Review

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Methods for selecting hypervirulent biocontrol agents of weeds: why and how

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Abstract

A considerable number of plant pathogens have been studied for their possible use in weed control. Some have proven virulent enough to control weed species and to compete commercially with chemical herbicides. However, most pathogens of weeds are not useful in their wild form because they are not sufficiently host-specific and/or virulent. The authors believe that these barriers can be overcome. The present research has focused on the inhibitory effects of certain amino acids on the growth and development of specific plants. Pathogens that overproduce these selected amino acids can be easily selected from a pool of spontaneous mutants. Such mutants can have increased pathogenicity to their target weed and enhanced field performance as biocontrol agents. Enhancement of biocontrol efficacy in three separate pathogen–host systems, two with *Fusarium* and one with *Pseudomonas*, has already been reported. It is proposed to use the same technology to enhance the biocontrol efficacy of the two species of Fusarium that are host-specific pathogens of the broomrape group of parasitic weeds. The stepwise approach outlined can lead to obtaining enhanced biocontrol agents capable of producing inhibitory levels of selected amino acids *in situ*. It is proposed that these approaches, in combination with other methods of virulence enhancement, will lead to sustainable systems of biological control of parasitic weeds.

Keywords: amino acid; *Orobanche*; *Striga*; virulence enhancement; plant pathogens; mycoherbicides; frenching disease; *Fusarium oxysporum*

1 INTRODUCTION

Most weed biocontrol projects have been based on the premise that, given due diligence, natural biological control agents that are capable of controlling a weed population will be found. Unfortunately, few pathogens meet standards for host specificity, effective kill rate and ease of production and application. The authors believe that these barriers may be overcome by technological and biological approaches to virulence enhancement. The observation that all plants, including parasitic weeds, are inhibited by one or more amino acids is important to this strategy. Different plants are more or less sensitive to different amino acids. Understanding these phenomena will provide new weapons for the biocontrol of weeds by improving the efficacy of biocontrol agents.

In this paper, research with biocontrol pathogens that excrete phytoinhibitory amino acids is reported. The effects of application of single amino acids or combinations of amino acids on growth of noxious and parasitic weeds are also reported. It is proposed that these approaches, in combination with other methods of virulence enhancement, will lead to sustainable systems of biological control of parasitic weeds.

2 RATIONALE

2.1 Frenching diseases

James Oglethorpe first described 'frenching disease' of tobacco in the American colony of Georgia in the seventeenth century.[1] Affected seedlings showed symptoms of chlorosis, wilting, stunting and leaf distortion, yet none of the bacteria or fungi recovered from the symptomatic seedlings was known to be a pathogen in the classical sense of invading tissue. Steinberg demonstrated that isoleucine would produce similar physiological symptoms in tobacco.[2] In 1950, he proposed that frenching disease of tobacco was actually a physiological disorder or disease syndrome caused by saprophytic rhizosphere bacteria [*Pseudomonas fluorescens* Mig. or *Bacillus subtilis* (Ehrenberg) Cohn] with the unusual trait of isoleucine excretion.[3] Frenching symptoms resulted from the uptake of the free isoleucine into the tobacco plant.[3] Isoleucine was suggested to inhibit the activity of acetolactate synthase (ALS), also known as acetohydroxyacid synthase (AHAS), the first enzyme of the branched-chain pathway for biosynthesis of leucine, valine and isoleucine, and of pantothenic acid, vitamin B5.

