals, but only at particular life stages during weed growth, so there would be little danger. If a biocontrol agent containing such a gene does not enter the food chain and it is no less dangerous to the applicator than conventional herbicides, there is good reason to attempt the regulatory hurdles.

Not all agents are at the soft and hard extremes; lytic enzymes that eat holes in the weed may be very similar or identical to the same GRAS enzymes used in processing of food and beverages. Also genes producing already commercial and safe herbicides might be in the middle.

5.1. Soft genes

5.1.1. Transgenically overcoming host defenses

Much is known about pathogen genes encoding enzymes that degrade crop phytoalexins or constitutive fungal toxins, enhancing virulence. For example a *Fusarium* tomatinase degrades α -tomatine of tomato (Roldan et al. 1999), and *pdal* gene of *Nectria* encodes a cytochrome P450 that degrades pisatin in peas (Ruan and Straney 1996). Overexpression of such genes could enhance virulence of mycoherbicides. Only rarely have the phytoalexins of weeds been determined (Sharon et al. 1992b) allowing knowledgeable choice of chemicals to suppress of phytoalexin production (Sharon et al. 1992a).

Often such phytoalexin-degrading genes are turned on by the phytoalexins themselves, and the isolated promoters are specific to the phytoalexins (Khan and Straney 1999). Sands and Pilgeram (2001)

has suggested that such promoters be used to empower other hypervirulence genes, ensuring host specificity of the hypervirulent biocontrol agent (unless a related crop makes the same phytoalexin).

5.1.3. Transgenically causing hormone imbalance in weeds

At present there is scant evidence to know what genes will work, but there is circumstantial evidence to say that some should work. For example, it was presumed that organisms overproducing the auxin IAA should enhance virulence. This is based on the findings that: (a) Exogenously-applied IAA can lead to cellulase-catalyzed cleavage of hemicellulose, resulting in wall loosening (Taguchi et al. 1999) and membrane leakage, thereby stimulating the loss of water and nutrients (Brandl and Lindow 1998); (b) Synthetic auxins such as the herbicide 2,4-D bind auxin receptors; (c) Many microorganisms associated with plants as symbionts or parasites directly alter the auxin content of the host; the auxin imbalance benefits the microorganisms (Gaudin et al. 1994). Fungal-infected tissue can contain ten times more auxin than the level found in healthy apices (Gruen 1959); (d) Pathogen strain virulence directly correlates with the levels of IAA, suggesting that IAA has a positive function in the infection process. Plants pre-infected with a pathogen caused auxin production, which then overcame the hypersensitive defense response of plants subsequently infected by an incompatible pathogen (Robinette and Matthyse 1990). IAA deficient mutant strains are not pathogenic, but virulence was restored when these mutants were transformed with genes for IAA synthesis (Comai and Kosuge 1982); and (e) Chitinase produced by plant tissues as a defense against pathogens was blocked by auxin, enhancing the virulence of the pathogens (Shinshi et al. 1987).

Thus it is worth testing the effects of overproduction of any and all plant hormones in mycoherbicidal organisms based on the assumption that plants have evolved to have near the optimal levels of hormones. Any vast hormone oversupply should at least cause imbalances that will facilitate pathogen establishment, growth, and development.

In two cases devoted to biocontrol of a weed, there was a modicum of success. *Fusarium* spp. transformed with two genes of the indole-3-acetamide pathway leading from tryptophan to IAA produced significantly more IAA than the wild type and were more effective in suppressing the number and size of *Orobancha* shoots than the wild type (Cohen et al. 2002). The same genes greatly enhanced the activity of a *Colletotrichum coccodes* attacking *Abutilon*, but only when augmented with tryptophan (Amsellem and Gressel, unpublished results, 2002). This demonstrates that having an enzyme may not be sufficient, if substrate for the enzymes is lacking.

Similarly, when the gene *ipt* or other genes inducing cytokinin biosynthesis were introduced into plants causing an overproduction, the plants were dwarfed with small leaves (Hlinkova et al. 1998). Hormone levels were modified by inserting rice homeobox OSH1 into tobacco, again severely disrupting growth processes with minimal amounts of added hormone (Kusaba et al. 1998). Would the same happen with these genes in fungi, and would such a disruption of hormonal balance enhance mycoherbicide action?

5.1.2. Dissolving host defenses

As described in Section 3, cell wall degradases have been used to enhance virulence against insects and pathogens. The importance of phospholipases in fungal pathogenesis in mammalian systems has been extensively reviewed (Ghannoum 2000), and extracellular lipases are part of virulence of some fungi attacking plants (Eddine et al. 2001) and perhaps their up regulation could enhance virulence.

A rapid increase in callose biosynthesis is typically seen in compatible responses to *Colletotrichum* infection. Lignin-like material later becomes embedded in the callose, sterically preventing its degradation by glucanases, and thus inhibiting the progression of infective fungi (Kauss 1992). Callose synthase can also be inhibited rather specifically by deoxyglucose, enhancing the virulence of a pathogen (Stanghellini et al. 1993). Alas this is only effective when applied through cut surfaces, the anti-metabolite does not penetrate plant cuticles.

The enzyme callose synthase has calcium as an obligate cofactor, and the activity of this enzyme *in vitro* can be blocked by chelators (Kauss 1992). Fungi have very low requirements but high affinity for calcium. The requirement for calcium is so low that *Colletotrichum coccodes* could be grown for generations on putatively calcium-free media containing EGTA, a very strong calcium specific chelator. It was hypothesized that infecting a weed with a compatible *Colletotrichum* mycoherbicide together with a calcium chelator would enhance infectivity (Gressel et al. 2002). As most calcium chelators are too hydrophilic to penetrate plant cuticles, EGTA derivatives were synthesized with hydrophobic tails and applied with *Colletotrichum* to *Abutilon*. Some of these compounds as well as calcium-complexing oxalic acid, doubled the number of infection sites (Gressel et al. 2002). Concomitantly, microscopic analysis showed that far less callose was present after such treatments. A similar enhancement of mycoherbicide virulence was found by augmenting the formulation with oxalate (Briere et al. 2000; Watson and Ahn 2001). Thus, calcium deprivation increased infectivity while decreasing callose content. This correlation fits the hypothesis but does not prove it, as calcium deprivation can have many effects in plants. Still, there is considerable evidence that oxalate is naturally used by fungi as part of their pathogenesis process (Dutton and Evans 1996). Oxalate can be synthesized in fungi: (a) by cleavage of oxaloacetate from Krebs' cycle (TCA cycle) by the enzyme oxaloacetase (Müller 1986); (b) oxidation of glyoxylate by glyoxylate NADP-1 oxyreductase (glyoxylate dehydrogenase) (Akamatsu 1993); and (c) by oxidation of ascorbate analogs; erythroascorbate and its galactoside by ascorbate oxidase (Loewus 1999). These genes could be considered for transgenically enhancing virulence. They are ubiquitous in fungi yet not all seem to be producing oxalate, so there is either a question of regulation and/or secretion.

A database search suggests that the genes responsible for oxalate production in fungi have not been isolated. The enzyme glyoxylate dehydrogenase has been completely purified, and is dauntingly large (331,000 D) (Tokimatsu et al. 1998). The gene will probably not be large, as the enzyme is composed of six identical homopolymers.

It is valid to ask whether increasing hypervirulence through oxalate production by the biocontrol agent should be transgenic, based on two arguments: (a) Perhaps it is metabolically and economically "cheaper" to provide commercial oxalate in the formulation than produce it from metabolites in the biocontrol agent. Such production can be at the expense of growth; and (b) providing oxalate in the formulation confers transient hypervirulence. The spores later formed do not have greater virulence. Using exogenous oxalate can act as a failsafe mechanism and preclude the necessity to register a transgenic organism. These arguments may be valid for any gene product, and should be part of the considerations for any over production. Oxalate is especially inexpensive to provide, compared to enzymes and protein toxins.

