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A Reflection on My Research in Weed Biological Control: Using What We Have Learned for Future Applications

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When I began my foray into the field of biological control of weeds in 1971, the concept of deliberately using pathogens to control weeds was novel and untested and met with skepticism and resistance. Soon, a worldwide network of plant pathologists, weed scientists, microbial technologists, formulation specialists, and regulatory personnel came together to study, develop, and apply pathogens in safe and effective ways of control of a variety of weeds in crops and natural areas. Several new weed–pathogen systems were studied; a few dozen products and pathogens were brought to use, albeit on a very small scale compared to conventional weed-control products; and along the way, some valuable lessons were learned in phytopathology and weed ecology. A seminal body of information was published on the etiology and epidemiology of several diseases of weeds, many new pathogens were discovered and described, and methods were developed for mass production, formulation, and storage of pathogens. Numerous pathogen-produced herbicidal metabolites were discovered and characterized. Protocols were developed, tested, and applied for safe importation and release of exotic pathogens and for registration of microbial herbicides. Spectacular success was achieved with some pathogens used as classical biocontrol agents, and a new class of herbicide, the bioherbicides, came on the scene. Yet some key opportunities were missed. Notably, weed biocontrol research remained largely preoccupied with agent or product development and deployment while great strides were made during this period in phytopathology to understand the genetic–molecular basis of virulence, host range, host specificity, host response to infection, cell death, and pathogen population structure. Nevertheless, the accomplishments in the field of weed biocontrol by pathogens are truly significant. Certainly, we are poised to apply the knowledge gained toward discovery and development of additional weed-control pathogens, but increased effort should be directed also at using pathogen genes, gene products, and genetic mechanisms for weed control. An investment in the latter could help us gain insights into genetically programmed host–pathogen interactions that may be exploited to kill weeds, restrain weed growth, or knock out traits for invasiveness. In our continuing struggle to manage weeds, biocontrol with pathogens should remain a major thrust. Here I present perceptions I have gained from the work that my students, postdoctoral and technical associates, colleagues, and I have done with several weed–pathogen systems.
Key words: Biological control, pathogen, bioherbicide, classical biocontrol, biocontrol challenges, biocontrol accomplishments.

Cuando empecé mi andar en el campo del control biológico de malezas en 1971, el concepto de uso deliberado de patógenos para controlar malezas era una novedad no probada y me topé con escepticismo y resistencia. Pronto, una red mundial de especialistas patólogos vegetales, científicos de malezas, tecnólogos especialistas en microbiología, especialistas en formulaciones y demás personal regulador se unieron para estudiar, desarrollar y aplicar los patógenos de una manera más segura y efectiva para controlar una variedad de malezas en cultivos y áreas naturales. Muchos de los nuevos sistemas de control de malezas con patógenos fueron estudiados. Algunas docenas de productos patógenos fueron traídos para su uso, aunque en una escala muy pequeña comparada a los productos convencionales en este campo. A lo largo del camino, aprendí algunas lecciones valiosas sobre fitopatología y ecología de malezas. Un creativo cuerpo de información fue publicado sobre etiología y epidemiología de varias enfermedades que atacan a las malezas. Muchos nuevos patógenos fueron descubiertos y clasificados y los métodos se fueron desarrollando para la producción masiva, la formulación y el almacenamiento de patógenos. Numerosos herbicidas metabólicos basados en patógenos fueron descubiertos y clasificados. Los protocolos fueron desarrollados, evaluados y autorizados para la importación y liberación segura de patógenos exóticos y también para su registro como herbicidas microbiales. Se alcanzó un éxito espectacular con algunos patógenos utilizados como agentes clásicos de bio-control, y un nuevo tipo de herbicidas, los bio-herbicidas, salieron al mercado, Aún así, algunas oportunidades claves hacían falta. Notablemente, la investigación sobre el control biológico de malezas permaneció mayormente concentrada en el desarrollo de agentes o productos y con su comercialización, mientras grandes avances se hacían durante este período en fitopatología para entender la base genética molecular de la virulencia, el rango y especificidad de las hospederas, la respuesta de las hospederas a la infección, la muerte celular y la estructura de la población de patógenos. Sin embargo, los logros en el campo del control biológico de malezas utilizando patógenos son verdaderamente significativos. Ciertamente, estamos en el momento oportuno de aplicar los conocimientos obtenidos hacia el descubrimiento y desarrollo de patógenos adicionales. Sin embargo, los esfuerzos también deben ser enfocados al uso de genes de los patógenos, el desarrollo de productos derivados de estos genes y los mecanismos genéticos para el control de malezas. Una inversión en el último concepto podría ayudarnos a ganar terreno en el campo de las investigaciones genéticamente programadas de interacciones de patógenos hospederos que pueden ser explotados para matar malezas, restringir su crecimiento o aniquilar las amenazas de invasión en los cultivos. En nuestra continua lucha para un mejor manejo de malezas, el control biológico con patógenos debe permanecer seguro. En este documento, presento especificaciones que he adquirido del trabajo que mis estudiantes, asociados post-doctorales y técnicos, colegas y yo hemos hecho con varios sistemas de patógenos para el control de las malezas.