Two fungi can also elicit frenching symptoms in tobacco. *Aspergillus wentii* Wehmer produces 1-amino-2-nitro-cyclopentane-1-carboxylic acid (ANCPA), an effective antagonist of leucine.[4,5] ANCPA is taken up by the plant, causing morphological changes and stunting in susceptible plants at very low concentrations.[5] The effects of ANCPA can be reversed by leucine but not by valine. In contrast, frenching symptoms caused by isoleucine can be reversed by valine.[2] ANCPA or *A. wentii* will also induce frenching disease symptoms in Chrysanthemum, where the disease is called yellow strapleaf.[6] The soilborne fungus *Macrophomina phaseolina* (Tassi) Gold has also been shown to cause frenching symptoms on

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inoculated tobacco seedlings.[7] Other plants (petunia, eggplant, ragweed, sorrel and squash) developed frenching symptoms when exposed to frenching soil.[8] However, frenching symptoms were not elicited on pepper (*Capsicum frutescens* L.) or on the weeds *Galinsoga parviflora* Cav., *Polygonum pennsylvanicum* L., *Portulaca oleracea* L. or several genera of the grass family. Tomato little leaf is a frenching disease with unknown etiology.[9] It should be noted that frenching symptoms are highly dependent on the environment and often fail to develop even after inoculation with the described organisms.

2.2 Amino acid overproduction in bacteria

Frenching disease is not the only case where microbes overproduce and excrete amino acids. Several industries routinely employ amino-acid-overproducing mutants of bacteria or fungi for production of lysine, tryptophan and threonine for use in food and feed supplements.[10,11] Selection of amino-acid-overproducing mutants is a relatively straightforward process.[12] Synthesis of an excess of any given amino acid is energy expensive. Thus, enzymes for amino acid synthesis are tightly regulated by feedback inhibition. Feedback-inhibition-insensitive mutants can be isolated by selecting bacterial variants that grow in the presence of high concentrations of an inhibitory amino acid or in the presence of toxic amino acid analogues. The inhibitory effects of the toxic analogue can be overcome by overexpression of the corresponding amino acid, effectively diluting the toxin.

2.3 Amino acid synthesis in plants

Several amino acid synthesis pathways in plants and microbes have multiple amino acid end-products [aromatic amino acids (tryptophan, phenylalanine and tyrosine), branched-chain amino acids (valine, leucine and isoleucine) and aspartate-derived amino acids (lysine, threonine and methionine)]. Such pathways are generally regulated by feedback inhibition of a controlling enzyme by one or more of the amino acid end-products. For example, isoleucine is synthesized from threonine via α-ketobutyrate, and leucine and valine are synthesized from pyruvate (Fig. 1). The first common enzyme in the convergent pathway is AHAS. In barley, AHAS is feedback inhibited by isoleucine, leucine and valine.[13] In *Arabidopsis*, this enzyme is inhibited by leucine and valine.[14] Thus, an excess of valine in either of these plants results in feedback inhibition of AHAS, resulting in a toxic shortage of leucine and isoleucine.

2.4 Herbicidal sites of action are similar

It is no coincidence that the herbicide industry has focused on inhibiting the enzymes 'at the top' of amino acid biosynthetic pathways. Five different classes of chemical herbicides (sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinylthiobenzoates and sulfonylamino-carbonyltriazolinones) target AHAS, blocking synthesis of the branched-chain amino acids.[15] The inhibitory effects of these herbicides can be overcome by supplementation with valine, leucine and isoleucine.[16] Imidazolinone-resistant plants have an altered AHAS that is not inhibited by imidazolinone.[17]

Glyphosate kills plants by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, a key enzyme in synthesis of the aromatic amino acids (phenylalanine, tyrosine and tryptophan).[18,19] The suppressive activity of glyphosate can be reversed by exogenous application of aromatic amino acids,[20] or overexpression of EPSP synthase.[19]

2.5 Amino acid overproduction in plants

Analogue resistance can also be used to select plants that have increased concentrations of a given amino acid. Many plants accumulate free proline in response to stresses such as low temperature, drought and high salinity. Proline-analogue-resistant mutants of spring canola have increased concentrations of free proline and enhanced cold tolerance compared with the parental lines.[21] *p*-Fluorophenylalanine-resistant plants have increased phenylalanine, resulting in increased resistance to insect pests.[22] Analogue resistance has also been used to select for plants with increased concentrations of essential amino acids,[23] including lysine (rice),[24] threonine (barley,[25] tobacco[26]), tryptophan (potato[27]) and methionine (*Arabidopsis*,[28] soybean[29]).