There is always the possibility that a target weed will evolve resistance to oxalate overproducers, or to added oxalate. The oxalate catabolic enzyme oxalate decarboxylase from *Collybio velatipes* (a basidiomycete) has significant sequence homology with germin-like proteins from *Arabidopsis*, tomato, and rice (Kesarwani et al. 2000). Oxalate can also be oxidized in plants by oxalate oxidase. Engineering poplars to over-produce oxalate oxidase rendered the plants resistant to *Septoria* (Liang et al. 2001). Conversely, overexpression of a calcium binding protein (Sebghati et al. 2000) might have the same effect as oxalate, and will be harder to overcome.

5.2. Hard genes encoding toxins

Fermenter produced fungal toxins have been proposed for use as stand alone "biocontrol" agents. The term biocontrol is best limited to the use of a living organism, and the possibility of using separately produced fungal derived phytotoxins alone or as adjuvants to enhance virulence of mycoherbicides will not be discussed, and the reader is referred to (Abbas et al. 2001; Bailey et al. 2000b; Duke et al. 2001;

Vurro 2001; Vurro et al. 2001b) among others. This chapter will deal only with toxins produced by the mycoherbicide itself that enhances its virulence.

Complex fungal toxins pre-dated herbicides, and were the lead compounds for herbicide discovery (see (Gressel 2002)– Table 2.1.). The genes encoding their production and that of novel toxins (Kimura et al. 2001; Vurro 2001) can be considered for enhancing mycoherbicide virulence, if indeed the genes are clustered, which seems to be the case in many instances (Kimura et al. 2001; Walton 2000). Another, easier approach is to use peptide toxins that can be produced in a single step from a single gene. Indeed, virulence was increased 9 fold without losing host specificity by introducing *nep1*, a gene encoding a phytotoxic protein, to an *Abutilon theophrasti*-specific, weakly mycoherbicidal strain of *Colletotrichum coccodes*. The parent strain was at best infective on juvenile cotyledons of this intransigent weed. The transgenic strain was lethal through the three-leaf stage, providing the time window of control of this asynchronously germinating weed (Amsellem et al. 2002).

A major problem in the engineering of mycoherbicides for toxin production may well be in obtaining expression. Some of the most potent toxin producing fungi, produce their toxins only when cultured on media, but not when they are pathogenic. We found that culture filtrates of a strain of *Fusarium oxysporum* were toxic to tomato, yet the fungus grows on the tomato rhizoplane, specifically attacking *Orobanche* spp. (broomrapes) before and after they parasitize the tomato (Cohen et al. 2002). Similarly, *Myrothecium verrucaria* produces milligrams of tricothecenes per gram medium in culture. Highly sensitive technologies could not find traces of these toxins when the pathogen infected kudzu (*Pueraria montana*) (Abbas et al. 2001). If the toxins are necessary for fungal virulence, they must be made in a very localized area for a very short time; when needed for establishment and toxin production could be under a contact inducible promoter. Additionally, transgenes encoding toxin degradation under control of a senescence activated promoter could be introduced into mycoherbicides to clear dead weeds of toxins, as an additional failsafe mechanism (as discussed later).

Even before such specific efficient promoters are found, it seems that it may be possible to use native promoters, but in distantly related species. Various *Fusarium* and other fungal species produce phytotoxic NEP1 proteins. The *nep1* gene was reintroduced with high expression promoters and made high levels of NEP1 on artificial media; so much so that NEP1 was considered for use as a “natural herbicide” (Bailey et al. 2000a) or as a separately produced additive to enhance virulence of another mycoherbicidal organism (Bailey et al. 2000b). The over expressing transformants did not have enhanced virulence when introduced into *Fusarium* used to infect a weed, only making toxin on artificial media (Bailey et al. 2002). The same construct enhanced virulence ten fold (i.e. a tenth as much inoculum was needed compared to wild type) when introduced into a *Colletotrichum coccodes* specifically pathogenic on *Abutilon theophrasti* (Amsellem et al. 2002). The *nep1* *Colletotrichum* rapidly killed through the three leaf stage, where at best, the wild type slowly killed plants with cotyledons or at most in the true leaf stage. An example of one hardly proves that heterologous overexpression and hypervirulence is more likely than homologous overexpression, but it does point to a direction worthy of consideration.

5.2.1. Are toxin producing mycoherbicides inherently dangerous?

There can be cases where toxin production may be thought to restrict the use of a mycoherbicide, i.e. when the toxin has high mammalian toxicity and may find its way into the food chain, as in the case of tricothecene production in a gorse-controlling agent (Morin et al. 2000). Pathogenic strains were found that did not produce these toxins. As described above, tricothecene-producing strains did not produce

toxin when infecting the target weed, only when they were in artificial culture. Such results suggest that toxicology must be based on real world field data, and not artificial media. If an organism produces toxins only when in artificial culture and the agricultural produce is toxin free, the organism is only hazardous to the producer of the mycoherbicide. This poses a conundrum. Some contend that such strains should not be used. Others counter by reminding that a very non-toxic herbicides such as glufosinate is produced from very dangerous organophosphates, and the only danger is to the production workers. Safe production facilities can be constructed to produce inoculum, just as safe factories produce glufosinate.

5.3 Should we look at up-regulated genes in pathogenesis?

Well before the terms genomics and proteomics were coined, researchers were trying to ascertain what genes and gene products were “turned on” in plants during pathogenesis. If they were not turned on in non-pathogenic strains, especially in mutants, they were considered to be pathogenesis-related. The question to be asked in our context is; will there be hypervirulence if the expression of genes that encode enzymes that counteract PR proteins is transgenically turned up? And then, what other genes must be turned up? And then, is there a controlling element that can be introduced into an organism that will up-regulate the endogenous genes, without a necessity to add more genes? Thus, one could up regulate the genes for a branched amino acid biosynthesis, without the need of a mutagen. With advanced knowledge, such enhancement could be performed by site-directed chimeraplasty (Zhu et al. 2000) without leaving transgenic traces.

Another open question is whether it will be simpler to transform a biocontrol agent than to up-regulate a silenced gene. For example, in a recent effort to enhance the toxicity of the *Fusarium oxysporum* that attacks *Orobancha* spp. by transforming it with the *nep1* toxin producing gene, it was found by PCR that the putative biocontrol organism has an (apparently) silenced *nep1* gene in it (Z. Amsellem and J Gressel, unpublished). Will this be the case with the genes controlling oxalate production and other genes that could potentially be effective in overcoming weed defenses?

6. RISKS FROM TRANSGENIC BIOCONTROL AGENTS

While little thought and effort have been previously focused on risk analysis of transformed mycoherbicides, there has been considerable discussion on the use of “transgenic arthropods as biocontrol agents of other arthropods” (arthropod natural enemies). Hoy (2000) summarizes at length the potential risk issues discussed in a workshop devoted to that subject that should be dealt with before releasing transgenic arthropods. The issues brought up are valid for all types of biocontrol agents and should be a part of any risk analysis.

6.1 Constraints on using molecularly-enhanced biocontrol fungi

In nature, genes can move among fungal species by whatever means. Thus, there is good reason to assess the risks of such movement as there are many good reasons to desire that organisms will not transfer hypervirulent transgenes from a biocontrol agent to a pathogen of a crop or of a beneficial insect or fungus. If there is such transfer, then there is reason to desire that the transferred transgene will not become established in a non-target population.

Many fungi with the potential to act as biocontrol agents exist as narrow host-range-specific pathogens that are related to pathogens of beneficial species. These *formae specialis* or pathovars have often been shown by DNA comparisons to have evolutionarily diverged a very long time ago from related pathogens. A case in point is the ability to show that most divergences in the imperfect *Fusarium oxysporum* with its thousands of *formae specialis* and even more vegetative compatibility groups occurred eons ago (Baayen et al. 2000). In contrast to this ancient evolution, there is evidence

that parasexual recombination between closely related species and supposedly incompatible strains of the same species is more prevalent when environmental stresses are exerted (Julian et al. 1999; Molnar et al. 1990). Indeed, incompatibility between strains and even between species can be overcome by the mutation of a single gene (Leslie and Zeller 1997). This has occurred in both imperfect strains of *Fusarium* and *Rhizoctonia* (Basidiomycetes) species that include major crop pathogens as well as host-specific pathogens of weeds, some of which could be used as biocontrol agents if their virulence were to be enhanced. Some imperfect *Fusarium* spp. can be physiologically stimulated to sexuality (with the sexual forms called *Gibberella* spp.) and a gene for hypervirulence could then be moved sexually among strains that do not parasexually recombine by heterokaryon formation (Plattner et al. 1996). Interspecific protoplast fusion with the appearance of stable hybrids is not unknown e.g. between an insect-killing *Beauveria* sp. and a toxigenic species. Some of the somatic hybrids formed are hypervirulent (Viaud et al. 1998).