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The Early Years

In 1970, the University of Florida, Institute of Food and Agricultural Sciences (IFAS) made a bold investment to explore the use of plant pathogens as biological control agents for weeds. The impetus for this novel program came from the realization that aquatic weeds, particularly waterhyacinth [*Eichhornia crassipes* (Mart.) Solms-Laub.] and hydrilla [*Hydrilla verticillata* (L.f.) Royle], which were choking Florida's lakes and waterways, needed a multipronged approach for management, and in the area of biological control, pathogens as a group had not been explored. With ample funding from state and federal sources, F. William Zettler and the late T. Ed Freeman initiated an exploratory program. As a first step, they reviewed the state of knowledge and found that except for a few seminal studies, little was known in general about pathogens of aquatic and other weeds or their use as biocontrol agents (Zettler and Freeman 1972). I was hired in 1973 as an assistant professor with 100% responsibility for biological control of weeds by plant pathogens. It was unique then, as it is even today, for a member of a plant pathology faculty to be assigned solely to study ways to kill plants (weeds in this case) with pathogens; this is contrary to the primary mission in the field of plant pathology, i.e., to protect plants from the ravages of pathogens and diseases. Nonetheless, I signed on to play the contrarian, helped by strong support from Zettler, Freeman, and the department chair, Lawrence ''Hank'' Purdy. Without a doubt, the University of Florida/IFAS, through its leadership in instituting this biological weed control program, made a key early contribution to the development of this field.

During my first decade in this field, there was general skepticism about using plant pathogens as weed-control agents. Claims that ''it wouldn't work'' and ''it's playing with fire'' and similar incredulous remarks were common. Nonetheless, a popular article I wrote in *Agrichemical Age* (Charudattan 1975) brought a surprising and encouraging number of inquiries from growers. It also put me in contact with George E. Templeton and Roy J. Smith, Jr., respectively from the University of Arkansas and the U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS) (regrettably both now deceased), who at that time were well into development of a fungal bioherbicide (mycoherbicide) for northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.], a weed of rice in Arkansas. Concurrently, Harry C. Burnett and William H. Ridings (also both deceased) of the Florida Division of Plant Industry were making significant progress in developing another fungal bioherbicide for strangler vine [*Morrenia odorata* (Hook. & Arn.) Lindl.], a weed of Florida citrus. I gained the much-needed peer support for my research endeavors from this foursome as well as from other academic and industrial scientists who were then beginning to enter this field. Also about this time, the USDA-ARS invested considerable resources in this field with new hires at Stoneville, MS; Fort Detrick, MD; and a few other locations. The U.S. Environmental Protection Agency formalized the registration requirements for biopesticides, and the Inter-Regional Project 4 was given the federal mandate to fund as well as to facilitate registration of biopesticides. These developments, funded and spurred by federal agencies and programs, provided a strong sense of legitimacy to the emerging field of biological control of weeds by pathogens and further motivated research and development efforts in the United States and abroad.

Skepticism did not disappear altogether; my attempts to find and release exotic pathogens for classical biological control in Florida repeatedly met with bureaucratic and regulatory hurdles at both state and federal levels, the latter at the ''Technical Advisory Group'' (TAG), described by Klingman and Coulson (1982) and Miller and Aplet (2005). Looking back, the TAG did not seem inclined towards pathogens as weed-biocontrol agents. Although the USDA–Animal and Plant Inspection Service–Plant Protection and Quarantine had permitted us to import candidate biocontrol pathogens into an approved quarantine facility in Gainesville, FL, the most promising pathogens could not be moved past quarantine even after host-range and other tests were completed that confirmed host specificity and safety. The prevailing attitude of apprehension about use of pathogens as weed-control agents and the consequent unwarranted extreme caution prompted Ed Freeman to coin the term ''pathophobia'' (Freeman and Charudattan 1985). In my view, the prevailing pathophobia created negative perceptions about risk as well as about the definition of acceptable risk in the use of pathogens for weed control. Funding for research on pathogens and administrative/political support for programs suffered as a result. It was not easy to dispel the ghost of pathophobia, despite attempts at ghost-busting with good science. Even now, the ghost is still around.

As my early encounters with regulatory agencies portended a difficult trek ahead for research and deployment of exotic pathogens, I decided to abandon my involvement in classical biological control in favor of bioherbicides in the interest of my academic career. The latter seemed to offer more chances for support and success. The registration of the first two bioherbicides in the early 1980s, DeVine [based on *Phytophthora palmivora* (Butler) Butler sensu lato] for the control of strangler vine) and Collego [based on *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. f.sp. *aeschynomene* for the control of northern jointvetch], both indigenous pathogens, brought believers to the field and recognition by the Weed Science Society of America, the American Phytopathological Society, and the U.S. Environmental Protection Agency. I cannot stress enough the importance of support from these three entities to the history and growth of bioherbicides in the United States.

Although the decision was made to work on bioherbicide candidates, the numerous foreign surveys I undertook to find prospective classical biological control agents did yield some scientifically useful material, including the discovery and characterization of a new species of *Potyvirus*, *Araujia mosaic virus* (ArjMV), a pathogen of strangler vine, moth plant (*Araujia sericifera* Brotero), and other milkweed vine species native to Argentina and Uruguay (Charudattan et al. 1980b; Hiebert and Charudattan 1984). Although our research proved that ArjMV is host-specific to plants in the Asclepiadaceae (Charudattan et al. 1980b), it could not be released for the control of strangler vine because of an arbitrary decision that introduction of an exotic plant virus, particularly a member of the aphid-transmitted *Potyvirus*, into

Florida might be too risky for unforeseeable reasons. Recently, ArjMV was characterized by genomic sequencing (Massey et al. 2007) as part of a consideration of this virus for biological control of moth plant in New Zealand. However, ArjMV is unlikely to be used in New Zealand because of the possibility of endangering a susceptible milkweed plant that is a food source for monarch butterfly [*Danaus plexippus* (L. 1758)] larvae (Elliott et al. 2009). Although monarch butterfly is nonnative to New Zealand, it is an iconic species to the public.

Surveys in Argentina also resulted in the ''rediscovery'' of waterhyacinth rust, caused by *Uredo eichhorniae* Fragoso & Ciferri (Charudattan and Conway 1975). Because of its inconspicuousness and patchy distribution in nature as well as its apparent extinction from the original country of its taxonomic description, the Dominican Republic (Ciferri and Fragoso 1927), the rust had eluded the attention of early biocontrol workers, although its occurrence in Argentina and Brazil had been known to mycologists (Lindquist 1982; Viégas 1961). Information on biology and potential of this rust for the biological control was gathered, but because its life cycle could not be fully delineated, no postquarantine studies could be undertaken (Charudattan and Conway 1975; Charudattan et al. 1976, 1981).