3 FRENCHING ENHANCEMENT OF BIOCONTROL AGENTS

The frenching disease hypothesis could be applied to biocontrol of weeds in a robust manner, either by direct application of amino acids to target weeds or *in situ* application by agents that overproduce the selected amino acids. Interestingly, the phenomenon of amino acid inhibition has also been observed in plant pathogens, and could be exploited in biocontrol of plant diseases.[30] A clearer understanding of the molecular genetic basis of these inhibitions/stimulations is necessary if they are to be fully, safely and sustainably exploited.

3.1 *Fusarium*

It has been demonstrated that amino acid overproduction can be used to enhance the virulence of host-specific formae speciales of *Fusarium*, as well as a pathovar of *Pseudomonas syringae* van Hall.[31] *Fusarium oxysporum* is a candidate pathogen for virulence enhancement because its formae speciales are host specific and the pathogen is easy to culture and to manipulate. *Fusarium oxysporum* can grow on minimal medium without amino acids, indicating that it is capable of synthesizing all 20 amino acids necessary for protein synthesis. Furthermore, specific forma have been isolated that are pathogenic to parasitic plants in the genera *Striga*[32] and *Orobanche*.[33] Valine-excreting mutants of *F. oxysporum* f. sp. *cannabina* Noviello & WC Snyder have been isolated and characterized.[31,34] These valine-overproducing variants showed increased virulence (percentage kill and kill rate) to cannabis plants (Table 1). The host range of these variants was not altered.

3.2 *Pseudomonas*

This frenching approach has also been used to enhance the virulence of the bacterial pathogen *Pseudomonas syringae* pv. *tagetis* (Hellmers) Young Dye & Wilkie, a potential biocontrol agent for Hound's tongue (*Cynoglossum officinale* L.),[35] by selecting for valine excretion.[31]

4 ENHANCEMENT OF BIOCONTROL OF PARASITIC WEEDS

Parasitic plants offer unique challenges to biocontrol. Biocontrol, or chemical control, must affect the parasite but not the attached host. *Orobanche ramosa* L. seed germination and growth are sensitive to specific amino acids (Table 2).[36] However, the

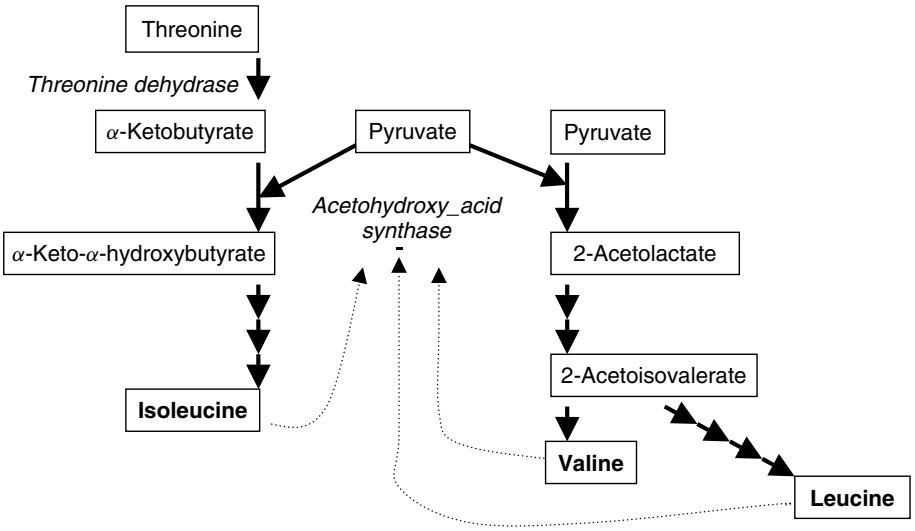

Figure 1. Branched-chain amino acid biosynthetic pathway.