6.2 Is horizontal gene transfer a risk?

The possibility of horizontal gene transfer (asexual exchange among different species or incompatible strains) has been suggested as the cause for many anecdotal appearances of improbable genes or gene sequences in various fungi. The ‘implied’ cases and the supporting evidence have recently been reviewed by (Rosewich et al. 1999; Rosewich and Kistler 2000). They endeavored to exclude cases in which parasexual heterokaryon formation and protoplast fusion might be the cause, but as seen above, mutations and stress can alter sexuality and compatibility. For this reason it was recently proposed to refer to this ‘gray’ area between vertical (sexual or parasexual) and horizontal exchange between closely related strains and organisms as ‘diagonal’ gene transfer (Gressel 2000). An example of what might be true horizontal gene transfer is the circumstantial EST (expressed sequence tag) evidence for the appearance of a bacterial chymotrypsin in a fungus (Screen and St. Leger 2000). Chymotrypsins were previously unknown in fungi, and sequence analysis showed that the intron-free gene of the fungus and that of a soil bacterium are related (Screen and St. Leger 2000). Rosewich and Kistler, after exhaustively discussing all the extant reports, conclude that horizontal gene transfer in fungi has “not been proven beyond reasonable doubt” (Rosewich and Kistler 2000), but diagonal gene transfer is a clear possibility, especially when organisms are under stress.

6.3 Potentially acute constraints

Three potential acute biological constraints have limited the interest in developing transgenic mycoherbicides: (a) The possibility that the agent would persist in the environment and spread, affecting non-target hosts that were not assayed when checking host range; (b) they could mutate to a change in host range. Such evolutionary change in host range is potentially more hazardous in a hypervirulent organism than in the native pathogen; and (c) the mycoherbicide might sexually or asexually introgress and recombine with a related pathogenic species that attacks desirable species, and confer it with hypervirulence. Fungi can sexually mate or asexually conjugate or form heterokaryons and thus transfer genetic material to closely related species by ‘diagonal’ gene transfer.

7. PREVENTION OF PERSISTENCE AND SPREAD OF TRANSGENIC MYCOHERBICIDES

Various methods can be considered to prevent persistence and spread. For example, transgenic gene disruption can also be used to limit the ability of a mycoherbicide to exist in nature, but be used as a biocontrol agent. For example, *Psy1* disruptants of a *Trichoderma* could still be used as biocontrol agents against damping off agents, but lack the siderophores (encoded by this gene) that allow them to compete in natural, low iron environments (Wilhite et al. 2001). The gene could be knocked out in the transgenic mycoherbicide to prevent persistence and used

in antisense or suppressive overexpression in a construct with hypervirulence genes to debilitate any organism that introgresses the hypervirulence construct, as described below.

A scheme to obviate the spread of native or host-range mutated agents, and an additional scheme to mitigate introgression are described (Fig. 1). The general concepts described may be broadly applicable to biocontrol agents against other pest types. The specific examples presented are more limited to mycoherbicides. Fungi typically spend parts of their life cycles in dormant, resting structures that are resistant to heat, cold, desiccation, or other environmental tribulations. These same resting propagules (spores, conidia, sclerotia, pycnidia, ascospores, etc.) are a major form of dispersal, whether by wind, water, or animal movement. The suppression of spore formation whether by mutation (Sands and Miller 1993) or transgenically in hypervirulent biocontrol agents can prevent both persistence and spread (Fig. 1). Non-sporulating mutants are not hard to isolate; it is probably best in such a case to use a physical mutagen (gamma or neutron radiation) that causes a loss of gene fragments. Point mutations caused by ultraviolet light or most chemical mutagens can revert, whereas deletions cannot. Experiments using mycoherbicidal levels of *Sclerotinia* highlighted the necessity to use non-sclerotia forming mutants; the half life of sclerotia was always more than 6 months, and it would take four years for the densities to decay to those found in pristine soils (Bourdote et al. 2001).

It was recently shown for two fungal species that chopped mycelia could be dried, stored for over a year and rehydrated (Amsellem et al. 1999). The rehydrated mycelia were more virulent than spores of the same species, because the mycelia establish more quickly in the pest (Amsellem et al. 1999). This procedure has an added asset insofar as it is usually far more efficient to produce mycelia in liquid culture than spores in liquid or on solid media.

The spread mycoherbicidal agents can also be prevented by rendering them transgenically asporogenic (Fig 1a). This could be performed by antisense type strategies or preferably by gene targeting (Shiotani and Tsuge 1995) and knockout. Many pathogenic species seem to require melanized spores or structures for pathogenicity (Butler et al. 2001; Perfect et al. 1999). The germinating spores often develop melanized appressoria that attach tightly to the host, forming an infection peg that penetrates the host. Occasionally, the same species can attack both via melanized appressoria, as well as by mycelial penetration through stomates in the leaves (Latunde-Dada et al. 1999). Thus, if appressoria are not needed in the target weed for biocontrol, losing the ability to make appressoria would preclude some host range changes.

Mycelia themselves are not always pathogenic. Where only spores are pathogenic, the spread of a transgenic hypervirulent biocontrol agent could be prevented by a more complex strategy akin to the 'terminator' strategy (Crouch 1998; Oliver et al. 1998). Transgenes that could potentially suppress sporulation could be engineered into the biocontrol organism under the control of a chemically-inducible promoter. Sporulation genes in antisense configuration or in high over-expression so as to cause co-suppression would suppress the sporulation. Spores or mycelia to be used as inoculum could then be treated with the chemical inducer, before application to the target pest. The chemical inducer could be in the micropellet containing used for application (Amsellem et al. 1999), or the chemical inducer could be an endogenous, specific compound in the pest host. Thus, the biocontrol agent could be contained to the single, purposely-infested weed population. The use of transgenic sporulation suppression is less appealing than the use of physical deletion mutations because of the possibility of transgene silencing, allowing the organism to revert back to wild type.

7.1 Obviating recombination between mycoherbicidal agents and crop pathogens

The above strategies can be used to prevent persistence and spread, but would not preclude introgression with organisms from the same or related species (after sexual conjugation or heterokaryon formation). Such conjugation might provide genes that support spore formation, resulting in

hypervirulent, persistent, and spreading pathogens. Thus, means are needed to mitigate the possibility that recombined, introgressed, hypervirulent organisms could become ‘superbugs’ attacking non-targeted species.

A concept of using analogous tandem constructs was recently proposed as a failsafe to mitigate introgression of transgenes from crops to weeds (Gressel 1999) and has been modified to deal with biocontrol agents (Gressel 2001a). It was proposed that the important hypervirulence gene be flanked with transgenic mitigator (TM) genes that are positive or neutral to the biocontrol agent in the form to be used, but would be detrimental to any recombinant (Fig. 1b.). In the simplest form, as an example, the primary hypervirulence gene(s) could be flanked by one or two of the TM genes listed in Table 2, if the TM genes do not adversely affect virulence on the target weed. These genes, in the antisense or co-suppressive form would affect one of the processes leading to the ability to recombine, to form viable spores, or to make efficient infection structures. Some of these genes may have other deleterious effects that may render them inappropriate for this purpose. Clearly there are many known as well as yet to be discovered genes that would be appropriate additions to this list.

An antisense gene suppressing sporulation (Table 2) should prevent sporulation in a heterokaryon or other recombinant organism. The genes that control melanin biosynthesis and/or conidiation might only be applicable for biocontrol agents that do not need spores or melanin for pathogenicity. Spores without melanin do not have the viability in, or resistance to harsh environments. Thus, if some spores do form, they would be without vigor. Interestingly, related genes can have different functions. Of the three genes described coding for G α proteins subunits, deletion of *magC* had no effect on mycelial growth or appressorium formation, unlike *magA* or *magB* (Liu and Dean 1997; Truesdell et al. 2000).

