

Enhancing the Efficacy of Plant Pathogens for control of weeds

Short title: Enhancing biocontrol

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Abstract- There are many plant pathogens that attack weeds, but only a few have proven virulent enough to control weed species [1] and compete with chemical herbicides. One might surmise that there has been strong selection against highly virulent host-specific pathogens, as survival of the pathogen depends upon survival of the host. Total eradication of the host weed would not benefit the pathogen, an impasse that challenges researchers to develop innovative strategies using formulation, genetics, and synergy to enhance the effectiveness of biocontrol pathogens. Our research has capitalized on the inhibitory effects of certain amino acids on plant growth and development. Biocontrol pathogens that overproduce selected amino acids have increased virulence to the target weed and enhanced field performance. We report enhancement of virulence in three separate pathogen-host systems, two with *Fusarium* and one with *Pseudomonas*.

Keywords: Canada thistle, *Poa annua*, plant pathogen, amino acid, virulence

1. Introduction: Plant disease epidemics.

Severe disease epidemics are rarely observed in native plant or dispersed weed populations. Epidemics are more frequently observed in monocultures that lack genetic diversity and distance between susceptible plants.

Small changes in the fitness or susceptibility of a plant or small changes in the virulence of a pathogen can drastically alter the severity of a plant epidemic. Changes in crop plant resistance can occur rather rapidly due to breeding and more recently genetic engineering. In contrast, changes in pathogen populations result from random mutations. Disease-resistant monocultures may enhance selection for pathogens with increased virulence driving further selection of disease resistance.

There is decreased selection pressure in native plant/pathogen populations. First, native plant populations have greater genetic diversity and tend to occur over much larger distances with variable density. Plant disease, regardless of severity, may be contained simply by distance between susceptible hosts.

Weed populations are intermediate in diversity between native plant populations and agricultural crops. Early in a weed infestation, plants are dispersed. However, many weed infestations (spotted knapweed, leafy spurge) rapidly progress to monoculture providing uniformly susceptible or resistant host populations. The invasiveness of a weed is often correlated to its adaptation to a new environment and the inability of pathogens and insect pests to match its rapid expansion in the new environment. Disease within a plant population should become more prevalent with decreasing diversity within the plant population.

Biocontrol researchers have exerted a tremendous effort to find naturally-occurring pathogens capable of controlling noxious weeds. There are pathogens that will attack weeds. However, there are very few pathogens that suppress weed expansion, much less actually eradicate a weed population. In pathogen-host interactions virulence is expensive and eradication of the host is suicidal. Therefore, parasitism becomes the more beneficial interaction for the pathogen, ensuring longer term survival of the pathogen.

In our research we have found that every weed so far examined is inhibited by at least one amino acid. This observation leads to the conclusion that weeds have a weakness that can be readily exploited. Subsequent studies have found that the virulence and efficacy of bioherbicides can be greatly enhanced by selecting for variants of weed

pathogens that overproduce and excrete amino acids that are inhibitory to a target plant [2]. The host range of the enhanced pathogen remains unaltered and very few plants within the population have been observed that are tolerant of such an amino acid imbalance. Alternatively, the fitness of a weed, and therefore its resistance to plant pests, can be reduced by direct application of inhibitory amino acids.

2 Enhancement of Bioherbicides

2.1 Criteria for selection of biocontrol agents.

Classical biocontrol has proven successful in a few situations including biocontrol of rush skeletonweed with *Puccinia Chondrilla* in Australia [3] and *Acacia saligna* by the rust fungus *Uromycladium tepperianum* in South Africa [4]. These successes have utilized obligate pathogens that are highly host-specific, highly virulent, and capable of naturally spreading from a focal inoculation point. Such pathogens are few and far between. Since virulence of a pathogen can be increased, we can focus only on those pathogens with host specificity and a disseminative nature. Unfortunately, there are still few candidates available for most weeds. There are a number of genera of plant pathogenic fungi and bacteria where there are forma speciales or pathovars that display narrow host specificity including fungi (*Fusarium oxysporum*, several species of *Phomopsis* and *Colletotrichum*, and the rust fungi) and bacteria (*Ralstonia*, *Pseudomonas syringae* and *Xanthomonas*). These agents offer the added advantage of being easily disseminated.