Table 1. Valine excretion and virulence of wild-type and valine-overproducing variants of *Fusarium oxysporum* f. sp. *cannabina* to *Cannabis sativa*

Strain[a]	Description	Valine excretion (mg L^{-1})	Disease development (weeks)[b]	Kill (%)
C95	Wild-type	0–0.18	6–8	25
4nv	Norvaline R	2.84	2–3	70
6pa	Penicillamine R	2.48	2–3	90
8pa	Penicillamine R	9.93	2	90

[a] Spontaneous mutant strains were selected for their resistance to successively higher levels of valine analogues. Strain 4nv is resistant to norvaline, and strains 6pa and 8pa are resistant to penicillamine.
[b] Disease development is the duration between inoculation and the first appearance of severe disease symptoms or death (greenhouse studies).

Table 2. Effect of different L-amino acid concentrations on the germination of stimulated *Orobanche ramosa* seeds[36]

Amino acid concentration (mM)	Germination (%)[a] Methionine	Lysine	Arginine
4.0	0.0e	0.0c	0.0b
2.0	24.3d	10.3c	0.0b
1.5	42.3c	38.3b	0.0b
1.0	70.3b	81.0a	0.0b
0.5	85.7a	89.7a	77.3a
Control	84.7a	84.7a	84.7a

[a] Values within a column with no letters in common differ significantly at $P = 0.05$.

question still remains as to whether these inhibitory amino acids can be effectively delivered to the plant parasite by parasite-specific pathogens, and as to whether the excess amino acid concentrations will adversely affect the crop plant.

5 DIRECT APPLICATION OF AMINO ACIDS AS HERBICIDES

A straightforward approach to the use of amino acid inhibition to control weeds is direct application of inhibitory amino acids. This approach has been effective for control of emerged Canada thistle, red bromegrass, kudzu and cannabis. The effect of amino acid application on the viability of the weed seed bank or underground structures such as tubers and root networks needs to be determined in a variety of climates and soils. The persistence of the applied amino acid in the soil profile has not yet been evaluated. The assumption is that the amino acid will rapidly be degraded or assimilated by the soil microflora. The rate of degradation or assimilation will depend upon the microbial community, the soil matrix and environmental conditions such as temperature and moisture. To date, this use of amino acids for weed control has not been put into practice.

Many homeowners and neighborhood organizations restrict the use of chemical pesticides in order to minimize exposure. Organic producers only have limited tools to control weed infestations, especially in post-emergent crops. Amino acids are green and non-toxic. Direct application of an inhibitory amino acid may provide economic and safe control of specific weeds, minimizing application of synthetic herbicides.

6 EXPERIMENTAL METHODS

The stepwise process for development of enhanced virulence in host specific pathogens is as follows:

1. Determine which amino acids inhibit the target plant, what concentrations are efficacious and whether the inhibition is reversible by the other amino acids of the same biosynthetic family.[36]
2. Determine whether there are host-specific pathogens of the target weed that are able to synthesize the amino acid.[30] Pathogens will be screened for growth on medium lacking the target amino acid. Growth of the pathogen indicates that the pathogen is capable of *de novo* synthesizing of the amino acid.
3. Select appropriate amino acid analogues to inhibit the pathogen, enabling a search for resistant mutants (Table 3).