A third prospective biocontrol agent, a Dutch isolate of *Fusarium culmorum*, (W.G. Sm.) Sacc., isolated from crab's-claw (*Stratiotes aloides* L.), was also discovered and shown to be pathogenic to hydrilla and a few other submerged aquatic plants in laboratory tests (Charudattan and McKinney 1978; Charudattan et al. 1980a). The pathogen appeared promising in small-scale studies, but when tested in large aquaria, practical challenges emerged that required an understanding of the epidemiology of this pathosystem. However, due to lack of funding, these studies, which are discussed in the next section, remained unresolved for more than a decade.

Although I stopped my work on classical biocontrol agents for exotic weeds in Florida by the late 1980s, my group contributed to a classical biological control program in Australia. In 1989, under a contract from the Alan Fletcher Research Station of the Queensland Primary Industries and Fisheries, we undertook a survey of pathogens attacking eastern baccharis (*Baccharis halimifolia* L.), a plant native to the United States and very common in Florida. It is a declared pest plant in Queensland and New South Wales. By the late 1980s, with 35 separate introductions of insect biocontrol agents providing mixed results, attention was given to pathogens. Our surveys and evaluations in Florida yielded three prospective agents: *Dothidea puccinioides* (DC.) Fr., an unidentified *Phomopsis* sp., and a rust fungus, *Puccinia evadens* Harkn. (Charudattan et al. 1995). A host-range study by Uma Verma provided the basis for introduction of the rust into Queensland, where it has spread throughout the Australian range of eastern baccharis (called groundsel bush in Australia) and produced variable levels of damage (Sims-Chilton et al. 2009; Verma et al. 1996). The rust is reported to be more abundant and effective on larger plants, particularly at high levels of rainfall (Sims-Chilton et al. 2009). Our unpublished observations in Florida indicate that young seedlings of eastern baccharis growing under infected mature plants can become heavily infected and may be killed. Together, these results and observations lead me to place *P. evadens* in the category of partially successful classical biocontrol agents (Charudattan 2005b).

From Water to Land: Bioherbicides for Weeds in Horticultural Crops

The focus of my research during the first decade was on aquatic weeds, mainly waterhyacinth and hydrilla. By the mid-1980s, the Florida group consisting of Kenneth E. Conway, Ed Freeman, and myself worked with Abbott Laboratories to develop *Cercospora rodmanii* (= *Cercospora piaropi*; Tessmann et al. 2001) as a bioherbicide for waterhyacinth. Field tests of a commercial formulation under an experimental use permit produced promising results, but further studies for commercial development were abandoned for economic and marketing reasons. Although hydrilla was still a major target for our principal funding agency for aquatic weeds, the U.S. Army Corps of Engineers, the best candidate pathogen for hydrilla control at that time was our isolate of *F. culmorum* from the Netherlands. Although the fungus was allowed to be tested inside a greenhouse, the tests could be done only under suboptimal and unnatural conditions. Nonetheless, the tests revealed some limitations to the use of this pathogen as a bioherbicide. For instance, the estimated amount of inoculum needed for control of a submerged weed like hydrilla on a large scale, e.g., a reservoir, was very large (our laboratory and field application standard was 100,000 spores/ml of the treated volume). Clearly, it would be expensive to produce such a large amount of inoculum. It was also of concern that a large dose of inoculum would produce an unacceptable microbial load in water. Also, the *Fusarium* functioned like a contact herbicide with no secondary disease cycles. So, inoculative applications to establish disease foci would not produce an epidemic. These limitations were subsequently confirmed by Margaret Smither-Kopperl in models of settlement, adhesion, and movement dynamics of spores in the hydrilla–*Fusarium culmorum* system (Smither-Kopperl et al. 1998, 1999).

Unlike *F. culmorum*, *Mycoleptodiscus terrestris* (Gerdemann) Ostazeski, a pathogen of hydrilla and Eurasian watermilfoil (*Myriophyllum spicatum* L.) is capable of epidemic spread from initial infection sites because it can sporulate and produce secondary inocula on diseased plants. This was reported in 1993, when Uma Verma, working under a contract from EcoScience Corporation, Worcester, MA, confirmed both the susceptibility of hydrilla to *M. terrestris* and secondary disease development beyond the initial points of infection on inoculated hydrilla and Eurasian milfoil plants (Verma and Charudattan 1993). This ability to spread from primary infection foci makes *M. terrestris* a better bioherbicide candidate for hydrilla than *F. culmorum* (see Shearer [2010] for more on *M. terrestris*).

Collectively, this experience and the limited choice of biocontrol targets among aquatic weeds prompted me to leave water (aquatic weeds) for upland (crop weeds) which I did by the end of the 1980s. Nonetheless, the early years provided me

a broad foundation for continued engagement in biological control of weeds by plant pathogens.

Lessons Learned

Successful as well as failed endeavors add valuable lessons to the quilt of career-long learning, and it is difficult to single out a few lessons from this broad, interlaced realization. Nevertheless, here are a few lessons that I consider important for the future of research and development in biological control of weeds by plant pathogens.

1. Initial Inoculum is Critical, but It Alone is Not the Answer. An underlying precept in the bioherbicide strategy is that by matching inoculum availability to a susceptible host stage (generally the seedling stage or when the annual growth cycle beings) or to optimal environmental conditions for disease development (generally spring, early summer, or fall), an epidemic could be initiated. Bioherbicide literature is full of examples of attempts to provide a properly timed high amount of inoculum (i.e., inundative application) of a promising candidate pathogen in hopes of creating an epidemic of weed-suppressive strength. While theoretically this is true, particularly for foliar pathogens, initial inoculum alone will not help if the pathogen lacks sufficient aggressiveness (i.e., level of virulence) or has poor capacity for secondary inoculum production that is essential for increase in pathogen population and disease spread. Even with an aggressive pathogen that has capacity for secondary spread, a host plant may be able to compensate for disease-induced losses. Furthermore, environmental constraints may limit disease spread in space or time. Thus, such factors can foil the desired outcome from use of high rates of an initial inoculum.