2.2 Selection of biocontrol agents that excrete target amino acids

The virulence and efficacy of bioherbicides is enhanced by selection of variants of the pathogen that overproduce and excrete amino acids that are inhibitory to the target plant [2, 5]. This approach is modeled after "Frenching disease", a naturally occurring disease of tobacco [6]. Steinberg et al. [7] discovered that saprophytic bacteria 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 in turn disrupted essential

protein metabolism. Interestingly, several modern chemical herbicides mimic this strategy by inhibiting single biosynthetic enzymes in plants, rendering treated plants incapable of producing a metabolite essential for plant growth [8].

The growth of *Cannabis sativa*, an illicit crop and a noxious weed, is inhibited by the amino acid valine. We isolated variants of *F. oxysporum* f. sp. *cannabis* that were resistant to valine analogs. When analyzed these variants excreted 10-55 times more valine than their wild type parent (Table 1) [2]. Subsequently, valine-excreting strains of *F. oxysporum* f. sp. *cannabis* were more virulent to *C. sativa* than the wild type parent (Table 1). The wild type strain resulted in 25% control of the target plant, while the valine mutants increased control to 70-90%. In addition, the development of wilt 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.

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

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

a. Spontaneous mutant strains were selected for their resistance to successively higher levels of valine analogs. Strain 4nv is resistant to norvaline and strains 6pa and 8pa are resistant to penicillamine.

b. Valine excretion was bioassayed by spectrophotometric analysis of growth of *Pediococcus cerevisiae* ATCC 8042 in culture supernatant.

c. Mortality rate is the duration between inoculation and the first appearance of severe disease symptoms or death (greenhouse studies).

Thus, overproduction of an essential amino acid provided a highly effective means of enhancing the virulence of a biocontrol agent and has been used to enhance the virulence of *Fusarium oxysporum* f. sp. *cannabis* [9], *F. oxysporum* f. sp. *papaveris* [2], *Pseudomonas syringae* pv. *tagetis* (N. Zidack, personal communication), *Fusarium oxysporum* for control of *Orobancha* [5] and *Xanthomonas campestris* pv. *poae* (A. Pilgeram, personal communication).

2.3 INHIBITION OF WEEDS BY AMINO ACIDS

Amino acids, when applied to plants or seeds have a definite effect on plant health [5]. In all cases where noxious weeds have been analyzed for amino acid sensitivity an amino acid has been found that negatively affects the health of the plant. Inhibitory effects vary and include necrosis, wilting and stunting of growth. Certain amino acids actually enhance the growth and vigour of certain plants. Amino acids are applied to the soil at the base of the plant or drenched over the entire plant. In *Poa annua*, methionine stopped growth of the weed within days of application of the amino acid (Fig. 1). Similarly when lysine is applied to Canada thistle, necrosis was observed on the leaves within days. Application of methionine plus to Canada thistle resulted in yellowing on new leaf buds as well as necrosis. Other amino acids had little or no effect on the plants.