Amino-acid-overproducing and -excreting strains of each pathogen can be selected by exposure to specific amino acid analogues. For example, if the target weed is inhibited by lysine, a pathogen that overproduces lysine needs to be selected. Lysine-overproducing mutants are isolated by exposing the pathogen to *S*-aminoethyl-cysteine (AEC), a lysine analogue.[12] Resistant colonies can be selected using a well zone–diffusion assay on CUTS minimal medium (Czapek-Dox Agar; Difco, St Louis, MO) supplemented with ammonium sulfate (0.5 g L^{-1}), uracil (20 mg L^{-1}), thiamine (4 mg L^{-1}) and 100 mg L^{-1} of crushed generic daily vitamin pills. The zone diffusion plates for variant selection are prepared by cutting a plug from the center of the CUTS plate with a sterile cork borer. The plates are then spread with a suspension of the pathogen (10^3–10^7 CFU $plate^{-1}$). A sterile solution of the amino acid analogue (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 h. At that time, an additional 0.1 mL of the analogue solution can be added to the well. The plates are then incubated at 28 °C and monitored daily. Zones of inhibition will appear near the wells containing the analogue, and resistant colonies will appear within the zone of inhibition. AEC-resistant mutants are isolated and screened for lysine production. This analogue protocol can be repeated several times using increased concentrations of the amino acid analogue in order to select for maximum lysine excretion. Five or more selection cycles are commonly used in order to obtain high and higher levels of excretion. An additional benefit of analogue resistance is that the resistant strains have a selectable marker, facilitating studies on the fate and survival of the pathogen simply by using selective medium supplemented with the specific analogue.

4. Screen mutants for normal growth rates and bioassay for specific amino acid excretion.[12] After each one or two cycles of selection, analogue-resistant isolates of the pathogen are screened for lysine excretion. Amino acid excretion is bioassayed using a bacterial auxotroph [*Leuconostoc mesenteroides* (ATCC 4043)].[12] This bacterial strain is auxotrophic for most if not all amino acids. The bacterial auxotroph is seeded into defined synthetic medium lacking the target amino acid prior to solidification. This medium is allowed to solidify and then inoculated with the analogue-resistant pathogen. Subsequent growth of the auxotroph in the medium is dependent upon the amino acid excreted by the analogue-resistant pathogen. The growth of the auxotroph appears as a halo surrounding a pathogen that is producing the amino acid. The diameter of the halo is proportional to the quantity of amino acid being excreted. For example, in order to assay valine, *L. mesenteroides* (ATCC 4043) is seeded into valine assay medium (Difco, St Louis, MO). The auxotroph will not grow unless exogenous valine is added to the medium. Colonies of valine analogue-resistant plant pathogenic fungi or bacteria are subcultured onto the seeded medium. The plates are incubated at 28 °C for 2–3 days. If the analogue-resistant microbes excrete valine, there will be a zone of auxotroph growth surrounding the subcultured colony. The size of the zone is an indication of the magnitude of valine excretion. A standard dose–response curve can be generated by placing discs containing various levels of amino acid onto the auxotroph-seeded agar and determining the diameter of auxotroph growth. This simple and straightforward bioassay is a tool that enables the researcher to screen numerous mutants in search of variants that excrete an amino acid. Alternatively, any bacterium or yeast that is auxotrophic for a specified amino acid can be used in this assay system.
5. Continued selection for increased excretion of selected amino acid. Steps 3 and 4 can be repeated in order to select for variants with increased amino acid excretion. In the authors' laboratory, mutants with maximum levels of amino acid excretion and normal growth rate are selected. It was postulated that a pathogen with reduced growth rates would be less competitive in root colonization and less invasive in host tissue. *In situ* or '*in planta*' production of amino acids is linked both to level of excretion per cell and to extent of parasitism dependent on growth rate. However, the effect of the growth rate of an amino acid overproducer on plant inhibition has not been evaluated.
6. Greenhouse testing of pathogens for enhanced virulence. The virulence of amino-acid-overproducing pathogens is screened in greenhouses or growth chambers using protocols appropriate for the given pathogen and weed. *F. oxysporum* is cultured on autoclaved barley or canola seeds. Inoculated seed is cultured at 28 °C. When the seed is completely colonized by the mycelia, the inoculum is dried under a laminar flow hood (~24 h). The dried inoculum is stored at room temperature in brown paper bags. Greenhouse plants are inoculated by placing inoculum at the crown of the plant. The inoculated plants are maintained in the greenhouse and monitored for plant disease and death. Controls need to include non-inoculated plants and plants inoculated with the wild-type pathogen. The efficacy of promising pathogens is then evaluated under field conditions. In the authors' laboratory, increased virulence in the greenhouse has carried over to increased field virulence. However, actual disease rates vary considerably between the greenhouse and field owing to variable environmental conditions (water, temperature, soil type) and microflora.
7. Greenhouse and field testing as approved by regulatory agencies. Most countries closely monitor release of biocontrol agents. Researchers must prove efficacy against the target weed and safety to non-target plants. Researchers must also demonstrate that the pathogen does not negatively impact upon humans or animals. Pathogens obtained by spontaneous mutant selection are generally not considered to be regulated differently from wild types because such mutations are similarly occurring in nature. Over time, plant pathogens that constitutively overproduce an amino acid cannot compete with more efficient wild-type organisms, and their population will decline.