The interaction of pathogen aggressiveness and secondary spread is well illustrated by the success of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. f.sp. *aeschynomene*, a fungus previously registered as 'Collego' and now reregistered as 'Lockdown' (Andersen 2006). It is highly aggressive on northern jointvetch, sporulates readily and profusely on infected plants, and spreads rapidly and widely from initial infection foci. Its efficient dispersal is aided by several agents and mechanisms such as green treefrogs, grasshoppers, high infectivity, abundant sporulation, dispersal by rain, and high rate of disease increase operating in the field (Yang and TeBeest 1992a,b, 1993, 1994). Compared to *C. gloeosporioides* f.sp. *aeschynomene*, *Alternaria cassiae* Jurair & Kahn, another foliar fungal pathogen and a bioherbicide candidate for sicklepod [*Senna obtusifolia* (L.) H.S. Irwin & Barneby], kills seedlings that are sprayed POST with a spore suspension (Charudattan et al. 1986; Pitelli and Amorim 2003; Walker and Boyette 1985). However, the pathogen population does not increase and disease does not spread appreciably from initial infection foci, and consequently, the effect from POST application of this bioherbicide agent is similar to that of a contact herbicide. After considerable effort, commercial development of *A. cassiae* was abandoned in the United States, but there is continuing interest in Brazil where De Simoni et al. (2006) found soil incorporation of *A. cassiae* on fungus-colonized sicklepod seeds to be a promising method of application. As an alternative to POST spraying, a combination of POST spraying and soil incorporation of *A. cassiae* merits further consideration.

2. Formulation is Essential, But the Pathogen Holds the Key to Bioherbicide Efficacy. Whereas inoculum availability can be controlled by synchronizing application to a susceptible host stage or with favorable environmental conditions, or both, such can be difficult to achieve in practice. Environmental constraints, particularly the need for moisture for fungal spore germination on foliar surfaces, can be overcome by formulation technology, and we explored in this area with several weed–pathogen systems (Chandramohan and Charudattan 2001; Chandramohan et al. 2002; Kadir et al. 2000; Shabana et al. 1997; Vincent and Charudattan 1999). Although the type of formulation (liquid concentrate, wettable powder, emulsion, solid substrate, etc.) using appropriate materials (oils, gels, starch, cellulose, etc.) is essential for assuring bioherbicide efficacy, handling, and shelf life, the primary objective of formulation should be to improve weed control efficacy and consistency of performance. Handling characteristics and shelf life are important secondary considerations, as are the economics of formulation; any increase in the cost of the bioherbicide product will have to be weighed against what the market will bear. The key to success however is having a pathogen with an innate capacity to kill or severely cripple the target weed; it is simply not possible to make a weak pathogen into a super killer solely with the aid of formulation.

3. Despite Setbacks, Weeds Can Outgrow Disease Pressure. Another stark reality in several weed–pathogen systems we studied is the capacity for weeds to overcome disease pressure (i.e., the cumulative stress imposed on a plant by a disease) through rapid vegetative growth and vigorous regeneration following disease-induced loss of productivity. This capacity may be amplified if a weed has more than one mode of reproduction (i.e., sexual and vegetative, the latter by bulbs, tubers, turions, stolons, rhizomes, and others) or if it is a perennial. In addition to multiple modes of reproduction, many aquatic weeds are capable of spreading by vegetative fragmentation, a means by which uninfected plant propagules can escape from a disease focus by simply floating away or through transport by water currents (Charudattan 2005a). To overcome this challenge of compensation for disease losses, a bioherbicide pathogen should be capable of killing the weed outright or inflicting a near-lethal level of disease stress (i.e., chronic stress) during critical periods of weed growth and reproduction. The latter is possible only if the level of initial disease and the rate of secondary disease increase combined cause a net loss in weed biomass and reproduction. In our experience with waterhyacinth, hydrilla, purple nutsedge (*Cyperus rotundus* L.), cogongrass [*Imperata cylindrica* (L.) Beauv.], and several weedy grasses, nonlethal foliar fungal pathogens applied in POST sprays have not been effective in controlling weeds at a practical level, because these species, after sustaining the initial loss from disease, simply outgrow disease pressure.

It is possible to augment the level of damage by formulating pathogens (i.e., spores, mycelium, or both) with phytotoxic or

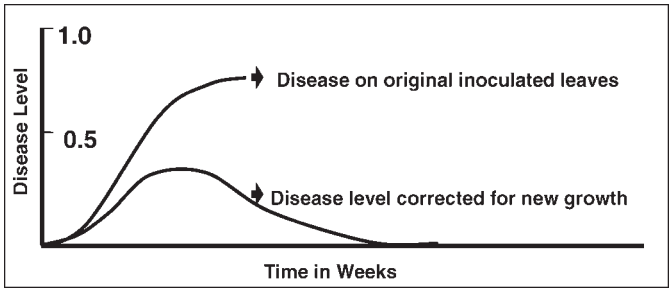

Figure 1. A diagrammatic representation of the *Cercospora piaropi*–waterhyacinth system in which the initial disease level established by spraying the fungus on the leaves is diminished by rapid plant growth. The disease level on the original inoculated leaves when corrected for new growth over a 6-wk period shows a drastic decline in the disease level because the disease fails to spread to the new leaves that replace the original inoculated leaves.

growth-suppressive agents (e.g., oil emulsion or sublethal concentration of a phytotoxin, a chemical herbicide, or a foliar desiccant) or by handicapping the weed through multiple applications of a bioherbicide, combinations of pathogens applied in a bioherbicide cocktail, or through competition from crop plants (Chandramohan and Charudattan 2001, 2003; Chandramohan et al. 2002; Cook et al. 2009; Evidente et al. 2006a,b; Morales-Payan et al. 2003; Pitelli et al. 1998; Vincent and Charudattan 2000; Wyss et al. 2004; Yandoc et al. 2004, 2005, 2006). However, the need for such measures to augment disease damage betrays the lack of sufficient aggressiveness of the pathogen. Furthermore, the practical and economic feasibility of these approaches requires a case-by-case assessment in the case of bioherbicides. Conversely, using a combination of two or more pathogens in a classical biocontrol program is much more feasible and cost effective, and may be highly desirable.

