Enhancing the Efficacy of Bioherbicides

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1. INTRODUCTION: BIOLOGICAL CONTROL, THE MISSING COMPONENTS;

If a scientist were funded today to develop a biological control agent against a specific weed, using the current technologies, he or she would probably fail to achieve efficacious and economically justifiable control of the weed. We in this field don't like to hear this, because it interferes with our vision and with our ability to compete for grants. We need new concepts, paradigms, and algorithms involving virulence enhancement, genetics of host range determinants, dissemination, and the biochemistry and evolution of the host/parasite interaction. We will attempt to cover these subjects in this paper. The more conventional incremental approach would be boring, fruitless and redundant.

2. AMINO ACID EXCRETION ENHANCES THE VIRULENCE OF PLANT PATHOGENS

The virulence and efficacy of bioherbicides can be greatly enhanced by selecting variants of the weed pathogen that overproduce and excrete amino acids that are inhibitory to the target plant (Tiourebaev, 1999)]. Our approach to enhancing the virulence of bioherbicides is modeled after "Frenching disease", a naturally occurring disease of tobacco (Steinberg, 1946). Steinberg et al. (1950) discovered that saprophytic bacteria (*Pseudomonas fluorescens* and *Bacillus subtilis*), growing on the roots of symptomatic plants overproduced a single amino acid, isoleucine. Isoleucine is synthesized in plants via the branched chain amino acid pathway. The end products of the pathway (valine, leucine, and isoleucine) allosterically regulate

the activity of acetolactate synthase (ALS). The enzyme is differentially inhibited by these amino acids in different plant species. In "Frenching disease", overproduction of isoleucine by the saprophytic bacteria inhibited the activity of ALS in the tobacco, shutting down synthesis of valine and leucine, which disrupted essential protein metabolism. Free-living bacteria have complex systems of regulating their amino acid biosynthesis, permitting them to tolerate unusual imbalances in external amino acids. In contrast, plants are less well regulated, seemingly because they are self contained and independent of external amino acid sources. A plant's regulation may be considered "degenerate" in that one amino acid may be the regulatory factor for the whole group.

Interestingly, several modern chemical herbicides also target biosynthesis and inhibit single biosynthetic enzymes in plants, rendering treated plants incapable of producing a metabolite essential for plant growth. For example, ALS, the key enzyme in the branched chain amino acid biosynthesis, is inhibited by the sulfonylurea herbicides. The herbicide glyphosate inhibits 5'enolpyruvylshikimate 3-phosphate synthase (EPSPS), the key enzyme in the shikimic acid pathway (Amrhein, 1986)].

Given this knowledge, we screened our target weed for amino acid inhibition and determined that it was inhibited by mMolar amounts of the amino acid valine. We then selected variants of our pathogen, *Fusarium oxysporum* f. sp. *cannabis,* that were resistant to valine analogs. Some of these resistant variants excreted 10-55 times more valine than their wild type parent and were more virulent to the target weed (Table 1) (Tiourebaev, 1999). The wild type strain controlled 25% of the target plant, while the valine excretion increased control to 70-90%. In addition, the development of disease was more rapid in the plants infested with the valine overproducers. Limited studies on fourteen other plant species did not reveal a change in host range. Thus, overproduction of an essential amino acid provided a highly effective means of enhancing the virulence of a biocontrol agent (Sands *et al.* 1999). This technology has also been used to enhance the virulence of *Fusarium oxysporum* f. sp. *papaveris*, *Pseudomonas syringae* pv. *tagetis*, and *Xanthomonas campestris* pv. *poae* .

Table 1. Valine excretion and virulence of valine overproducing variants of *F. oxysporum* f. sp. *cannabis*.

Strain	Description	Valine Excretion[a] (mg/l)	% Kill	Mortality Rate[b] (weeks)
C95	Wild-type	0-0.18	25	6-8
4nv	Norvaline^R	2.84	70	2-3
6pa	Penicillamine^R	2.48	90	2-3
8pa	Penicillamine^R	9.93	90	2

a. Valine excretion. Valine excretion was determined by spectrophotometric analysis of growth of *Pediococcus cerevisiae* ATCC8042 in culture supernatant.
b. Mortality Rate. Mortality rate is the duration between inoculation and the first appearance of severe disease symptoms or death.

3. GENERAL METHODOLOGY

3.1 Determination of amino acids that are most inhibitory to the target weed.

Surface sterilized seed are placed on plates of water agar (1.5% agar, 1 mM Tris, pH 6.8) that have been supplemented with a single amino acid (2-5 mM l-form). Amino acid(s) that decrease seed germination, inhibit shoot growth or cause necrosis are further evaluated (For example, tryptophan is inhibitory to growth in Fig. 1). Certain plants may be more inhibited by combinations of amino acids rather than single amino acids. Combinations of all the amino acids derived from a single pathway typically are not inhibitory.