Recommendations for the listed steps:

- Use L-forms of the amino acids.
- Most amino acids can be safely autoclaved, with the exception of tryptophan.
- Amino acid analogues are expensive, and many do not inhibit and are not useful for selection of excretion mutants. The authors check for inhibitory amino acid analogues in the recent literature, and order small amounts from the listed source.
- The analogues are generally sterilized with ethanol.
- Selection can be by use of antibiotic discs on agar plates or in broth of minimal medium (i.e. lacking amino acids).
- Agars used need not be the expensive, highly purified types.
- If vitamins are required for pathogen growth, they can be purchased as common vitamin pills. The vitamin pills are crushed and sterilized with ethanol.

Table 3. Amino acid analogues that have been utilized to select amino acid overproduction in plants, bacteria or fungi. A more comprehensive list of amino acid analogues is available[37]

Amino acid	Analogue	Microbe	Reference
Lysine	*S*-2-aminoethyl-L-cysteine	Saccharomyces	38
Methionine	Ethionine	Corynebacterium	39
		Saccharomyces	40
Threonine	Hydoxynorvaline	Saccharomyces	41
Valine	2-Thiazole alanine	Bacteria	42
	DL-norvaline	Fusarium	31
Isoleucine	L-*O*-methylthreonine	Arabidopsis	43
Leucine	5′,5′,5′-trifluoroleucine	Saccharomyces	44
	β-hydroxyleucine	Brevibacterium	45
Tryptophan	5-methyltryptophan	Saccharomyces	46
	6-fluorotryptophan	Pseudomonas	47
Proline	Azetidine-2-carboxylic acid	Saccharomyces	48

- Selection of high-excretion mutants is a serial process sometimes requiring 5–10 cycles of selection using progressively higher concentrations of analogue.

7 SPECULATIONS AND ALTERNATIVE APPROACHES FOR ENHANCEMENT OF VIRULENCE AND FOR IMPROVED CONTROL OF PARASITIC PLANTS

7.1 *Fusarium* as a model system

Fusarium oxysporum f. sp. are attractive as biocontrol agents because they are highly virulent and their host range is usually limited to one plant species. Furthermore, the fungus is persistent in the soil for long periods of time and is able saprophytically to colonize the roots of many non-host plants. Fusarium mutants can readily be obtained and characterized. Fusarium also produces other pathogenicity factors: jasmonic acid,[49] hydrolytic enzymes,[50,51] necrosis peptides,[52] fusaric acid,[53–55] effector proteins[56] and plant hormones.[57] For this reason, these pathogens are viewed as 'Trojan horses' able to carry enough new warrior genes into the battle cumulatively to tilt the playing field against parasitic plants. Some examples are given below.