The effect of rapid plant growth on the level of disease caused by *Cercospora piaropi* on waterhyacinth is illustrated in Figure 1. Although *C. piaropi* has good capacity for producing secondary inoculum and can spread rapidly from the initial disease foci, the waterhyacinth often outgrows disease pressure (Charudattan et al. 1985). A similar situation occurs in the *Dactylaria higginsii* (Luttrell) M.B. Ellis–purple nutsedge system. In a greenhouse study, when *D. higginsii* spores were applied once at 10^6 spores/ml, purple nutsedge growth components, as measured from shoot weight, tuber number, and tuber weight, were barely reduced (0 to 0.1%) when compared to the fungus-free control. Two or three applications of spores caused reductions of plant growth variables in the range of 68 to 83% and 72 to 80%, respectively (Kadir et al. 2000). Thus, at least three applications of the bioherbicide agent may be needed to reduce purple nutsedge growth and interference during the critical period of weed–crop competition (Kadir et al. 2000). Pablo Morales-Payan and colleagues confirmed this further in a field trial. When *D. higginsii* was applied to a field-grown bell pepper crop, highest pepper yield was seen in purple nutsedge–free, hand-weeded plots when compared to purple nutsedge–infested, untreated plots, which had the lowest yield. One application of *D. higginsii* 8 d after weed emergence reduced purple nutsedge growth and increased overall bell pepper yield and the proportion of large and extra-large fruit when compared to untreated, purple nutsedge–infested pepper. Two applications of *D. higginsii* (8 and 18 d after emergence) resulted in the same yield of large and medium-size fruit as in the purple nutsedge–free crop, although the yield of extra-large ''fancy'' fruit was lower than in the weed-free crop (Morales-Payan et al. 2003). The need for repeated applications of *D. higginsii* to control purple nutsedge under field conditions also illustrates the difficulty in controlling a weed with a robust capacity for vegetative regeneration. From an economic standpoint, the feasibility of a triple application of a bioherbicide agent is doubtful.

4. Genetic Heterogeneity in Weeds is a Challenge. We came upon the challenge of dealing with genetically diverse weed species and genotypes in Erin Rosskopf's work on the *Phomopsis amaranthicola*–pigweeds system (Rosskopf et al. 2000b). This fungus, a new species described by Rosskopf et al., is a genus-specific pathogen of pigweeds (*Amaranthus* spp.) that causes leaf spots, leaf abscission, and stem lesions (Rosskopf et al. 2000a, 2005a). The latter can girdle and kill infected plants. Both mycelium and spores of *P. amaranthicola* are infective and the inoculum is easy to produce. It produces abundant inocula on infected plants and spreads readily within the field. Typically, 85 to 100% control of young seedlings of susceptible *Amaranthus* spp., from the cotyledon stage up to the two-leaf stage, can be obtained with one or two POST applications of *P. amaranthicola*. Several troublesome pigweed species including *Amaranthus hybridus* L., *Amaranthus lividus* L., *Amaranthus retroflexus* L., and *Amaranthus spinosus* L. are susceptible. However, some species tested had intraspecific differences in susceptibility, i.e., different accessions within a species reacted differently to the pathogen), which is a complicating factor in this bioherbicide system (Rosskopf et al. 2005a). This experience with the pigweed–*Phomopsis* system illustrates the difficulty in controlling a species with genetic heterogeneity. A plant species that consists of genetically heterogeneous members or one that maintains genotypic plasticity through outcrossing will tend to have a genetically heterogeneous mixture of host-specialized pathotypes in the pathogen's population structure. It is difficult and perhaps impossible to find a single strain of a pathogen that is effective against all of the weed genotypes. As a corollary, weed species that have become invasive from a small number of introduced individuals should be good targets for control with a single pathogen isolate, because they are likely to be genetically homogeneous as a result of the founder effect.

Host genetic variability was an important theme in Alana den Breeyen's work on cogongrass (den Breeyen 2007). With the view to develop a pathogen-based biocontrol for cogongrass in the southeastern United States and West Africa, Alana examined genetic variability in cogongrass accessions from Florida, West Africa, and South Africa by using intersimple sequence repeat markers and sequence analysis of internal transcribed spacer and the noncoding chloroplast *trnL-trnF* spacer DNA. The purpose was to interpret the data in relation to implementation of biological control strategies using the two fungi, *Bipolaris sacchari* (E.J. Butler) Shoemaker and *Drechslera gigantea* (Heald & Wolf) Ito, in Benin, West Africa. Biological control may prove to be ineffective if