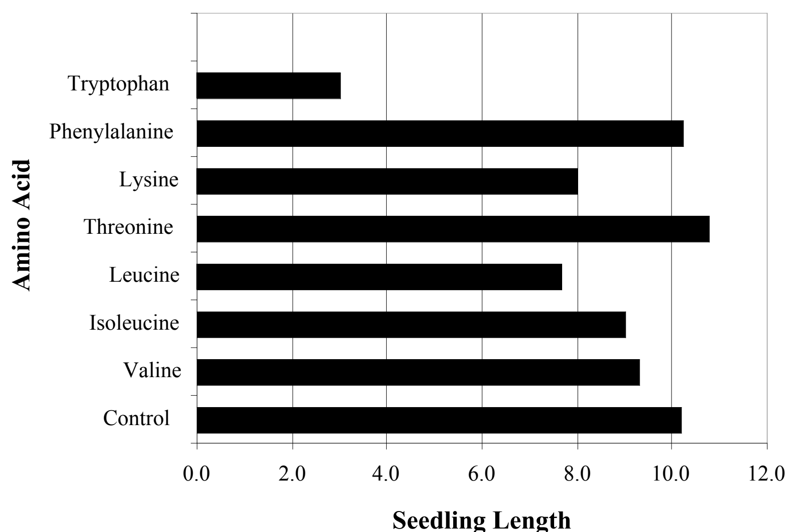

Figure 1. Inhibition of the growth of field bindweed seedlings by selected l-amino acids. Growth was measured 14 days after placing the seed on the amino acid plates.

3.2 Selection of variants of the bioherbicide resistant to analogs of the selected amino acid.

Amino acid overproducing strains of each fungus or bacterium are selected by exposure to specific amino acid analogs (Table 2). For example, if the target weed is inhibited by lysine, then pathogens for control of that weed will be exposed to lysine analogs to select mutants that overproduce lysine. Resistant colonies can be selected using a well zone–diffusion assay on CUTS minimal medium (Czapek-Dox Agar (Difco) (35 g/l) supplemented with ammonium sulfate (0.5 g/l), uracil (20 mg/l), thiamine (4 mg/l) and a vitamin mixture (100 mg of crushed Sesame Street Complete Vitamins). The zone diffusion plates are prepared by cutting a plug from the center of the plate. The plates are then inoculated with 10^6 - 10^7 fungal spores, a suspension of 10^3 – 10^5 mycelial fragments, or a suspension of 10^7 - 10^8 bacteria. A sterile solution of the amino acid analog (0.1 ml of a100 mM solution) is then added to the well. The plates are incubated in a laminar flow hood for 4 hours and an additional 0.1 ml of the analog solution is added to the well. The plates are then incubated at 28C and monitored daily for the appearance of zones of inhibition and resistant colonies within the zone. Resistant colonies are isolated and analyzed for amino acid excretion. This selection may be repeated several times using increasing concentrations of analog and/or different analogs.

Table 2. Amino acid analogs.

Amino Acid	Analog(s)
Valine	Valine hydroxamate, norvaline, penicillamine
Lysine	Aminoethyl-cysteine, hydroxylysine, aminocaproic acid
Methionine	selenoethionine, ethionine, selenomethionine,
Tryptophan	5-Methyltryptophane
Arginine	Canavanine
Proline	Hydroxy-proline, dehydro-proline
Leucine	Norleucine

3.3 Assay for amino acid excretion.

Bacterial auxotrophs are used to assay amino acid excretion (Sands and Hankin, 1974). The auxotroph is seeded into media lacking the amino acid required for growth. Subsequent growth of the auxotroph in the media is dependent upon and proportional to the quantity of added amino acid. For example, in order to assay valine, a valine auxotroph of *E. coli* is seeded into CUTS medium. The auxotroph will not grow unless exogenous valine is added to the medium. Colonies of the plant pathogenic fungi or bacteria that are resistant to a valine analog are sub-cultured onto the seeded medium and incubated for 2-3 days. If the resistant variants excrete

valine, there will be a zone of auxotroph growth surrounding the colony. The size of the zone is proportional to the amount of valine excreted. A standard dose-response can be determined by placing discs containing various levels of amino acid onto the agar.

3.4 Testing virulence and host range of the amino acid overproducing variants in growth chamber studies.

The virulence (rate of kill and % mortality) of amino acid producing variants of each pathogen are evaluated by inoculating target weed plants with each amino acid excreting variant and its respective wild type parent. Amino acid excreting variants that are more virulent than the parent should be further evaluated in host range experiments and scale-up experiments.

