

Amino Acid Excretion Enhances Virulence of Bioherbicides

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Abstract

Numerous host-specific plant pathogens suppress weed species, but do not provide control comparable to chemical herbicides. We have developed a novel method to increase the virulence of these plant pathogens. Certain amino acids, such as valine, are inhibitory to plants. Spontaneous *Fusarium oxysporum* Schlecht. mutants that are resistant to toxic analogs of the amino acid valine excrete high levels of valine. Growth chamber pathogenicity studies of these mutants showed increased pathogenicity to the target weed. Target plants inoculated with these mutants demonstrated symptoms similar to freching disease of tobacco, a disease caused by an amino acid-excreting root saprophyte. The host range of these mutants was not altered. In addition, resistance of the mutant strains to the toxic analog marks the strains for field release. Thus, we propose this method as a means to enhance the virulence of all mycoherbicides.

Keywords: Mycoherbicide, Bioherbicide, Amino Acid, *Fusarium oxysporum*

Science proceeds in fits and starts. Instead of smooth, unimpeded progress, scientists frequently encounter obstacles or even cascades of obstacles. Biological control of pests is a discipline where this trend is exemplified. Biological control researchers can only point to a few cases where biocontrol agents actually reduce weed populations significantly and few of these products have reached the market (Cook *et al.* 1996). In short, biocontrol agents have been unable to compete with chemical herbicides.

The disparity arises because host specificity, generally a stringent requirement for biocontrol agents, goes hand-in-hand with non-lethality (Miller *et al.* 1989; Gressel *et al.* 1996). Chemical herbicides have no such limitation and are selected on the basis of efficacy alone. Although chemical herbicides have some serious environmental drawbacks, they produce results. Given this situation, we clearly must find a way to obtain more lethal pathogens without altering their host-specific nature. We describe here a novel selection method to accomplish this task. This methodology should be effective with a broad range of host specific bioherbicides.

Fusarium oxysporum Schlecht., a plant pathogenic fungus, is an excellent candidate for biocontrol of weeds (Boyette *et al.* 1993; Murasheva and Sizova 1995; Sands *et al.* 1997; Thomas *et al.* 1998). There are over ninety known forma speciales of *F. oxysporum*, each attacking one genus or even one species of host plants (Brayford 1993). New forma speciales are discovered with great frequency, often in association with weedy plant species. These forma cause vascular wilts, leading to death of the plants. All are soil borne, survive as chlamydospores, and are capable of growing as saprophytic mycelia on the roots of non-host plants. When the mycelia encounter their host plant, they invade the vascular

tissue, stunting or killing their host. *Fusarium oxysporum* generally causes low mortality even under ideal conditions. For example, *F. oxysporum* f. sp. *cannabis* killed 25% of infested *C. sativa* plants within six weeks in greenhouse studies (Tiourebaev 1999). Mortality rates are similar under field conditions. For annual herbaceous weeds, this is not an effective “knockdown” rate. We have developed several methods to greatly improve the applicability of these fungi as host specific biocontrol agents for weeds.

Materials and Methods

Isolation of Analog Resistant Mutants of *Fusarium oxysporum*. The inhibitory effects of valine and seven valine analogs were tested against strain Cs95 using a well zone-diffusion assay on a minimal medium. The gradient plates were prepared as follows: a 10 mm disk of the solidified medium was cut from the center of the plate and filled with 0.5 ml of a sterile solution of either valine or a valine analog (1000 :g/ml sterile distilled H₂O). Microconidia of *Fusarium oxysporum* (5 X 10⁶ spores) were spread onto each plate. The plates were then incubated at 28° C. Resistant colonies were observed within inhibition zones within 3-5 days and were transferred to minimal agar medium containing 500 :g/ml analog. Analog resistance colonies of *F. oxysporum* were further evaluated.

Assay of Excreted Valine. Amino acid excretion was bioassayed using *Pediococcus cerevisiae* ATCC 8042, a bacteria requiring valine for growth (Sands and Hankin 1974). The mutant colonies of *F. oxysporum* were patched onto valine assay media seeded with *Pediococcus* and incubated for 2-3 days at 28° C. The diameter of the *Pediococcus* growth zones around the *Fusarium* mutants were measured and used to calculate valine excretion.

Virulence Testing of Pathogens on the Target Host. The virulence of the wild type strain and mutant strains of Cs95 was evaluated in environmental growth chambers (28° C, 19 hours day and 5 hours night). *Cannabis sativa* plants with two - three pairs of true leaves were infested with the pathogen by placing fungal formulation at the crown of each plant (Sands et al. 1997). Control plants were inoculated with autoclaved formulation. Each treatment was duplicated five times. Treatments consisted of ten individual *C. sativa* plants grown in 16-cm pots. Individual plants were evaluated daily for symptoms of vascular wilt and were rated for disease severity on a scale of 1 to 5, where 1 = no disease, 2 = minor wilting of lower leaves, 3 = wilting symptoms observed on 25-50% of leaves, 4 = >75% leaves show severe wilting symptoms, 5 = dead plant. Disease Average Index (DAI) for each treatment was calculated as = (1 n + 2 n + 3 n + 4 n + 5 n) divided by Total n, where n = number of plants with symptoms from 1 through 5. Disease Incidence Percent (% disease) was estimated for each treatment as = a/b x 100, where a = number of *C. sativa* plants with severe disease symptoms (from 3 through 5) and b = total number of *C. sativa* plants per treatment. Diseased tissues were plated onto PDA and minimal media supplemented with the appropriate analog (500 :g/ml) to recover *Fusarium oxysporum* f. sp. *cannabis* strain Cs95.