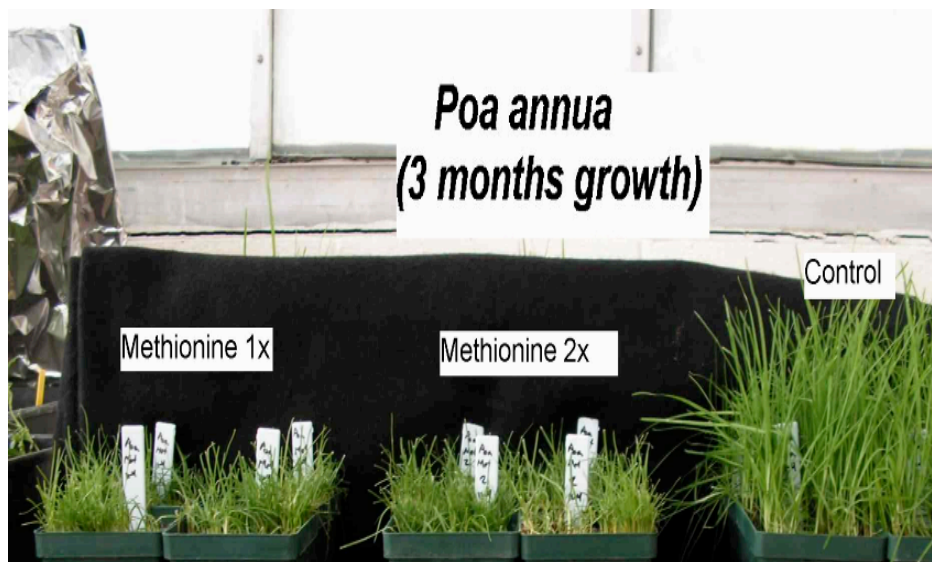


Figure 1. Growth of *Poa annua* 3 months after the application of methionine at 50mmol (1X), 100mmol (2X), or 0 mmol (Control) concentrations.

3. General Methodology

3.1. DETERMINATION OF AMINO ACIDS OR COMBINATIONS OF AMINO ACIDS THAT ARE MOST INHIBITORY TO THE GROWTH AND DEVELOPMENT OF 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). The inhibitory effects of amino acids in the branch chain pathway (valine, leucine, isoleucine), the aspartate pathway (lysine, threonine, and methionine) and the aromatic pathway (tyrosine, tryptophan, phenylalanine) can be evaluated as amino acid(s) that decrease seed germination, inhibit shoot growth or cause necrosis. (Fig. 2). Effects may be seen with single or combinations of amino acids depending on the plant involved.

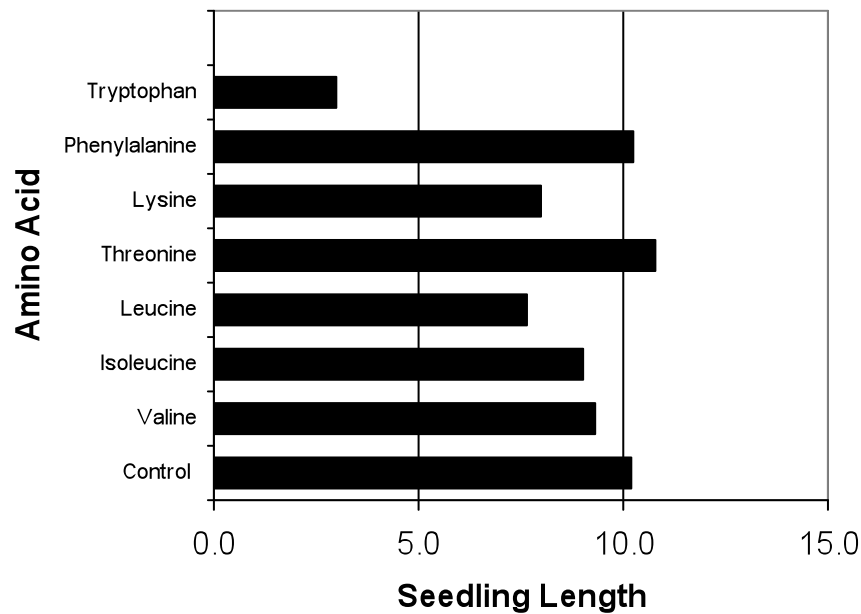


Figure 2. Inhibition of the growth of field bindweed seedlings by selected l-amino acids. (Seedling growth was measured 14 days after placing the seed on a water agar plate supplemented with amino acid.)

The lowest inhibitory concentrations of amino acids that are inhibitory to a target plant are determined by placing surface-sterilized seed on water agar that has been supplemented with increasing concentrations of the selected amino acid(s) (0 mM, 0.01 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, 3 mM, 4 mM).