cogongrass populations are genetically heterogeneous within and between the U.S. and African populations. Included in this study were *Imperata brevifolia* Vasey, *Imperata brasiliensis* Trin., and the ornamental variety *I. cylindrica* var. *rubra*. Possibly due to introgression as well as overlapping morphological characters, it is often difficult to distinguish between cogongrass and *I. brasiliensis*. Den Breeyen's study indicated that the U.S. and West/South Africa *I. cylindrica* populations were geographically and genetically separated and defined by three distinct groups. Among the Florida cogongrass accessions were several genetic distinctions, confirming that *I. cylindrica* was introduced in the United States at different times and sources. *Imperata brasiliensis* was not genetically distinct from the U.S. *I. cylindrica* population as a whole but rather was related as a sister species to the Florida *I. cylindrica* accessions. *Imperata cylindrica* var. *rubra* was more closely related to the West African and South Africa populations. *Imperata brevifolia* was genetically distinct from all the *Imperata* populations within this study. When the U.S. isolates of *B. sacchari* and *D. gigantea*, which had been previously determined to be prospective bioherbicide agents for cogongrass (Yandoc et al. 2004, 2005), were compared with West African isolates of these pathogens for pathogenicity to the West African *I. cylindrica*, there was low variation among the isolates. Thus, the Florida isolates of *B. sacchari* and *D. gigantea* could be used against the U.S. and West African cogongrass, but all isolates of these pathogens, from Florida or West Africa, lacked the necessary aggressiveness or reproductive capability to be able to control cogongrass (Den Breeyen 2007).

5. Scale-Up and Mass Production: A Severe Bottleneck. A common problem with fungi is the difficulty of mass-producing spores for both precommercial field trials and commercial product development. With a few fungi, both mycelium and spores can be used as inoculum (e.g., *Alternaria eichhorniae* Nag Raj & Ponnappa, *C. piaropi*, and *P. amaranthicola*), whereas others require spores (Freeman and Charudattan 1984; Rosskopf et al. 2005b; Shabana et al. 1995; Tessmann et al. 2008). Generally, spores are more difficult to mass produce than mycelial biomass. Some that require spores, such as *A. cassiae*, *B. sacchari*, *D. gigantea*, *Exserohilum longirostratum* (Subram.) Sivan., and *Exserohilum rostratum* (Drechsler) Leonard & Scuggs could be produced with substantial effort on a laboratory scale, but *D. higginsii* was particularly problematic. A slow-growing fungus, *D. higginsii* also sporulates poorly, which is an intractable problem for mass production of this bioherbicide agent.

The preferred industrial method for production of fungi is liquid fermentation. This approach is not suitable for all fungi because most do not produce spores in submerged culturing. A biphasic system, modified from the method of Walker (1980), was a good standby in our work, but it is labor-intensive and expensive for large-scale production (Chandramohan and Charudattan 1998). Autoclaved grain and dried plant biomass were good natural substrates for laboratory-scale solid-state production of fungal spores (Wyss et al. 2001; Yandoc et al. 2005). As an offshoot of the solid-state production, we examined various plant materials as both substrates and carriers of fungal inocula in field studies

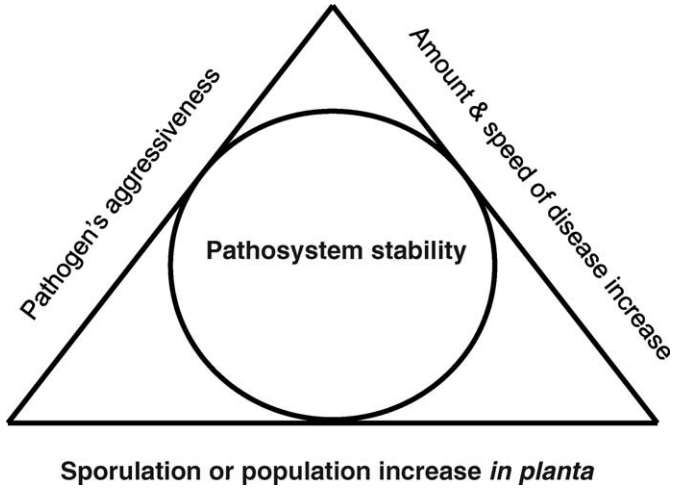

Figure 2. Essential attributes of a highly effective bioherbicide system.

(Shabana et al. 2007). Based on our results and those of De Simoni et al. (2006), the use of pathogen-infested plant substrates appears to be practical and deserves further evaluation. Interestingly, compared to the various fungi we have attempted to mass produce, a bacterium [*Ralstonia solanacearum* (Smith) Yabuuchi et al.] and a virus (tobacco mild green mosaic virus [TMGMV; *Tobacco mild green mosaic tobamovirus*]), both bioherbicide candidates for tropical soda apple (*Solanum viarum* Dunal), have been far easier to mass produce (Charudattan and Hiebert 2007; DeValerio and Charudattan 1999).

6. Wanted: Natural Born Killers. The revelation from all of this takes me back to the thesis of this paper: that highly efficacious and successful bioherbicides contain pathogens that are killers that get the job done (Charudattan 1990, 2005b). I call them ''natural born killers'' because they are characterized by having a high level of aggressiveness with the ability to kill or inflict a near-lethal level of disease severity. A pathogen's lack of ability to kill outright may be compensated for by its capability to sporulate or otherwise multiply profusely in planta, produce rapid secondary disease increase, and spread from initial infection. Also, in successful cases, the weed–pathogen system is stable in terms of the pathogen's traits as well as the weed's genotypic homogeneity (i.e., the pathogen is stable in its virulence or aggressiveness, sporulation, reproduction, and other pathogenicity traits and the weed is genetically homogeneous and lacks genotypic plasticity). These attributes are diagrammatically represented in Figure 2.

In an analysis published in 2005, I reported that nearly 20.5% of the classical biocontrol and bioherbicide projects were partially or fully successful, 25.3% were of uncertain outcome and possibly too early to judge, and a slight majority, 54.2%, were ineffective or simply abandoned before field release. Within the subset of bioherbicide projects, only 8.1% were successful, leaving 91.5% of the projects as uncertain, untried, or ineffective (Charudattan 2005b). To improve the success rate of bioherbicides it would seem necessary to choose

for development only pathogens that have the necessary killer traits, namely those that fit the model in Figure 2.