Greenhouse Host Range Study. The host range of selected mutants and wild type isolates was evaluated for virulence in environmental growth chambers on the following crop plant species: *Lycopersicon esculentum* (cv. Bonnie Best), *Zea mays* (cv. Sakota), *Triticum aestivum* (cv. Penewawa), *Hordeum vulgare* (cv. Clark), *Phaseolus vulgaris* (cv. Blue Lake Bush), *Festuca arundinaceae* (cv. Apache), and Blue Bunch wheatgrass (*Agropyron spicatum*), *Cucumis sativus* (cv. Straight eight), *Daucus carota* (Burpee), *Gossypium hirsutum* (cv. Stoneville), *Pisum sativum* (cv. Alaska), *Raphanus sativus* (cv. Cherry Bomb), *Melilotus indica* (Burpee). Control plants were infested with autoclaved

inoculum. Plants were maintained in environmental growth chambers with computer controlled conditions: 28° C (19 hours day, 5 hours night). One-week-old plants were infested by placing fungal inoculum at the base of each plant. Each treatment consisted of five individual plants, replicated twice. Plants were watered daily for 2 minutes by an automatic sprinkler. The plants were evaluated daily for symptoms of vascular wilt. Tissue from plants exhibiting disease symptoms was plated as described earlier in the section. At the end of the experiment, one plant from the wild type isolate treatment was arbitrarily chosen and assessed for the presence of *Fusarium* by plating plant tissue on Komada's medium (Komada 1975). Similarly, one arbitrary plant from each treatment with mutant strains was assessed for the presence of *Fusarium* by plating plant tissue on minimal medium supplemented with the corresponding amino acid analog.

Results

Isolation of Analog Resistant Mutants of *Fusarium oxysporum*. Of all analogs tested, only norvaline and penicillamine were inhibitory to *Fusarium oxysporum*. After several days of incubation, a clear zone of inhibition was formed. Several days later, single mutant colonies appeared within the zone and were transferred to plates containing minimal media supplemented with 500 µg/ml of the corresponding analog. Single celled mutant colonies capable of wild type growth in the presence of the inhibitory analog were transferred to toothpicks and stored for future analysis.

Assay of Excreted Valine. The resultant mutant colonies were screened for their ability to secrete valine into the medium. Preliminary data showed that mutants excrete 10-55 times as much valine in liquid nitrate-containing minimal medium as the wild type parent after an incubation period of 14 days at 28C (Table 1).

Table 1.
Valine Excretion by Wild Type and Valine Analog Resistant Mutants of *F. oxysporum* Strain Cs95

Valine-excreting Mutants	
Isolate/Mutant	Valine excretion (mg/l)
Cs95 (wild type)	0-0.18
4nv	2.84
6pa	2.48
8pa	9.93

Virulence Testing of Pathogens on the Target Host.

The virulence of wild type *Fusarium oxysporum* f. sp. *cannabis* Cs95 and its valine excreting mutants (4nv, 6pa, 8pa) was evaluated on *C. sativa*. The average disease ratings of plants inoculated with *F. oxysporum* mutants were significantly higher than disease ratings of plants inoculated with the parent wild type isolate ($p < 0.05$) (Table 2). The wild type

strain controlled 25% of the target plants, while the mutants controlled 70-90%. The development of wilt disease symptoms in treated plants was faster in the case of the mutant strains than the wild type (Table 2). *Cannabis* plants inoculated with wild type parent strain were dead or severely wilted in a period of 6-8 weeks, while mutant treated *C. sativa* plants showed severe wilt and death within 2-3 weeks.

Interestingly, in addition to wilt symptoms, plants infected with the valine-excreting mutants had leaf distortion, loss of stem apical dominance, and occasionally, stunted

Table 2.
Virulence of Valine Excreting Mutants of
Fusarium oxysporum f. sp. *cannabis* Cs95.

Isolate/Mutant	DAI ¹	% Disease	Kill Rate ²
Non-inoculated	1.35 a ³	0%	Na
Cs95 (wild type)	2.40 b	25%	6-8 weeks
4nv	3.75 c	70%	2-3 weeks
6pa	4.45 cd	90%	2-3 weeks
8pa	4.60 d	90%	2 weeks

1. Mean disease average index. Disease average index was rated using a 1 (no disease) to 5 (dead plant) rating scale.
2. Kill Rate. Elapsed time after inoculation that plant death was observed.
3. Means with the same letter are not significantly different ($p \geq 0.05$). (LSD (0.05) = 0.775)

growth. The symptoms of leaf distortion can be characterized by chlorosis of leaflet margins of some of the younger leaves, loss of the characteristic cannabis coarse saw-toothed edge and pointed tip. Re-isolation of the pathogen from infected plant tissues on the media supplemented with the corresponding valine analogs confirmed that survival of the plants was not due to plant escape from the pathogen.

Greenhouse Host Range Study. Symptoms of *Fusarium* wilt were not observed on any of the test plant species inoculated with *Fusarium oxysporum* f. sp. *cannabis*, with exception of *Cannabis sativa*.

Discussion

We examined the mode of action of several modern chemical herbicides and set out to mimic their specific site of action using metabolites excreted by our fungi. In the case of synthetic glyphosate and sulfonourea-type herbicides, the first enzyme of an amino acid biosynthetic pathway is inhibited. The amino acid endproducts of these pathways inhibit the same enzymes. Using amino acid analogs, we have been able to obtain high levels of valine and tryptophan excretion from spontaneous mutants of *F. oxysporum*. These strains have shown a much faster kill rate and higher mortality than the wild type isolate (Table 1). The host range of the mutant was unaltered.

In conclusion, we propose amino acid excretion as a method of improving the virulence of mycoherbicides. Incremental increases in virulence can be made by selection for additional excretion of the same or different amino acid. In addition the resistance of the mutant strains to various amino acid analogs enables the strains thus to be marked for their field release.

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