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ALFRED COFRANCESCO

AQUATIC PLANT CONTROL
RESEARCH PROGRAM

MISCELLANEOUS PAPER A-86-1

BIOENGINEERING TECHNOLOGY MEETING

September 28-29, 1983
US Army Engineer Waterways Experiment Station
Vicksburg, Mississippi

by

Judith C. Pennington

Environmental Laboratory

DEPARTMENT OF THE ARMY
Waterways Experiment Station, Corps of Engineers
PO Box 631, Vicksburg, Mississippi 39180-0631

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The use of genetic engineering technology is being considered for application in the area of aquatic plant control. To determine the feasibility of establishing a work effort toward that end, a conference was convened at the US Army Engineer Waterways Experiment Station (WES). Three noted scientists in the field were invited to meet with WES staff working on the general area of biological control of aquatic plants. It was determined that existing technology in bioengineering is sufficiently advanced to produce a microorganism for effective control of nuisance aquatic plants.

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Preface

This report summarizes the results of a workshop meeting held at the US Army Engineer Waterways Experiment Station (WES) during 28-29 September 1983. The conference was sponsored by the Aquatic Plant Control Research Program (APCRP) under the area of research studying biological control of aquatic plants.

The conference was organized, conducted, and the report prepared by Mrs. Judith C. Pennington under the direct supervision of Dr. Dana R. Sanders, Sr., Wetland and Terrestrial Habitat Group, Dr. Hanley K. Smith, Chief. The work was accomplished under the general supervision of Dr. Conrad J. Kirby, Chief, Environmental Resources Division, and Dr. John Harrison, Chief, Environmental Laboratory. Mr. J. Lewis Decell was Manager, APCRP. Mr. Dwight Quarles was Technical Monitor for the Office, Chief of Engineers, US Army. The report was edited by Ms. Jamie W. Leach of the WES Publications and Graphic Arts Division.

Director of WES was COL Allen F. Grum, USA. Technical Director was Dr. Robert W. Whalin.

This report should be cited as follows:

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Contents

BIOENGINEERING TECHNOLOGY MEETING

Introduction

Background

Eurasian watermilfoil (Myriophyllum spicatum L.) and hydrilla (Hydrilla verticillata (L.f.) Royle) are two noxious submersed aquatic plants that interfere with drainage, irrigation, boat traffic, and recreational use of waterways. Eurasian watermilfoil is distributed worldwide and is present in every state in the United States. It reaches most severe proportions in the northern tier of states, gulf and Atlantic coasts states, the southeast, and a few of the mid-Atlantic states. Hydrilla occurs in 100,000 to 150,000 acres* of the Nation's waterways. Most extensive growth occurs in the gulf and Atlantic coast states, but it has been found in isolated sites as far north as Delaware and Iowa. It has, therefore, tremendous potential for expanding its present range.

The conventional approach for finding control agents for such exotic nuisance species is to conduct searches for natural enemies in the species' country of origin. After 10 years of searching, no effective control agents have been made available. Overseas as well as domestic searches continue, but many years will be required to develop any agents found. If no successful agents are found, conventional approaches will have been exhausted and no biocontrol agents will be available. Furthermore, chemical and mechanical methods have not proven cost-effective in providing the desired level of control.

For these reasons, it has become necessary to explore less conventional approaches for development of biocontrol agents for these noxious submersed aquatic plants. Application of genetic engineering technology is one possible approach. Through existing genetic engineering technology, microorganisms have been produced and some are commercially available to address problems in such diverse areas as interferon production, treatment of oil spills, control of agricultural pests, and production of pharmaceuticals. To examine the feasibility of applying existing technology to the development of biocontrol agents of aquatic plants, a small conference was conducted at the US Army Engineer Waterways Experiment Station, Vicksburg, Miss. Three outstanding scientists involved in different aspects of genetic engineering were invited to attend. This report presents the results of that meeting.

Purpose

The purpose of the conference was to determine the feasibility of using genetic engineering technology for the development of microorganisms as biological agents for aquatic plant control.

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* To convert acres to square metres multiply by 4,046.873.

Objectives

Specific objectives were to:

(a) Identify and describe applicable tools already available.

(b) Define needs, if any, not met by state-of-the-art technology.

(c) Outline an approach for the research effort.

(d) Estimate a time frame for each research element identified.

(e) Develop cost estimates for each research element.

(f) Ascertain whether or not a full-scale workshop is warranted for further examination of the topic.

(g) Catalog sources from which further specific information may be obtained.