8. RISK ANALYSIS AND LIMITATIONS OF THESE FAILSAFES

Risk analysis must be separately performed for each transgenic biocontrol agent considering two types of issues: (a) the limitations on the failsafe mechanisms that can be used; and (b) the biology of the pathogen and its relatedness to other pathogens.

A safety aspect that must be clearly ascertained early in development of asporogenic mutants or by antisensing spore formation, is that all types of sporulation are suppressed. For example, only light-induced conidiation is precluded in some asporogenic fungal mutants, but not starvation-induced sporulation (Horwitz et al. 1985). Some organisms make more than one type of spore; many *Fusarium* species can produce micro and macro conidia as well as chlamyospores. Each is produced under different environmental conditions. It would be interesting to ascertain whether each of the genes that control spore stalk development (Table 2) can (when antisensed) suppress all types of spore forms. The *stuA* transcription factor does control both sexual and asexual reproduction in *Aspergillus nidulans*, so genes are known suppress all spore types (Wu and Miller 1997). It will be easier to load more or simpler failsafe mechanisms into organisms that do not require appressoria for penetration. This includes mycoherbicides that attack through stomates or other inter or intra cellular penetration.

More complex methods such as the modified ‘terminator’ technology will also have to be considered for organisms that use melanized appressoria, or where hyphae are not typically pathogenic. Not all organisms (e.g. *Alternaria alternata*) utilize melanization of appressoria as part of infection (Kawamura et al. 1997). One can consider using a spore-specific promoter for anti-melanin genes where hyphae form appressoria-requiring melanin. Thus, failsafe mechanisms will perforce be more complex with the melanized appressoria-utilizing *Colletotrichum* species (Perfect et al. 1999). Still, some *Colletotrichum* spp. can attack plants by stomatal penetration (Latunde-Dada et al. 1999). Many regulatory genes that are activated during sporulation are known (Adams et al. 1998; Marshall and

Timberlake 1991). Such genes could be used to activate melanin suppressing or other anti-sporogenesis genes to render them more spore specific.

8.1. Risk considerations based on pathogen biology

Background knowledge about the possibility of a pathogen mutating its host specificity and its ability or inability to sexually or asexually conjugate ‘diagonally’ with related organisms will govern the required number and level of failsafe mechanisms. Thus, one must consider the possibility of mutation causing pathogenicity to a broader host spectrum of a host-specific biocontrol agent, e.g. a specific pathovar of *Fusarium oxysporum*. There are no documented cases of a member of this species mutating its host range. The species is sub-divided into hundreds of known *forma specialis*, each with its own host specificity. There must have been evolution to different hosts, even if not documented cases, suggesting that more caution is needed here than for (example) the *Fusarium arthrosporioides* that is known to be pathogenic on *Orobanche* species (Amsellem et al. 2001). This *Fusarium* does not have known *forma specialis* (pathovars) on known crop species where it was isolated. Still, the possible existence of alternate crop hosts must be considered.

Some species easily conjugate with close relatives forming heterokaryotic mycelia with mixed nuclei (e.g. *Trichoderma*), and the mycelia have mixed properties that can enhance pathogenicity and host range (Harman and Donzelli 2001). Problems might ensue where spores are multinucleate or where there is recombination among nuclear chromosomes. Further generations will carry the heterokaryotic complemented properties. In a multigenerational experiment with hundreds of millions of (uninucleate) spores, there was no recombination among complementing nuclei that allowed a heterokaryon of two different *Trichoderma* auxotrophs to live on minimal media. No spore formed on these heterokaryons that could exist on this minimal medium (E. Galun, unpublished results). This demonstrates that even closely-related or con-specific organisms have impenetrable barriers to prevent recombination with ‘alien’ genomes in nature, even when the traits could be beneficial or even vital for existence. Imperfect (asexual) fungi have less capacity to transfer traits than perfect (sexual) fungi. Still, it is impossible to ‘prove’ that an imperfect fungus does not have a rare sexual form that appears only in highly special conditions.

Scientists and regulators interested in risk assessment would be advised to set up a decision tree mechanism that will allow an organized, less biased mechanism to formally answer a series of questions that will assist in categorizing risk. Such decision trees have been constructed for evaluating the analogous situation of introgression of transgenic traits from crops to related weeds (Gressel and Rotteveel 2000). It is imperative that containment (Kahn and Mathur 1999) be considered when working with transgenic hypervirulent organisms that can spread, until they are transgenically mitigated or otherwise deemed safe for release into the environment.

9. MARKING BIOCONTROL AGENTS

There are a variety of needs for devising simpler recognition methods for mycoherbicides and other organisms marketed in commerce; whether they are conventionally selected, mutant, or transgenic bacteria, fungi, plants or animals. The needs include: (a) *The need for protection for patented or other IP lines*, where IP takes on either designation: “Intellectual Property” or “Identity Preserved”. It is often hard to prove that a line has been ‘miss-appropriated’; (b) *The need to trace biocontrol and other inoculation systems in the environment*. If the use of mycoherbicides increases, there will be a need for tracing irrespective of whether the mycoherbicide is indigenous or transgenic. Many of the mycoherbicides are closely related to known pathogens and proof (against grower liability suits) will continually be needed that it did change its host range and attack valuable species. There are

complicated DNA fingerprinting techniques to accurately ascertain causality e.g. (Hintz et al. 2001; Inglis et al. 2001), but they cannot be used to answer the general question: “I suspect a mycoherbicide – which is it?” and ; (c) *Labeling* Regulatory authorities and various consumer groups are demanding labeling of transgenic commodities. They spend vast sums typically probing for commonly used promoters or selectable marker genes and not for the trait genes. Even when transgenics are discovered with such ‘kits’, there is no information as to source.

Thus, there is a need for common recognition sequences for detecting transgenic or other organisms released into the environment, in a single test that will clear or implicate particular organism. The simplest detection system for differentiating a large number of products is the “bar code” system. A simple genetic analogy has been proposed (Gressel 2001). A set of two universal ‘nonsense’ (non-coding) nucleotide sequences is designed that can be detected by a set of universal PCR primers to recognize all bar codes. The universal primers are long enough that a few mutational changes in the initial universal sequence will still allow it to be recognized by a PCR primer. The bar code DNA can be co-transformed or spliced with the gene of choice. In other cases, an excisable selectable marker will be needed, so that just the bar code remains after transformation. The PCR amplified bar codes can be automatically sequenced and compared to the barcode database to ascertain the source of the organism. Should there be a possibility of introgression of the barcode from the initial organism into another strain or species, R or AFLP can be used to elucidate the target organism.

10. WEEDS WILL EVOLVE RESISTANCE TO TRANSGENICALLY-MYCOHERBICIDES

Weeds have been very successful in evolving partial resistance to the pathogens and arthropods attacking them, in a long-term evolutionary sense. Weeds are weeds because they are capable of replacing themselves in agroecosystems, despite generations of human intervention. In a thoughtful analysis “when is biological control evolutionary stable (or is it?)” Holt and Hochberg (1997) cogently ask why there is a lack of reports on the evolution of resistance to biocontrol agents. The mundane reasons for lack of resistance may reside in the fact that they have not been used long enough and to a large enough extent, or just poor reporting. Their discussion scrutinizes the long-term questions relating to ‘classical’ agents, but can be extended to transgenic hypervirulent agents. In many respects the factors impinging on the evolution of resistance are analogous to those discussed (Gressel, 2002) vis a vis the evolution of herbicide resistance. Their analysis (Holt and Hochberg 1997) is long and complex, and is discussed in the context of mycoherbicides in (Gressel 2002).

In the final analysis, the strong selection pressure of transgenic hypervirulent organisms has the potential of selecting for resistant weed populations. The choice of genes and the synergistic use of multigenes can temper this evolutionary process, and crop/conventional/biological herbicide rotation should further delay such evolutionary processes, retaining transgenic hypervirulent mycoherbicides in the farmers’ arsenal for a long time.