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 can be selected by exposure to specific amino acid analogs [10]. For example, if the target weed is inhibited by lysine, then pathogens for control of that weed are 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 (blank mm) plug from the center of the CUTS plate with a sterile cork borer. 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 a 100 mM solution) is then added to the well. The plates are incubated in a laminar flow hood for 4 hours. An additional 0.1 ml of the analog solution is added to the well. The plates are incubated until the analog solution is absorbed into the agar and an additional 0.1 ml of 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 (Fig 3). Resistant colonies are isolated and analyzed for amino acid excretion. This selection may need to be repeated several times using increasing concentrations of analog and/or different analogs.

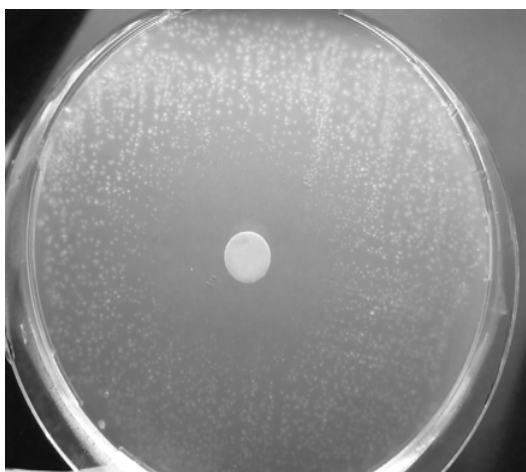


Figure 3. Zone diffusion assay for selection of variants resistant to an amino acid analog. The amino acid analog solution is placed on the disc in the center of the plate. Colonies that grow in the zone of inhibition are isolated and screened for amino acid excretion.

3.3 ASSAY FOR AMINO ACID EXCRETION

Amino acid excretion is measured assayed by growth of a bacterial auxotroph [10]. 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* (strain CAG18431) is seeded into CUTS media. The auxotroph will not grow unless exogenous valine is added to the media. Colonies of the plant pathogenic fungi or bacteria that are resistant to a valine analog are sub-cultured onto the seeded media. The plates are incubated at 28° C for 2-3 days. If the resistant variants excrete valine, there will be a zone of auxotroph growth surrounding the sub-cultured colony. The size of the zone is an indication of the magnitude of valine excretion. A standard dose-response can be determined by placing discs containing various levels of amino acid onto the auxotroph seeded 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 should be first evaluated in environmental growth chambers in order to eliminate as many external factors that may influence experimental results. In the initial studies, target weed plants are inoculated with each amino acid excreting variant and its respective wild type parent. Amino acid excreting variants that are more virulent than the parent are further evaluated in host range and scale-up experiments.

3.5 IMPROVING DISSEMINATION

A soil-applied pathogen will not be an efficacious mycoherbicide, even if it has specificity, sufficient lethality, and long term soil survival, unless it can be delivered in a cost effective manner. Fungi grown in liquid or solid-phase fermentation are inherently expensive when applied to large acreages at 10^4 spores per gram of soil. Conventional formulation methods with spore suspensions and food-based formulations [11, 12, 13, 14] did not provide enough spores in the root zone of the target weed. However, plant pathogenic fungi such as *Fusarium oxysporum* saprophytically colonizes the roots of many non-host plants [15,16] and thus, *Fusarium oxysporum* mycoherbicides could be delivered to farmer's

fields on non-host seed such as crops or grass, positioning the mycoherbicide directly in the rhizosphere of target weed [13, 2]. 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.

4 Conclusions

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 past, researchers have focused on lethality and host specificity as requirements for a successful agent. However, many pathogens that do not meet these criteria could be enhanced by synergistic additions or genetic modification. Embracing new methodologies may allow many "unsuitable" pathogens to be developed into successful biocontrol agents. Likewise embracing collaborations with scientists with other approaches to biocontrol may provide the necessary synergy to implement a successful biocontrol project.

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