BIOENGINEERING TECHNOLOGY MEETING
September 28-29, 1983

Agenda

WEDNESDAY

8:30-8:35	Call to Order and Introductions	Mrs. Judy Pennington
8:35-8:40	Welcome to Environmental Laboratory	Dr. John Harrison
8:40-9:00	Aquatic Plant Control Research Program (APCRP)	Mr. Lewis Decell
9:00-9:30	Biological Control of Aquatic Plants	Dr. Dana Sanders, Sr.
9:30-10:00	Microbiological Control of Aquatic Plants and Meeting Objectives	Mrs. Judy Pennington
10:00-10:15	Break	
10:15-10:45	Genetic Engineering of Fungi	Dr. O. C. Yoder
10:45-11:15	Genetic Engineering of Bacteria	Dr. George Lacy
11:15-11:45	Gene-Splicing Technology	Dr. Gerard Riedel
11:45-1:00	Lunch	
1:00-2:30	Session 1: Feasibility	
2:30-2:45	Break	
2:45-4:15	Session 2: Techniques	

THURSDAY

8:30-8:45	Summary of First Day	Mrs. Judy Pennington
8:45-10:15	Session 3: Approach	
10:15-10:30	Break	
10:30-12:00	Session 4: Practicality	
12:00-1:00	Lunch	
1:00-2:00	Session 5: Conclusions and Recommendations	

Participants

Guests

Dr. George H. Lacy, Virginia Polytechnic Institute and State University, Blacksburg, Va.

Dr. Gerard E. Riedel, Genetics Institute, Boston, Mass.

Dr. O. C. Yoder, Cornell University, Ithaca, N. Y.

Hosts

Mr. J. Lewis Decell, US Army Engineer Waterways Experiment Station (WES), Vicksburg, Miss.

Mrs. Judy Pennington, WES

Mr. William Rushing, WES

Dr. Dana R. Sanders, Sr., WES

Dr. George H. Lacy

Associate Professor, Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061.

Highest Academic Degree: Ph.D., Plant Pathology, University of California, Riverside, California.

Research: Dr. Lacy is currently involved in a research project entitled "Mechanisms for Plant Damage by Erwinia chrysanthemi: Cloning Genes for Pectolytic Activity." The long-term goal of this research is to define the genetic basis for pathogenicity in this species. The research will generate knowledge of the number and regulation of genes important in host-pathogen interactions, enhance understanding of pathogenic mechanisms, and provide a library of cloned DNA fragments for future studies.

Pertinent Publications:

1. Lacy, G. H., and Leary, J. V. 1975. "Transfer of Antibiotic Resistance Plasmid RP-1 into Pseudomonas glycinea and Pseudomonas phaseolicola in vitro and in planta," J. Gen. Microbiol., Vol 88, pp 49-57.

2. Lacy, G. H., and Leary, J. V. 1976. "Plasmid-mediated Transmission of Chromosomal Genes in Pseudomonas glycinea," Genet. Res. Camb., Vol 27, pp 363-368.

3. Lacy, G. H. 1978. "Genetic Studies With Plasmid RP1 in Erwinia chrysanthemi Strains Pathogenic on Maize," Phytopathology, Vol 68, pp 383-1330.

4. Lacy, G. H., and Leary, J. V. 1979. "Genetic Systems in Phytopathogenic Bacteria," Annu. Rev. Phytopathol., Vol 17, pp 181-202.

5. Lacy, G. H., and Sparks, R. B., Jr. 1979. "Transformation of Erwinia herbicola with Deoxyribonucleic Acid of Plasmid pBR322," Phytopathology, Vol 69, pp 1293-1297.

6. Lacy, G. H., Hirano, S. S., Victoria, J. I., Kelman, A., and Upper, C. D. 1979. "Inhibition of Soft-rotting Erwinia Strains by 2,4-dihyroxy-7-methoxy-2H-1, 4-benzoxazin-3(4H)-one in Relation to Their Pathogenicity on Zea mays," Phytopathology, Vol 69, pp 757-763.

7. Sparks, R. B., Jr., and Lacy, G. H. 1980. Purification and Characterization of Cryptic Plasmids pSL1 and pSL2 from Erwinia chrysanthemi," Phytopathology," Vol 70, pp 369-372.

8. Mount, M. S., and Lacy, G. H., eds. 1982. Phytopathogenic Prokaryotes, Vol 1 (541 pp) and Vol 2 (506 pp), Academic Press, New York, 1047 pp.

Dr. Gerard E. Riedel

Senior Scientist, Genetics Institute, 225 Longwood Ave., Boston, Massachusetts 02115.

Highest Academic Degree: Ph.D., Biology, Harvard University, Cambridge, Massachusetts, 1980.