11. CONCLUSIONS

Mycoherbicides will only be widely used in most row crop situations if virulence is transgenically enhanced to overcome the evolutionary barriers that limit achieving adequate weed control. Enough genes are already available and the proof of concept has been demonstrated. Concepts for transgenically limiting off target spread and mitigating effects of gene flow, as well as universally marking hypervirulent mycoherbicides have been delineated.

ACKNOWLEDGEMENTS

The author’s research on the assessment of gene introgression among species is supported by EU-INCO project ERB IC18 CT98 0391, the research on biocontrol by a DFG Trilateral Grant, and the

research on transgenic mitigation and biobarcodeTM by the Levin Fund. J. G. is the Gilbert de Botton Professor of Plant Sciences.

REFERENCES

- Abbas HK, Tak H, Boyette CD, Shier WT, Jarvis BB (2001). Macrocyclic trichothecenes are undetectable in kudzu (*Pueraria montana*) plants treated with a high-producing isolate of *Myrothecium verrucaria*. *Phytochemistry* 58:269-276.
- Adams TH, Weiser JK, Yu JH (1998). Asexual sporulation in *Aspergillus nidulans*. *Microbiol Mol Biol Rev* 62:35-54.
- Ahn I-P, Lee Y-H (2001). A viral double-stranded RNA up regulates the fungal virulence of *Nectria radiculicola*. *Mol Plant-Microbe Interact* 14:496-507.
- Akamatsu Y (1993). Enzymatic formation of oxalic acid from glyoxylic acid and cell-free extracts of *Tyromyces palustris*. *Mokuzai Gakkaishi* 39:860-862.
- Amsellem Z, Zidack NK, Quimby JPC, Gressel J (1999). Long term dry preservation of active mycelia of two mycoherbicidal organisms. *Crop Protect* 18:643-649.
- Amsellem Z, Kleifeld Y, Kerenyi Z, Hornok L, Goldwasser Y, Gressel J (2001). Isolation, identification, and activity of mycoherbicidal pathogens from juvenile broomrape plants. *Biol Contr* 21:274-284.
- Amsellem Z, Cohen BA, Gressel J (2002). Transgenically conferring hypervirulence on an inundative mycoherbicidal fungus for efficient weed control. Submitted.
- Arditi R, Berryman AA (1991). The biological control paradox. *Trends Ecol Evol* 6:32.
- Auld BA, Morin L (1995). Constraints in the development of bioherbicides. *Weed Tech* 9:638-652.
- Baayen RP, O'Donnell K, Bonants PJM, Cigelnik E, Kroon LPNM, Roebroek EJA, Waalwijk C (2000). Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. *Phytopathology* 90:891-900.
- Bailey BA, Collins R, Anderson J (2000a). Factors influencing the herbicidal activity of Nep1, a fungal protein that induces the hypersensitive response in *Centaurea maculosa*. *Weed Sci* 48:776-785.
- Bailey BA, Apel-Birkhold PC, Akingbe OO, Ryan JL, O'Neill NR, Anderson JD (2000b). Nep1 protein from *Fusarium oxysporum* enhances biological control of opium poppy by *Pleospora papaveracea*. *Phytopathology* 90:812-818.
- Bailey BA et al. (2002). *Phytopathology*:(in press).
- Bourdot GW, Hurrell GA, Saville DJ, De Jong MD (2001). Risk analysis of *Sclerotinia sclerotiorum* for biological control of *Cirsium arvense* in pasture: Ascospore dispersal. *Biocont Sci Tech* 11:119-139.
- Boyette CD, Quimby PC, Connick WJ, Daigle DJ, Fulgham FE (1991) Progress in the production, formulation and application of mycoherbicides. In: DO TeBeest, ed. *Microbial Control of Weeds*. New York: Chapman and Hall, pp 209-222.
- Brandl MT, Lindow SE (1998). Contribution of indole-3-acetic acid production to the epiphytic fitness of *Erwinia herbicola*. *Appl Envir Microbiol* 64:3256-3263.
- Briere SC, Watson AK, Hallett SG (2000). Oxalic acid production and mycelial biomass yield of *Sclerotinia minor* for the formulation enhancement of a granular turf bioherbicide. *Biocont Sci Tech* 10:281-289.
- Butler MJ, Day AW, Henson JM, Money NP (2001). Pathogenic properties of fungal melanins. *Mycologia* 93:1-8.

- Callahan FE, Rowe DE (1991). Use of a host-pathogen interaction system to test whether oxalic-acid is the sole pathogenic determinant in the exudate of *Sclerotinia-tripoliorum*. *Phytopathology* 81:1546-1550.
- Chen Z, Sun X, Hu Z, Li M, O'Reilly DR, Zuidema D, Vlak JM (2000). Genetic engineering of *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus as an improved pesticide. *J Invert Path* 76:140-146.
- Choi WB, Dean RA (1997). The adenylate cyclase gene MAC1 of *Magnaporthe grisea* controls appressorium formation and other aspects of growth and development. *Plant Cell* 9:1973-1983.
- Cohen B, Amsellem Z, Maor R, Sharon A, Gressel J (2002). Transgenically-enhanced expression of indole-3-acetic acid (IAA) confers hypervirulence to plant pathogens. *Phytopathology*:(in press).
- Comai L, Kosuge T (1982). Cloning and characterization of *iaaM*, a virulence determinant of *Pseudomonas savastanoi*. *J Bact* 149:40-46.
- Crouch ML (1998) How the terminator terminates: An explanation for the non-scientist of a remarkable patent for killing second generation seeds of crop plants
<http://www.bio.indiana.edu/people/terminator/html>. Edmond WA, USA: The Edmonds Institute.
- Culp DW, Dodge CL, Miao YH, Li L, Sag-Ozkal D, Borgia PT (2000). The *chsA* gene from *Aspergillus nidulans* is necessary for maximal conidiation. *FEMS Microb Lett* 182:349-353.
- Dangl JL, Jones JDG (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411:826-833.
- Dennill GB, Hokkanen HMT (1990). Homeostasis and success in biological control of weeds - a question of balance. *Agr Ecosys Envir* 33:1-10.
- Duke SO, Baerson SR, Dayan FE, Kagan IA, Michel A, Scheffler BE (2001) Biocontrol of weeds without biocontrol agents. In: M Vurro, J Gressel, T Butts, GE Harman, A. Pilgeram, RJ St. Leger and DL Nuss, eds. *Enhancing Biocontrol Agents and Handling Risks*. Amsterdam: IOS Press, pp 96-105.
- Dutton M, Evans C (1996). Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. *Can J Microbiol* 42:881-895.
- Eddine AN, Hannemann F, Schafer W (2001). Cloning and expression analysis of *NhL1*, a gene encoding an extracellular lipase from the fungal pea pathogen *Nectria haematococca* MP VI (*Fusarium solani* f. sp *pisi*) that is expressed in planta. *Mol Gen Gen* 265:215-224.
- Fan X, Shi X, Zhao J, Zhao R, Fan Y (1999). Insecticidal activity of transgenic tobacco plants expressing both Bt and CpTI genes on cotton bollworm (*Helicoverpa armigera*). *Chin J Biotech* 15:1-5.
- Flipsen JT, Mans RM, Kleefsman AW, Knebel-Morsdorf D, Vlak JM (1995). Deletion of the baculovirus ecdysteroid UDP-glucosyltransferase gene induces early degeneration of Malpighian tubules in infected insects. *J Virol* 69:7380-7381.
- Frank SA (2000). Specific and non-specific defense against parasitic attack. *J Theor Biol* 202:283-304.
- Fujii I, Mori Y, Watanabe A, Kubo Y, Tsuji G, Ebizuka Y (1999). Heterologous expression and product identification of *Colletotrichum lagenarium* polyketide synthase encoded by the *PKSI* gene involved in melanin biosynthesis. *Biosci Biotech Biochem* 63:1445-1452.
- Gaudin V, Vrain T, Jouanin L (1994). Bacterial genes modifying hormonal balances in plants. *Plant Physiol Biochem* 32:11-29.
- Ghannoum MA (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* 13:122-143.
- Gressel J (1999). Tandem constructs: preventing the rise of superweeds. *Trends Biotech* 17: 361-366.
- Gressel J (2000). Molecular biology of weed control. *Transgenic Res* 9:355-382.