7. The Perfect Bioherbicide? We came upon a ''natural born killer'' in 1999 while screening tropical soda apple, a noxious weed dubbed the ''plant from hell,'' for susceptibility to some plant viruses (Coile 1993; Pettersen et al. 2000). Initially, Mathew Pettersen established unequivocally that TMGMV killed tropical soda apple plants (Pettersen et al. 2000). This and subsequent studies revealed that tropical soda apple is killed by a highly unusual phenomenon of a virus-elicited delayed hypersensitive response (Charudattan et al. 2004; Charudattan and Hiebert 2007; Horrell 2007). The pathogen, a member of the genus *Tobamovirus*, typically causes a mild, green, mosaic symptom in susceptible hosts, almost all of which are in the Solanaceae. In tropical soda apple, TMGMV evokes a highly host-specific host response that kills the entire plant. Plants of all ages are killed. When properly applied, it is possible to kill the weed with 100% efficacy and 100% consistency in the field. The host–pathogen interaction in this system is easy to manage compared to that of a fungal bioherbicide system: (1) there is no moisture requirement for virus infection, (2) application can be made at any time of day after the morning dew has evaporated off the foliage, and (3) inoculation is possible over the entire growing season for tropical soda apple in the South. The only requirements are that the plant should be actively growing or metabolizing when treated, and that the virus has to infiltrate the leaf tissue. The latter can be accomplished by POST spraying at high pressure ($\geq$ 552 kPA [80 psi]) or with a wiper applicator that slightly abrades the foliage. The virus is easy to mass produce on an industrial scale and has an exceptionally long shelf life. Based on extensive host-range, dispersal, environmental persistence, and other studies, feasibility and safety of TMGMV as a bioherbicide were confirmed. A commercial formulation of the virus, *SolviNix* LC, was approved in 2007 for field trials under an experimental use permit and is awaiting full registration.

The molecular–biochemical mechanism of plant-kill in this pathosystem is still unresolved but the predictable course of plant response to TMGMV infection offers room for some speculation. Typically, a week after inoculation, the tropical soda apple plant develops local lesions, a hypersensitive resistance response, on a few leaves (Horrell 2007). This is followed by chlorosis and epinasty over the entire canopy by around 3 wk, rapid wilting in the next few days, and complete plant death in 3 to 5 wk (Figure 3). The rapid and complete wilting of the plant followed by death suggests that the virus-elicited hypersensitive host response (cell death) occurs in some or all living cells and tissues that are vital for plant survival. The root hairs, the phloem, the mesophyll, or all of these are likely sites of action. Alternatively, gene silencing might be at play, affecting some gene function (or functions) critical to plant survival. The TMGMV–tropical soda apple pathosystem is certainly the most extraordinary and exciting of the bioherbicide systems I have encountered. An important lesson from it is the need for researchers to keep an open mind and a watchful eye for unusual and surprising results, particularly in weed–pathogen systems that previously have not been well studied.

A Missed Opportunity

In hindsight, in our preoccupation with development of bioherbicide products, we did not and could not pursue studies either on the molecular–mechanistic or the ecological–population aspects of pathosystems we encountered. While we remained focused on methods and technology for product development, there were major advances within plant pathology in the areas of genetic–molecular basis of virulence/aggressiveness, host range, host specificity, host response to infection, and pathogen-elicited cell, organ, and plant death. An exception was Dauri Tessmann's work on the population structure of *Cercospora piaropi* that characterized pathogen variability in aggressiveness, cultural characteristics, toxin production, and fatty acid profile (Tessmann et al. 2001, 2008). It is well known that a plant in its native range will have a diversity of coevolved pathogen strains with differences in virulence/aggressiveness and other traits. Yet we rarely applied this principle in our search for the most effective bioherbicide isolates. In nearly all cases, when a prospective bioherbicide candidate was identified, we settled for the strain at hand, discovered through a chance encounter, and made little or no attempt to find the most aggressive strain of that pathogen for final development as a bioherbicide. The funding for research on bioherbicide generally did not allow extensive exploration and characterization of the population structure of the pathogen candidate. Since regulatory agencies (e.g., the U.S. Environmental Protection Agency in the United States) require a bioherbicide product to consist of a single, well-characterized strain, it is prudent to survey the pathogen population structure as much as possible before committing to a particular isolate for development.

The importance of this lesson was brought home in Dauri Tessmann's study of a collection nearly 70 isolates of *Cercospora* recovered from waterhyacinth exhibiting *Cercospora* leaf spot–like symptoms (Tessmann et al. 2001, 2008). The isolates were from various parts of the world, representing both the native and adventive ranges of waterhyacinth. As this study revealed, highly aggressive as well as nonaggressive isolates were found from within and outside the center of origin of waterhyacinth. Although the isolate chosen for bioherbicide development by Abbott Laboratories in the 1980s was among the aggressive isolates, it was clearly not the most aggressive. It might have been worthwhile to have surveyed at the beginning of the waterhyacinth program and assessed the extent of variability among *C. piaropi* isolates in order to find and develop the most aggressive isolate.

More recently, Alan Pomella followed a studied approach in his selection of a *Nimbya alternantherae* (Holcomb and Antonopoulos) E.G. Simmons & Alcorn isolate for biological control of alligatorweed [*Alternanthera philoxeroides* (Mart.) Griseb.] (Pomella et al. 2007). He compared several isolates of this fungus from Brazil, the United States, and Puerto Rico, and found no differences in virulence. However, the Brazilian isolates had lower dew requirement for infection, which is very desirable in a foliar pathogen intended as a bioherbicide.