Research: Dr. Riedel is responsible for coordination of the Institute's agricultural efforts, and the development of vector systems which allow the routine introduction of cloned genes into crop plants. For 1-1/2, years he worked as a visiting scientist at CSIRO, Division of Plant Industry, in Australia. His research there involved development of Agrobacterium vectors for DNA transformation of plant cells, and studies on the molecular genetics of nitrogen fixation.

Pertinent Publications:

1. Ausubel, F. M., Cannon, F. C., and Riedel, G. E. 1976. "Cloning of his and nif Genes from Klebsiella pneumoniae," Recent Developments in Nitrogen Fixation, W. Newton, J. R. Postgate, and C. Rodriques-Barrueco, eds., Academic Press, New York, pp 357-364.

2. Cannon, F. C, Riedel, G. E., and Ausubel, F. M. 1977. "Recombinant Plasmid that Carries Part of the Nitrogen Fixation (nif) Gene Cluster of Klebsiella pneumoniae," Proc. Natl. Acad. Sci. USA, Vol 74, pp 2963-2967.

3. Riedel, G. E., Margolskee, R., Cannon, F., Peskin, A., and Ausubel F. 1977. "The Nitrogen Fixation (nif) Operon of Klebsiella pneumoniae: Cloning nif Genes and the Isolation of nif Control Mutants," Molecular Cloning of Recombinent DNA, W. A. Scott and R. Werner, eds., Academic Press, New York, pp 115-132.

4. Ausubel, F. M., Riedel, G. E., Cannon, F., Peskin, A., and Margolskee, R. 1977. "Cloning of Nitrogen Fixation Genes from Klebsiella pneumoniae in vitro and the Isolation of nif Promoter Mutants Affecting Glutamine Synthetase Regulation," Genetic Engineering for Nitrogen Fixation, A. Hollaender, ed., Plenum, New York, pp 111-128.

5. Cannon, F. C., Riedel, G. E., and Ausubel, F. M. 1979. "Overlapping Sequences of Klebsiella pneumoniae nif DNA Cloned and Characterized," Mol. Gen. Genet., Vol 174, pp 59-66.

6. Riedel, G. E., Ausubel, F. M., and Cannon, F. C. 1979. "Physical Map of the Chromonsomal Nitrogen Fixation (nif) Genes of Klebsiella pneumoniae," Proc. Natl. Acad. Sci. USA, Vol 76, pp 2866-2870.

7. Janssen, K. A., Riedel, G. E., Ausubel, F. M., and Cannon, F. C. 1980. "Transcriptional Studies with Cloned Nitrogen Fixation Genes," Nitrogen Fixation," Vol 1, W. H. Orme-Johnson and W. E. Newton, eds., University Park Press, Baltimore, pp 85-93.

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9. Riedel, G. E. 1980. "The Use of Molecular Cloning to Study the Organization and Expression of the Nitrogen Fixation (nif) Genes of Klebsiella pneumoniae," Ph.D. Thesis, Harvard University, Cambridge, Mass.

10. Roberts, T. M., Swanberg, S. L., Poteete, A., Riedel, G., and Bachman, K. 1980. "A Plasmid Cloning Vehicle Allowing a Positive Selection of Inserted Fragments," Gene., Vol 12, pp 123-127.

11. Riedel, G. E., and Ausubel, F. M. 1981. "Certain Hybrid nif Plasmids Inhibit the Expression of Chromosomal Nitrogen Fixation Genes in Klebsiella pneumoniae," Current Perspectives in Nitrogen Fixation, A. H. Gibson and W. E. Newton, eds., Australian Academy of Science, Canberra, p 396.

12. Riedel, G. E., Brown, E., and Ausubel, F. M. 1983. "Nitrogen Fixation in Klebsiella pneumoniae is Inhibited by Certain Multicopy Hybrid nif Plasmids," J. Bacteriol., Vol 153, pp 45-56.

Dr. O. C. Yoder

Associate Professor, Department of Plant Pathology, 334 Plant Science Bldg., Cornell University, Ithaca, New York 14853.

Highest Academic Degree: Ph.D., Plant Pathology, Michigan State University, East Lansing, Michigan, 1971.

Research: Dr. Yoder's research involves the use of pathogen-produced toxins in genetic engineering of plants and pathogens. He is developing a transformation system for the toxin-producing fungal pathogen Cochliobolus heterostrophus based on complementation of adenine-requiring fungal protoplasts with cloned ADE gene from yeast. Construction of a cloning vector that can be maintained indefinitely by Cochliobolus cells is in progress.