- Gressel J (2001a). Potential failsafe mechanisms against the spread and introgression of transgenic hypervirulent biocontrol fungi. *Trends Biotech* 19:149-154.
- Gressel J (2001b). Method and system for identifying commercially distributed organisms. PCT.
- Gressel J (2002) *Molecular Biology of Weed Control*. London: Taylor and Francis.
- Gressel J, Rotteveel T (2000). Genetic and ecological risks from biotechnologically-derived herbicide resistant crops: decision trees for risk assessment. *Plant Breeding Reviews* 18:251-303.
- Gressel J, Vered Y, Bar-Lev S, Milstein O, Flowers HM (1983). Partial suppression of cellulase action by artificial lignification of cellulose. *Plant Sci Lett* 32:349-353.
- Gressel J, Michaeli D, Kampel V, Amsellem Z, Warshawsky A (2002). Ultralow calcium requirements of fungi facilitates use of calcium regulating agents to suppress host calcium-dependent defenses, synergizing infection by a mycoherbicide. (submitted).
- Gruen HE (1959). Auxins and fungi. *Annual Review of Plant Physiol* 10:405-440.
- Harman GE, Donzelli BGG (2001) Enhancing crop performance and pest resistance with genes from biocontrol agents. In: M Vurro, J Gressel, T Butts, GE Harman, A. Pilgeram, RJ St. Leger and DL Nuss, eds. *Enhancing Biocontrol Agents and Handling Risks*. Amsterdam: IOS Press, pp 114-125.
- Harman GE, Stasz TE (1991) Protoplast fusion for the production of superior biocontrol fungi. In: DO TeBeest, ed. *Microbial Control of Weeds*. New York: Chapman and Hall, pp 171-186.
- Harris LJ, Gleddie SC (2001). A modified *Rpl3* gene from rice confers tolerance of the *Fusarium graminearum* mycotoxin deoxynivalenol to transgenic tobacco. *Physiol Mol Plant Path* 58:173-181.
- Hintz WE, Becker EM, Shamoun SF (2001). Development of genetic markers for risk assessment of biological control agents. *Can J Plant Path* 23:13-18.
- Hlinkova E, Bert B, Filipp D (1998). Phenotypes of tobacco plants expressing genes for the synthesis of growth regulators. *Biol Plant* 41:25-37.
- Holt RD, Hochberg ME (1997). When is biological control evolutionarily stable (or is it?). *Ecology* 78:1673-1683.
- Horwitz BA, Gressel J, Malkin S, Epel BL (1985). Modified cryptochrome *in vivo* absorption in *Trichoderma dim* photosporulation mutants in *Trichoderma*. *Proc Natl Acad Sci USA* 82:2736-2740.
- Hoy MA (2000). Transgenic arthropods for pest management programs: risks and realities. *Exp Appl Acar* 24:463-495.
- Inglis PW, Teixeira EA, Ribeiro DM, Valadares-Inglis MC, Tigano MS, Mello SCM (2001). Molecular markers for the characterization of Brazilian *Cercospora caricis* isolates. *Curr Microbiol* 42:194-198.
- Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J* 8:97-109.
- Jennings JC, Apel-Birkhold PC, Bailey BA, Anderson JD (2000). Induction of ethylene biosynthesis and necrosis in weed leaves by a *Fusarium oxysporum* protein. *Weed Sci* 48:7-14.
- Julian MC, Acero J, Salazar O (1999). Mating type-correlated molecular markers and demonstration of heterokaryosis in the phytopathogenic fungus *Thanatephorus cucumeris* (*Rhizoctonia solani*) AG 1IC by AFLP DNA fingerprinting analysis. *J Biotech* 67:49-56.
- Kahn RP, Mathur SB (1999) *Containment Facilities and Safeguards*. Madison, WI: American Phytopathology Society.

- Kauss H (1992) Callose and callose synthase. In: DJ Bowles, S Gurr, M McPherson, eds. *Molecular Plant Pathology: Practical Approach to Molecular Plant Pathology*. Vol. 2. Oxford: Oxford University Press, pp 1-8.
- Kawamura C, Moriwaki J, Kimura N, Fujita Y, Fuji S, Hirano T, Koizumi S, Tsuge T (1997). The melanin biosynthesis genes of *Alternaria alternata* can restore pathogenicity of the melanin-deficient mutants of *Magnaporthe grisea*. *Mol Plant-Microbe Interact* 10:446-453.
- Kesarwani M, Azam M, Natarajan K, Mehta A, Datta A (2000). Oxalate decarboxylase from *Collybia velutipes*. Molecular cloning and its overexpression to confer resistance to fungal infection in transgenic tobacco and tomato. *J Biol Chem* 275:7230-7238.
- Khan R, Straney DC (1999). Regulatory signals influencing expression of the PDA1 gene of *Nectria haematococca* MPVI in culture and during pathogenesis of pea. *Mol Plant-Microbe Interact* 12:733-742.
- Kimura M, Anzai H, Yamaguchi I (2001). Microbial toxins in plant-pathogen interactions: Biosynthesis, resistance mechanisms, and significance. *J Gen Appl Microbiol* 47:149-160.
- Kistler HC (1991) Genetic manipulation of plant pathogenic fungi. In: DO TeBeest, Ed., ed. *Microbial Control of Weeds*. New York: Chapman and Hall, pp 151-170.
- Kronstad JW (1997). Virulence and cAMP in smuts, blasts and blights. *Trends Plant Sci* 2:193-199.
- Kubo Y, Takano Y, Endo N, Yasuda N, Tajima S, Furusawa I (1996). Cloning and structural analysis of the melanin biosynthesis gene *SCD1* encoding scytalone dehydratase in *Colletotrichum lagenarium*. *Appl Env Microbiol* 62:4340-4344.
- Kusaba S, Kano-Murakami Y, Matsuoka M, Tamaoki M, Sakamoto T, Yamaguchi I, Fukumoto M (1998). Alteration of hormone levels in transgenic tobacco plants overexpressing the rice homeobox gene OSH1. *Plant Physiol* 116:471-476.
- Kwon K-C (1998). Gene disruption to evaluate the role of fungal candidate virulence genes. *Curr Opin Microbiol* 1:381-389.
- Langfelder K, Jahn B, Gehringer H, Schmidt A, Wanner G, Brakhage AA (1998). Identification of a polyketide synthase gene (pksP) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. *Med Microbiol Immun* 187:79-89.
- Latunde-Dada AO, O'Connell RJ, Nash C, Lucas JA (1999). Stomatal penetration of cowpea (*Vigna unguiculata*) leaves by a *Colletotrichum* species causing latent anthracnose. *Plant Pathol* 48:777-784.
- Lau GW, Hamer JE (1998). Acropetal: A genetic locus required for conidiophore architecture and pathogenicity in the rice blast fungus. *Fungal Genet Biol* 24:228-239.
- Lee BN, Adams TH (1996). *FluG* and *flbA* function interdependently to initiate conidiophore development in *Aspergillus nidulans* through *br1A* beta activation. *EMBO J* 15:299-309.
- Leslie JF, Zeller K (1997). Mutants that blur the line between biological species & vegetative compatibility groups. *Cereal Res Commun* 25:539-542.
- Lev S, Sharon A, Hadar R, Ma H, Horwitz BA (1999). A mitogen activated protein kinase of the corn leaf pathogen *Cochiobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: Diverse roles for mitogen-activated protein kinase homologs in foliar pathogens. *Proc Natl Acad Sci USA* 96:13542-13547.
- Liang H, Maynard CA, Allen RD, Powell WA (2001). Increased *Septoria musiva* resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant Mol Biol* 45:619-29.
- Liu SH, Dean RA (1997). G protein alpha subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol Plant-Microbe Interact* 10:1075-1086.