7.2 Seed germination

Preconditioned *Striga* and *Orobanche* seed germination is stimulated by plant metabolites produced by the host plant.[58,59] In the absence of a susceptible host plant, seed germination of parasitic plants is suicidal. Trap crops and germination stimulants have been utilized to reduce the striga seed bank in infested soils.[59]

Fungal metabolites can also impact *Striga* and *Orobanche* seed germination. Trocothecenes produced by *Fusarium solani* (Martius) Sacc. inhibit *Striga* germination,[60] and metabolites from *Fusarium compactum* (Wollenw.) WL Gordon inhibit *Orobanche* germination.[61] Certain fungal metabolites also stimulate germination, including jasmonic acid[62] and ethylene.[63,64] Many *formae speciales* of *Fusarium oxysporum* and numerous other fungi produce some ethylene.[65] *Fusarium oxysporum* f. sp. *tulipae* Apt produces 1000-fold higher levels of ethylene than most fusaria, and the level of ethylene production of this pathogen was correlated with tulip virulence.[65] Ethylene production by pathogens of *Striga* or *Orobanche* could be correlated with virulence. It remains to be seen how much ethylene could be produced and whether or not it can be tolerated by the crop plant. *Orobanche* seed germination is less sensitive to ethylene stimulation than *Striga* seed germination. Thus, this approach may be limited to biocontrol of *Striga*.

Production of growth regulators by a pathogen such as inhibitors of flowering and male sterility induction have been described in other plant/pathogen interactions, and they may be applied to control of parasitic plants.[54] The impact of the described plant regulators on growth and development of parasitic plants is unknown.

7.3 Innovations in delivery systems of biocontrol agents

There is a great need to make biological control research relevant to field situations. Too often research stops short of practical and efficacious delivery to the field. There are some strong features of the chosen pathogen, *F. oxysporum*, that will enable it to be produced and disseminated effectively. It is host specific but often an aggressive colonizer of non-host seedling roots. In recent work, the authors used non-host seeds as a carrier system to disseminate *F. oxysporum* because of cost constraints of producing large quantities of spores in culture. The production of spores in the rhizosphere of non-host seedlings greatly decreased field application costs. The depth and size of the carrier root system effectively distributed the *F. oxysporum* throughout the soil profile. In the case of *Striga* in Africa, it is speculated that an aggressive *Striga* pathogen applied to maize or sorghum seeds might well suppress other pathogens, including *Fusarium graminearum* Schwabe, a problem now requiring expensive seed treatments. If seed treatments are still used, it would be important to ensure that pathogens are resistant to the fungicides or herbicides. Inoculum will be most useful if it can be cheaply delivered, possibly produced on an agricultural waste product such as bagasse produced by sugar cane processors, and if it can be recycled through several seasons by composting, passage through animal wastes, survival in the rhizosphere of alternate crops, etc.

7.4 Sustainability

Fusarium oxysporum successfully survives as a pathogen because of its ability to produce three very different spore types: microconidia, ephemeral but small enough to move efficiently through vascular tissues; macroconidia, capable of longer-range dissemination; and chlamydospores, capable of surviving for years in the soil. All three spore types can serve as inocula, and all three types may be needed for successful biocontrol of parasitic plants in subsistence or sustainable agriculture systems.

8 SUMMARY

Control of parasitic plants represents one of the greatest enigmas for modern agriculture. Is it possible to kill a parasitic plant that is so physically and biochemically attached and associated with its host plant without also inhibiting the host as well? The more diverse the approaches that can be integrated into parasitic weed control, the greater will be the chance of staying ahead of the countermutation and adaption that can be expected to follow. One strong technological approach is presented here, specifically the use of amino-acid-excreting mutants of *Fusarium oxysporum* or other similar pathogens.

It would not be wise to deploy this frenching technology alone, as the seed banks of *Striga* and *Orobanche* could provide sufficient

numbers of mutants to overcome the temporary advantage afforded by this singular approach. This lesson has been learned many times over in the field of plant pathology, and only when a multiplicity of approaches has been combined will it be possible to rest. Such a multiplicity of approaches will most likely be achieved by a spirit of collaboration among a number of laboratories, with each contributing in their special areas of expertise. It is hard to envision a more urgent and pressing goal than that of biological control of *Orobanche* and *Striga*.

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