Pertinent Publications:

1. Scheffer, R. P., and Yoder, O. C. 1972. "Host-specific Toxins and Selective Toxicity," Phytotoxins in Plant Diseases, R. K. S. Wood, A. Ballio, and A. Graniti, eds., Academic Press, New York, pp 251-272.

2. Yoder, O. C. 1980. "Toxins in Pathogenesis," Ann. Rev. Phytopathol., Vol 18, pp 103-129.

3. Yoder, O. C. 1981. "Assay," Toxins in Plant Disease, R. D. Durbin, ed., Academic Press, New York, pp 45-78.

4. Leach, J., and Yoder, O. C. 1982. "Heterokaryosis in Cochliobolus heterostrophus," Exp. Mycol. (In press).

5. Leach, J., Lang, B. R., and Yoder, O. C. 1982. "Methods for Selection of Mutants and in vitro Culture of Cochliobolus heterostrophus," J. Gen. Microbiol., Vol 128 (In Press).

6. Leach, J., Tegtmeier, K. J., Daly, J. M., and Yoder, O. C. 1982. "Dominance at the Tox1 Locus Controlling T-toxin Production by Cochliobolus heterostrophus," Physiol. Plant Pathol., Vol 21 (In Press).

Mr. J. Lewis Decell

Program Manager, Corps of Engineers Aquatic Plant Control Research Program, US Army Engineer Waterways Experiment Station, Vicksburg, Mississippi 39180.

Highest Academic Degree, ME, Environmental Engineering Sciences, University of Florida, Gainesville, Florida.

As Program Manager, Mr. Decell is responsible for the planning, development, funding, and execution of the Corps of Engineers' nationwide research program for development of technology to control problem aquatic plants. In managing this program, Mr. Decell is also responsible for the Corps of Engineers' coordination with other Federal agencies, numerous universities, and many state and local agencies. Through the Director of the Waterways Experiment Station, Mr. Decell reports directly to the Office, Chief of Engineers, on program activities.

Mrs. Judy Pennington

Biologist, US Army Engineer Waterways Experiment Station, Vicksburg, Mississippi 39180.

Highest Academic Degree: M.Ed., Chemistry, Southeastern Louisiana University, Hammond, Louisiana.

Research: Biological control of nuisance aquatic plants with microorganisms.

Pertinent Publications:

1. Pennington, J. C., and Theriot, E. A. 1982. "Compatibility of a Cercospora rodmanii Formulation with Selected Herbicide Spray Additives," Miscellaneous Paper A-82-6, US Army Engineer Waterways Experiment Station, Vicksburg, Miss.

2. Pennington, J. C., and Theriot, E. A. 1983. "Compatibility and Infectivity of a Cercospora rodmanii Formulation with Enhancing Agents," Miscellaneous Paper A-83-6, US Army Engineer Waterways Experiment Station, Vicksburg, Miss.

3. Pennington, J. C. 1985. "Biological Control of Hydrilla verticillata (L.f.) Royle with Lytic Enzyme-Producing Microorganisms," Technical Report A-85-3, US Army Engineer Waterways Experiment Station, Vicksburg, Miss.

Mr. William N. Rushing

Assistant to the Program Manager, Corps of Engineers Aquatic Plant Control Research Program, US Army Engineer Waterways Experiment Station, Vicksburg, Mississippi 39180.

Highest Academic Degree: B.S., Millsaps College, Jackson, Mississippi; three years of graduate work in botany, Vanderbilt University, Nashville, Tennessee.

As assistant to the Program Manager, Mr. Rushing is responsible for review and recommendation of program elements during planning and development of the yearly program in aquatic plant research. Mr. Rushing coordinates fiscal matters of the program and acts as liaison with principal investigators on the research program.

Dr. Dana R. Sanders, Sr.

Plant Physiologist, US Army Engineer Waterways Experiment Station, Vicksburg, Mississippi 39180.

Highest Academic Degree: Ph.D., Plant Ecology, Iowa State University, Ames, Iowa, 1969.

Research: Biological control of nuisance aquatic plants with insects and plant pathogens, development of methods for identifying and delineating wetland boundaries, and assessment of wetland values.

Pertinent Publications:

1. Environmental Laboratory. 1981. "The Use of Insects to Manage Alligatorweed," Instruction Report A-81-1, US Army Engineer Waterways Experiment Station, Vicksburg, Miss.

2. Theriot, E. A., Theriot, R. F., and Sanders, D. R., Sr. 1981. "Evaluation of a Formulation of Cercospora rodmanii for Infectivity and Pathogenicity of Waterhyacinth," Technical Report A-81-5, US Army Engineer Waterways Experiment Station, Vicksburg, Miss.