- Loewus F (1999). Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi. *Phytochemistry* 52:193-210.
- Lorenz MC, Fink GR (2001). The glyoxylate cycle is required for fungal virulence. *Nature* 412:83-86.
- Lorito M, Woo SL, Garcia I, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F, Garcia I (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proc Natl Acad Sci USA* 95:7860-7865.
- Lorito M, Scala F, Zoina A, Woo SL (2001) Enhancing biocontrol of fungal pests by exploiting the *Trichoderma* genome. In: M Vurro, J Gressel, T Butts, GE Harman, A. Pilgeram, RJ St. Leger and DL Nuss, eds. *Enhancing Biocontrol Agents and Handling Risks*. Amsterdam: IOS Press, pp 248-259.
- Marshall MA, Timberlake WE (1991). *Aspergillus nidulans wetA* activates spore-specific gene expression. *Mol Cell Biol* 11:55-62.
- Molnar A, Sulyok L, Hornok L (1990). Parasexual recombination between vegetatively incompatible strains in *Fusarium oxysporum*. *Mycol Res* 94:393-398.
- Morin L, Gianotti AF, Lauren DR (2000). Trichothecene production and pathogenicity of *Fusarium tumidum*, a candidate bioherbicide for gorse and broom in New Zealand. *Mycol Res* 104:993-999.
- Motoyama T, Fujiwara M, Kojima N, Horiuchi H, Ohta A, Takagi M (1997). The *Aspergillus nidulans* genes *chsA* and *chsD* encode chitin synthases which have redundant functions in conidia formation. *Mol Gen Genet* 253:520-528.
- Müller H (1986). Utilization of gluconate by *Aspergillus niger*. II. Enzymes of degradation pathways and main end products. *Z Mikrobiol* 141:461-469.
- Oliver MJ, Quisenberry JE, Trolinder NLG, Keim DL (1998). Control of plant gene expression. US Patent 5,723,765.
- Pedras MSC, Biesenthal CJ (2000). Vital staining of plant cell suspension cultures: evaluation of the phytotoxic activity of the phytotoxins phomalide and destruxin B. *Plant Cell Reports* 19:1135-1138.
- Perfect SE, Hughes HB, O'Connell RJ, Green JR (1999). *Colletotrichum* - A model genus for studies on pathology and fungal-plant interactions. *Fungal Genet Biol* 27:186-198.
- Plattner RD, Desjardin AE, Leslie JF, Nelson PE (1996). Identification and characterization of strains of *Gibberella fujikuroi* mating population A with rare fumonisin production phenotypes. *Mycologia* 88:416-424.
- Puyesky M, Benhamou N, Noyola PP, Bauw G, Ziv T, Van Montagu M, Herrera-Estrella A, Horwitz BA (1999). Developmental regulation of *cmp1*, a gene encoding a multidomain conidiospore surface protein of *Trichoderma*. *Fungal Genet Biol* 27:88-99.
- Quimby PC, Zidack NK, Boyette CD, Grey WE (1999). A simple method for stabilizing and granulating fungi. *Biocont Sci Tech* 9:5-8.
- Rausher MD (2001). Co-evolution and plant resistance to natural enemies. *Nature* 411:857-864.
- Regev A, Keller M, Strizhov N, Sneh B, Prudovsky E, Chet I, Ginzberg I, Koncz-Kalman Z, Koncz C, Schell J, Zilberstein A (1996). Synergistic activity of a *Bacillus thuringiensis* delta-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Appl Envir Microbiol* 62:3581-3586.
- Robinette D, Matthyse AG (1990). Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of development of the hypersensitive response elicited by *Pseudomonas syringae* pv. phaseolicola. *J Bact* 172:5742-5749.
- Robinson M, Riov J, Sharon A (1998). Indole-3-acetic acid biosynthesis in *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. *Appl Envir Microbiol* 64:5030-5032.

- Roldan TA, Espinosa AP, Rubio-M R (1999). Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* defines a new class of saponinases. *Mol Plant-Microbe Interact* 12:852-861.
- Rosen S, Yu JH, Adams TH (1999). The *Aspergillus nidulans* *sfaD* gene encodes a G protein beta subunit that is required for normal growth and repression of sporulation. *EMBO J* 18:5592-5600.
- Rosewich UL, Kistler HC (2000). Role of horizontal gene transfer in the evolution of fungi. *Ann Rev Phytopath* 38:325-363.
- Rosewich UL, Pettway RE, McDonald BA, Kistler HC (1999). High levels of gene flow and heterozygote excess characterize *Rhizoctonia solani* AG-11A (*Thanatephorus cucumeris*) from Texas. *Fungal Genet Biol* 28:148-159.
- Ruan YJ, Straney DC (1996). Identification of elements in the PDA1 promoter of *Nectria haematococca* necessary for a high level of transcription in vitro. *Mol Gen Genet* 250:29-38.
- Sands DC, Miller RV (1993) Altering the host range of mycoherbicides by genetic manipulation. In: SO Duke, JJ Menn, JR Plimmer, eds. *Pest Control with Enhanced Environmental Safety*. Washington, DC: American Chemical Society, pp 101-109.
- Sands DC, Pilgeram A (2001) Enhancing the efficacy of biocontrol agents against weeds. In: M Vurro, J Gressel, T Butts, GE Harman, A. Pilgeram, RJ St. Leger and DL Nuss, eds. *Enhancing Biocontrol Agents and Handling Risks*. Amsterdam. IOS Press pp 3-13.
- Saupe SJ, Kuldau GA, Smith ML, Glass NL (1996). The product of the hetC heterokaryon incompatibility gene of *Neurospora crassa* has characteristics of a glycine-rich cell wall protein. *Genetics* 143:1589-1600.
- Schmitt MJ, Schernikau G (1997). Construction of a cDNA-based $K_1/K_2/K_{28}$. *Food Tech Biotech* 35:281-285.
- Screen SE, St. Leger RJ (2000). Cloning, expression, and substrate specificity of a fungal chymotrypsin. Evidence of lateral gene transfer from an actinomycete bacterium. *J Biol Chem* 275:6689-6694.
- Sebghati TS, Engle JT, Goldman WE (2000). Intracellular parasitism by *Histoplasma capsulatum*: Fungal virulence and calcium dependence. *Science* 290:1368-1372.
- Sewall TC, Mims CW, Timberlake WE (1990). *abaA* controls phialide differentiation in *Aspergillus nidulans*. *Plant Cell* 2:731-739.
- Sharon A, Amsellem Z, Gressel J (1992a). Glyphosate suppression of an elicited defense response. *Plant Physiol* 98:654-659.
- Sharon A, Ghirlando R, Gressel J (1992b). Isolation, purification and identification of 2-(*p*-hydroxyphenoxy)-5,7-dihydroxychromone: a fungal induced phytoalexin from *Cassia obtusifolia*. *Plant Physiol* 98:303-308.
- Shinshi H, Mohnen D, Meins FJ (1987). Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *Proc Natl Acad Sci USA* 84:89-93.
- Shiotani H, Tsuge T (1995). Efficient gene targeting in the filamentous fungus *Alternaria alternata*. *Mol Gen Genet* 248:142-150.
- St. Leger RJ, Screen S (2001) Genetic improvement of fungi for insect and weed control. In: TM Butt, C Jackson, N Magan, eds. *Fungal Biocontrol Agents: Progress, Problems and Potential.*. Wallingford, UK: CABI, pp. 219-237.
- St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1996). Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proc Natl Acad Sci USA* 93:6349-6354.
- Stanghellini ME, Rasmussen SL, Veandemark GJ (1993). Relationship of callose deposition to resistance of lettuce to *Plasmopara lactucae-radici*s. *Phytopathology* 83:1498-1501.