4. IMPROVING DISSEMINATION

The effectiveness of a soil-applied mycoherbicide depends upon the survival and movement of the pathogen from the formulation particle through the soil profile to the root and crown of the target plant (Fravel *et al.,*1996; Bailey *et al*.,1997, Ciotola *et al*., 2000). Spore suspensions and food-based formulations are common delivery systems for bioherbicides. However, this technique does not work well for *F. oxysporum,* as spores become bound by soil particles in the top few millimeters of the soil (Connick *et al*., 1998). *Fusarium oxysporum* saprophytically colonizes the roots of many non-host plants (Burgess, 1981). Thus, *Fusarium oxysporum* mycoherbicides could be delivered to farmers' fields on non-host seed such as crops or grass, positioning the mycoherbicide directly in the rhizosphere of the target weed (Sands *et al,* 2002; Tiorebaev, 1999; Ciotola *et al*., 2000). In addition, the multiplication of fungal biomass in the rhizosphere of the carrier seedling allows for application of low levels of the mycoherbicide, greatly reducing the cost of inoculum production. Many phytopathogenic bacteria such as *Pseudomonas syringae* and *Xanthomonas campestris* also have a saprophytic phase and could be delivered in a similar manner.

5. GENETICS: READY OR NOT

Genetic techniques have only recently been integrated into the field of biocontrol. When confronted with poor field trials, biocontrol researchers simply sought more efficacious formulations or looked for new pathogens. The following sections cover some of the efforts currently underway in the genetic improvement of mycoherbicides.

5.1 Genetic Marking.

Researchers often want to track the fate of a released biocontrol strain. Three types of genetic markers have been used to label bioherbicides: auxotrophy (Miller *et al*., 1989; Chacko *et al*., 1994; Yang and TeBeest, 1995), antibiotic resistance [Yang and TeBeest, 1995;Toyota *et al*., 1992; Holmstrom-Ruddick and Mortensen, 1995; Brooker *et al*., 1996) and visual markers such as *gus* and *gfp* (Cohen *et al,* 2002; Robinson and Sharon, 1999). Transformation with *lux* genes that confer bioluminescence is also a possibility for marking bioherbicidal bacteria (Kennedy, 1995; Kragelund *et al*., 1997). Amino acid analog resistant mutants for virulence enhancement have an unanticipated value in that the strains are marked by their resistance and can be easily be followed using selective media. Care has to be taken to select for mutants with normal growth and optimal virulence characteristics.

5.2 Contained Systems

Biocontrol agents must be judged to be safe prior to release into a new environment, because once an organism is released, there may be no way to get it back. Host range evaluation, human toxicity and environmental risk analysis can be used to reach a probability-based decision on safety. Possible genetic-containment systems include temperature sensitivity (species or mutants unable to survive the heat of summer or the cold of winter), antibiotic requiring mutants that can only grow in the presence of antibiotic, auxotrophy, or mutants unable to produce sexual or survival structures (Gressell, 2001; Miller *et al*., 1989a).

5.3 Virulence.

Besides the already mentioned virulence enhancement by amino acid excretion, other ways to enhance virulence also include overproduction of known virulence genes or genes whose products disengage host responses. The genes encoding production of the natural herbicide bialaphos have been cloned from *Streptomyces hygroscopicus* (Murakami, 1986) and transferred into the plant pathogen *Xanthomonas campestris* pv. *campestris* (Charudattan *et al*., 1996). This herbicide inhibits the enzyme glutamine synthetase and disrupts normal amino acid metabolism. The transformed bacterium retained pathogenicity to its natural hosts (broccoli and cabbage) but elicited a hypersensitive response on four nonhost plants, suggesting that the host range of the pathogen was broadened (Charudattan *et al*., 1996). The number of genes and size of the gene cluster necessary for bialaphos production may limit transfer of this technology to mycoherbicidal strains of plant pathogenic fungi. Cohen *et al*. (2002) transformed two bioherbicidal strains of *Fusarium* with two genes from the indole-3-acetimide (IAA) pathway. Transgenic strains of the fungi were more effective in suppressing the plant parasitic weed, *Orobanche*, than the parental wild-types. Thus, stimulating an auxin imbalance also enhanced pathogen virulence.

5.4 Isolation of Promoters.

DNA promoters can be isolated from plant pathogenic fungi (Weltring, 1995) or bacteria (Horwitz and Loeb, 1986) by screening libraries consisting of small DNA fragments that have been inserted into plasmids containing a promoter-less selectable marker. The plasmid library is transformed into the target pathogen and the transformants are screened for expression of the selectable marker. DNA fragments that drive expression of the marker are considered promoters. Inducible promoters can be isolated by differentially screening for expression under different conditions or in the presence of different inducers. Desirable promoters include host specific, tissue specific, temperature specific, or herbicide-inducible.