Applications for the Future

As a research endeavor, biological control of weeds with plant pathogens still has room to grow and evolve. I look at

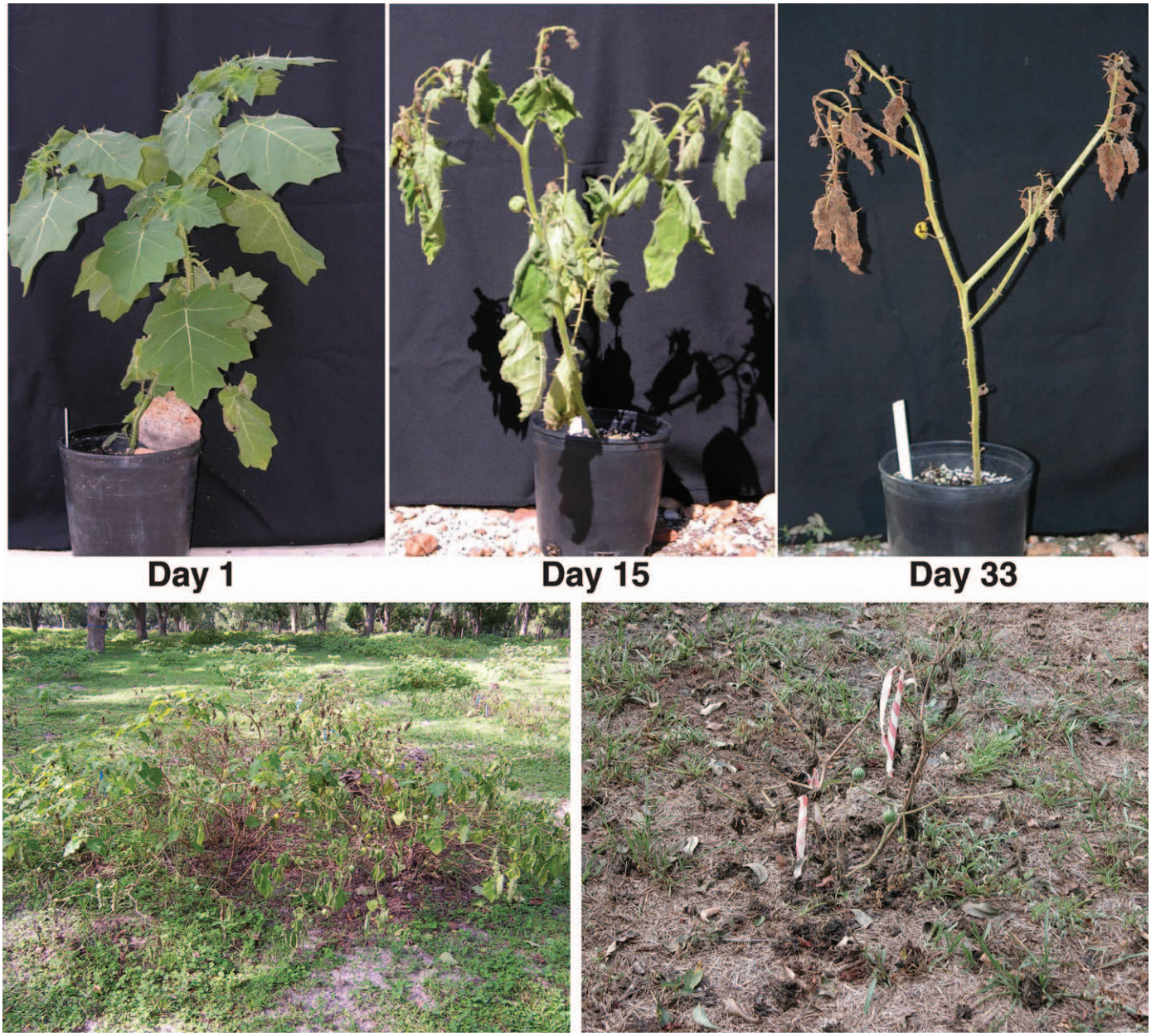

Figure 3. Progression of tobacco mild green mosaic virus–elicited plant death in tropical soda apple. Top row: a virus-inoculated tropical soda apple plant showing disease progression at days 1, 15, and 33 after inoculation. Bottom row: an inoculated tropical soda apple plant in the field showing chlorosis, epinasty, and early signs of wilting (left) and a completely killed plant (right).

the cup as half full, not half empty, and workers in this field worldwide deserve to be complimented for what they have accomplished. Valuable knowledge has been gained, and as we look to the future, we are well equipped to apply the knowledge gained over nearly four decades. Clearly, we can and should do better to improve the ratio of success with bioherbicide agents. However, success rate alone does not tell the whole story and certainly should not discourage further investment in this field. Unlike the vast number of leads available for screening for chemical herbicides, suitable candidates for biological control are relatively few, since only those pathogens that have been selected out in nature by coevolutionary forces are available for exploitation. Furthermore, the total investment of resources in plant pathogens is also miniscule compared to that for chemicals, and it is small relative to investments for insect biocontrol agents. Considering this, the rate of success with classical and bioherbicide projects involving pathogens is reasonable.

As for the future, first and foremost it is necessary to continue the search for promising leads, namely weed–pathogen systems that have elements for success (Figure 2). Finding and characterizing good, solid leads is fundamental for building on the foundation of knowledge and experience gained thus far. To assure continued funding, administrative support, and peer recognition for this field, each lead should be scientifically interesting and challenging. While commer-

cial feasibility and practical applicability will remain a driving force, good basic science should guide research.

Although fewer bacterial and viral pathogens may qualify relative to fungi, more effort should be directed at finding bacterial and viral pathogens. Application of pathogen genes, gene products, and genetic mechanisms for weed control should be emphasized. In this regard, two areas are worth pursuing: (1) plant hypersensitivity responses and (2) viral gene silencing. An understanding of genetically programmed host–pathogen interactions could lead to newer modes of plant pathogen-mediated herbicidal action that may be exploited and utilized to kill weeds, restrain weed growth, or knock out traits for invasiveness. In the continuing struggle to manage weeds in a safe and sustainable manner, research on biocontrol by pathogens should remain a primary thrust.

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