- Stephens KE, Miller KY, Miller BL (1999). Functional analysis of DNA sequences required for conidium-specific expression of the *spoCl-C1C* gene of *Aspergillus nidulans*. *Fungal Genet Biol* 27:231-242.
- Taguchi T, Uraguchi A, Katsumi M (1999). Auxin- and acid-induced changes in the mechanical properties of the cell wall. *Plant Cell Physiol* 40:743-749.
- Takano Y, Kikuchi T, Kubo Y, Hamer JE, Mise K, Furusawa I (2000). The *Colletotrichum lagenarium* MAP kinase gene *CMKI* regulates diverse aspects of fungal pathogenesis. *Mol Plant-Microbe Interact* 13:374-383.
- Talbot NJ, Kershaw MJ, Wakley GE, deVries OMH, Wessels JGH, Hamer JE (1996). *MPG1* encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*. *Plant Cell* 8:985-999.
- TeBeest DO, ed. (1991) *Microbial Control of Weeds*. New York: Chapman and Hall, 284p.
- Tokimatsu T, Nagai Y, Hattori T, Shimada M (1998). Purification and characteristics of a novel cytochrome c dependent glyoxylate dehydrogenase from a wood-destroying fungus *Tyromyces palustris*. *FEBS Lett* 437:117-121.
- Truesdell GM, Yang ZH, Dickman MB (2000). A G alpha subunit gene from the phytopathogenic fungus *Colletotrichum trifolii* is required for conidial germination. *Physiol Mol Plant Path* 56:131-140.
- Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ (1999). A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *J Bact* 181:6469-6477.
- Viaud M, Couteaudier Y, Riba G (1998). Molecular analysis of hypervirulent somatic hybrids of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria sulfurescens*. *Appl Envir Microbiol* 64:88-93.
- Vurro M (2001) Microbial toxins in biocontrol enhancement strategies. In: M Vurro, J Gressel, T Butts, GE Harman, A. Pilgeram, RJ St. Leger and DL Nuss, eds. *Enhancing Biocontrol Agents and Handling Risks*. Amsterdam: IOS Press, pp 28-37.
- Vurro M, Gressel J, Butts T, Harman G, Pilgeram A, St.-Leger R, Nuss D. (eds) (2001a) *Enhancing Biocontrol Agents and Handling Risks*. Amsterdam: IOS Press.
- Vurro M, Zonno MC, Evidente A, Andolfi A, Montemurro P (2001b). Enhancement of efficacy of *Ascochyta caulina* to control *Chenopodium album* by use of phytotoxins and reduced rates of herbicides. *Biol Contr* 21:182-190.
- Walton JD (2000). Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: An hypothesis. *Fungal Genet Biol* 30:167-171.
- Watson AK, Ahn B-S (2001) Better control of weeds with enhanced bioherbicides. In: M Vurro, J Gressel, T Butts, GE Harman, A. Pilgeram, RJ St. Leger and DL Nuss, eds. *Enhancing Biocontrol Agents and Handling Risks*. Amsterdam: IOS Press, pp 106-113.
- Weston VCM (1999). The commercial realization of biological herbicides. *Brighton Crop Protect Conf - Weeds*:281-288.
- Whitham SA, Yamamoto ML, Carrington JC (1999). Selectable viruses and altered susceptibility mutants in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 96:772-777.
- Wilhite SE, Lumsden RD, Straney DC (2001). Peptide synthetase gene in *Trichoderma virens*. *Appl Envir Microbiol* 67:5055-5062.
- Wu JG, Miller BL (1997). *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translation mechanisms regulating stunted gene expression. *Mol Cell Biol* 17:6191-6201.
- Xu J-R, Hamer JE (1996). MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Devel* 10:2696-2706.

- Yamada O (1999). Cloning and functional analysis of the *Aspergillus oryzae* conidiation regulator gene *br1A* by its disruption and misscheduled expression. *J Biosci Bioeng* 87:424-429.
- Zaghmout OF, Dang PD, Allen RD (1997). Expression of oxalate oxidase in transgenic plants provides resistance to oxalic acid and oxalate-producing fungi. *Plant Physiol* 114:1152-1152.
- Zhou T, Boland GJ (1999). Mycelial growth and production of oxalic acid by virulent and hypovirulent isolates of *Sclerotinia sclerotiorum*. *Can J Plant Path* 21:93-99.
- Zhu T, Mettenburg K, Peterson DJ, Tagliani L, Baszczynski CL (2000). Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. *Nature Biotech* 18:555-558.

Table 1. Simple genes that may be appropriate for enhancing biocontrol activity of weeds by microorganisms

Gene	Property	Ref.
<u>Soft genes</u>		
tomatinase/ <i>pdal</i>	Degrade phytoalexins	Roldan et al. (1999); Ruan and Straney (1996)
<i>iaaH</i>	Auxin biosynthesis	Robinson et al. (1998)
<i>iaaM</i>	Auxin biosynthesis	Comai and Kosuge (1982)
Xalicae	Inhibit callose synthase and other enzymes	Robinson et al. (1998)
<i>cbp1</i>	Binds calcium, depriving host	Sebghati et al. (2000)
<u>Intermediate^a</u>		
Branch chain amino acid	Secrete branch chain amino acids, effect sirr to ALS inhibitors	Sands and Pilgeram (2001)
<i>P450 sul</i>	Converts pro-herbicide to herbicide	Whitham et al. (1999)
<u>Hard</u>		
<i>Nep1</i>	Protein causes leaf necrosis	Bailey et al. (2000); Jennings et al. (2000)
<i>Yeast killer genes</i>	Will they kill plants?	Schmitt and Schernikau (1997)

^a See also Table 2.1 in (Gressel 2002) for organisms containing genes that are known to have plant-specific toxins

Table 2. Genes that might act as transgenetic mitigators (TM) if introgressed into non-target pathogens in gene dependent orientation and regulation

<u>Target Process</u>	(Presumed) mode of gene action	Ref.
<i>Potential TM gene</i>		
<u>Reproduction</u>		
<i>hetC</i>	Heterokaryon incompatibility	Saupe et al. (1996)
<i>sfaD****</i>	Repressing G protein β subunit	Rosen et al. (1999)
<i>stuA</i>	Transcription factor	Wu and Miller (1997)
<u>Appressorium formation*</u>		
<i>mpg1</i>	Hydrophobic surface recognition protein	Talbot et al. (1996)
<i>mac1***</i>	adenylate cyclase	Choi and Dean (1997)
<i>pmk1</i>	MAP kinase	Xu and Hamer (1996)
<u>Spore stalk formation</u>		
<i>fluG</i>	Unknown diffusible factor	Lee and Adams (1996)
<i>brl A</i>	Contains Zn finger motifs	Yamada (1999)
<i>chsA/chsE</i>	Chitin formation	Culp et al. (2000)
<i>chk1**</i>	Mitogen activated protein kinase	Lev et al. (1999)
<u>Viable spore formation*</u>		
<i>abaA</i>	Regulates phialide to spore transition	Sewall et al. (1990)
<i>magC</i>	G protein α subunit-decreases conidiation	Liu and Dean (1997)
<i>cmp1</i>	Spore surface protein	Puyesky et al. (1999)
<i>acr1**</i>	Prevents regulation of <i>mpg1</i> for spore maturation	Lau and Hamer (1998)
<i>chsA/chsD</i>	Chitin synthesis	Motoyama et al. (1997)
<u>Spore germination</u>		
<i>cmk1**</i>	MAP kinase (regulator gene)	Takano et al. (2000)
<i>ctg1</i>	G protein α subunit required for germination	Truesdell et al. (2000)
<u>Melanin formation</u>		
<i>alb1</i>	Polyketide synthase	Fujii et al. (1999); Motoyama et al (1997); Takano et al. (2000);

<i>arp1</i>	Scytalone dehydratase	Tsai et al. (1999); Wu and Miller (Kubo et al. (1996); Langfelder et (1998); Takano et al. (2000)
<i>arp2</i>	Hydroxynaphthalene reductase	(Takano et al. (2000)
<u>Mineral nutrition</u>		
<i>psy1</i>	Iron siderophore production	(Wilhite et al. (2001)

* no spores are produced when there are no spore stalks; ** also prevents appressorium formation; *** also reduces sporulation. Additionally, there are other spore-specific genes that are expressed, with yet unclassified phenotypes that may prove to be vital for spore function. These include *SpoCI-c1c*, (Stephens et al. 1999) **** must be used in the constitutive sense form to suppress reproduction. Source: Modified from Gressel (2001) by permission of Elsevier.

Figure Legend

Figure 1. Dual failsafes to prevent (Step 1) spread of biocontrol agents, and (Step 2) their introgression into other organisms.

a. chlamydospores; b. microconidia; c. macroconidia; d. ascus with ascospores; e. sclerotia; f. asporogenic mycelia

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