Plants produce a variety of secondary compounds. Some of these compounds are specific to certain plant species. For example, tomato produces an antifungal saponin called alpha-tomatine. *Fusarium oxysporum* f. sp. *lycopersici*, a pathogen of tomato, produces tomatinase, an enzyme that degrades the tomatine (Roldan *et al*., 1999). The promoter regulating expression of the tomatinase is induced by the tomatine, and is therefore, tomato-specific. This tomato-specific promoter may not prove useful in biocontrol. However, spotted knapweed produces cnicin, a secondary metabolite that inhibits the growth of other plants. A cnicin-specific promoter would allow targeting of hypovirulence genes to spotted knapweed.

6. SPECULATIONS: BOOLEAN ALGORITHMS, EVOLUTION, AND BIOHERBICIDES

Many of the more than 200,000 known species of plants are attacked by host-specific fungi or bacteria. Given this situation, where the diversity of the plant kingdom is paralleled by near equivalent diversity in plant pathogens, there might be some underlying principles of evolution whereby the host-parasite relationship was established. If we assume that each host-parasite relationship is completely unique, then we might expect that genetic analysis of each pair would reveal unique sets of genes conferring unique biochemistry. Studying all of these differences would require a lot of biochemists. On the other hand, if the host parasite relationship could partially be explained by a defined series of biochemical“ decisions or states” of the either/or type (i.e., Boolean (Wolfram, 2001), then some general and predictable kinds of genetic adaptations could account for host specificity. We report evidence for some “either /or” losses of options in the host and concomitantly in the homologous pathogen. We hypothesize that, in the case of host-specific plant pathogen relationships, a handful of variations in the regulation at branch points of intermediary metabolism may account for a significant amount of the biochemical differences leading to host-specificity.

At first glance, the host-specific but non-obligate plant pathogenic microbes and strict saprophytic microbes are very similar in their metabolism in that both grow on minimal media with simple carbon and nitrogen sources. But in closer examination,

there are significant differences. In strictly saprophytic microbes, amino acid biosynthesis is meticulously regulated because the external environment relative to nutrients such as amino acids can be quite variable. In the host-specific plant pathogenic pseudomonads much of this regulation does not exist (Sands and Zuker 1976)). Pathovars of *Pseudomonas syringae* are inhibited by certain single amino acids that shut down an entire pathway, even when not all end-products are available. This pattern appears to be pathovar dependent. Related saprophytes (*P. fluorescens* and *P. putida)* appear to be well regulated as they are not inhibited by single amino acids. Perhaps host-specific pathogens, limited to a specialized environment, do not have frequent occasion to regulate for certain amino acid imbalances. For instance, if a plant “milieu interior” is routinely high in the free amino acid lysine, then the lysine regulated aspartokinase in the pathogen would seldom if ever be active. One might presume that this unused enzyme, and the corresponding gene, might in time suffer losses in efficacy, leading eventually to deletion and permanent loss.

This suggests that the key regulatory point of a biosynthetic pathway can serve as an “either/or” gate. In Boolean algebra, more commonly used in decision trees in computer programming, this branch point could be referred to as a ”Boolean Gate”. Unless all gates are set correctly within a pathogen, there is no chance for protein biosynthesis and nor hence for growth of the pathogen to sufficiently high levels to cause disease. A handful of such gates operating in parallel would be sufficient to narrow the biosynthetic window of a pathogen to one or a few plants. Regardless of the relative merits of the structure of models, it is clear that both plants and their microbial pathogens are subjected to misregulation by an oversupply of some amino acids, usually at key branch points in their biosynthetic pathways. These points of misregulation may serve as determinants of host-pathogen specificity. For instance a plant always high in lysine might only be a host for pathogens without lysine regulation of their single aspartokinase. Possession of a single acetolactate synthase inhibitable by valine would render a pathogen only capable of attacking plants either low in free valine or high in isoleucine and leucine, the other two amino acids in that biosynthetic family. Alternatively a microbe that invades the vascular system of a plant and/or resides in high numbers in its rhizosphere and excretes a single amino in sufficiently high amounts may be expected to inhibit plant growth.

6. CONCLUSION

Over the last thirty years, numerous pathogens have been investigated as potential bioherbicides. Despite this intensive research effort, few pathogens have been successful as biocontrol agents. The inherent constraints associated with biological species are largely responsible for this failure, yet our preconceived ideas about these agents are also at fault. The authors believe that a paradigm shift must occur if bioherbicides are to enjoy wider success as a weed control method. In the largely unsuccessful past, researchers have required lethality and host specificity as requirements for a successful agent. However, many pathogens that do not meet

these criteria can be enhanced by simple selection as described in this paper, or through, perish the thought, recombinant genetics.

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