3. Sanders, D. R., Sr., Theriot, R. F., and Theriot, E. A. 1982. "Organisms Impacting Waterhyacinth in the Panama Canal," Miscellaneous Paper A-82-1, US Army Engineer Waterways Experiment Station, Vicksburg, Miss.

Questions to be Addressed in Each Session

Session 1: Feasibility

Can existing genetic engineering technology be applied to microorganisms to effect biocontrol of aquatic plants? What questions must be addressed to answer this?

Session 2: Techniques

What applicable tools are already available and what additional techniques must be developed? Can microorganisms already isolated from hydrilla and Eurasian watermilfoil be made effective biocontrol agents by genetic manipulation?

Session 3: Approach

What general areas of research should be pursued? What criteria for donor and recipient species must be satisfied? Must gene pools be established? What legal factors must be considered?

Session 4: Practicality

For each elements of the research approach developed in Session 3, what are the anticipated costs and time frame?

Session 5: Conclusions

Have all meeting objectives been met? If not, what options are available to complete objectives? Is a full-scale workshop justified? Where may further information be obtained, if needed?

Answers to Questions Addressed in Each Session

Session 1: Feasibility

Q: Can existing genetic engineering technology be applied to microorganisms to effect biocontrol of aquatic plants?

A: The technology to attempt this is certainly available. Microorganisms can be modified to increase production of metabolites that could function in pathogenicity. Some work has been done on the genetic aspects of pathogenicity that would be beneficial and applicable to the effort. The final effects of genetically modified microorganisms on the target aquatic plants cannot be predicted with certainty before actually being attempted.

Q: What questions must be addressed to answer the above question?

A: The following are questions suggested at this point. Examination of these questions and others has been incorporated into the "proposed" study plan.

(a) What are the candidate species? Answering this first and perhaps most crucial question will require extensive effort. The candidate must be host specific to the target plant and as many candidates as possible should be identified.

(b) To what substances or injurious mechanisms is the host plant susceptible?

(c) What molecular mechanisms characterize the relationship between the candidate microorganism and the target plant? If various genetic strains exist, the virulence of each must be assessed and the most virulent selected. An intensive study must be made of the host plant physiology with specific emphasis on mechanisms of host/pathogen interactions. All existing data should be synthesized and new research conducted to answer the following questions:

(1) Where is the candidate located in relation to the plant (leaves, stems, roots, etc.)?

(2) What is the life cycle of the candidate microorganism and what is the disease cycle?

(3) What are the genetic characteristics and behavior of the microorganisms?

(4) How intimately associated is the microorganism with the host and what is the nature of the association?

(5) What are the physical and chemical mechanisms of the plant/microorganism interaction?

(6) Does the microorganism exhibit a sexual cycle? (An organism having a sexual cycle is much easier to manipulate genetically than one having only an asexual cycle.)

(d) What mechanism can be used to screen for the activity of the modified microorganism?

Session 2: Techniques

Q: What applicable tools are already available and what additional techniques must be developed?

A: The necessary genetic engineering tools are available, but each microorganism is unique and will present its own set of problems to be solved. For example, nucleases in the microorganism may be incompatible with introduced genetic material. The same three basic steps have been accomplished for many microorganisms. They are (1) obtain the proper DNA, (2) transform the DNA (mutants), and (3) develop protoplasts to reinsert the modified gene(s) into the candidate microorganism. All of these things have been done for many species, so all the basic technology is available.

Modifications of current technology may be necessary for application to specific systems, but should not require extensive work.

Q: Can microorganisms already isolated from hydrilla and Eurasian watermilfoil be made effective biocontrol agents by genetic manipulation?

A: Yes, provided that they are host specific. Host specificity probably involves several genes, and, if those genes happen to be widely separated on the chromosome(s), the engineering job increases tremendously in difficulty and the likelihood of success plummets. All the genes involved would also have to be expressible. It is impractical at this time to consider engineering host specificity into a candidate microorganism.

Session 3: Approach

The following approach was outlined:

Phase I

A. Isolate as many culturable microorganisms as possible that are specifically associated with the target plant.

B. Screen for substances or mechanisms injurious to the target plant including fungi, bacteria, and chemicals (hormones, toxins, etc.). (optional)

Phase II

A. Study the biology of host-specific microorganism(s) and target plant interactions to discover potential mechanisms amenable to bioengineering.

B. Obtain gene(s) from eukaryotes or prokaryotes to act as shuttle vectors, i.e., genes for producing and/or regulating the injurious effect(s) defined by B in Phase I.

Phase III

Develop assays to determine effects of added traits on the target plant

and the recipient microorganism. (Phases I and II must interact with Phase III.)

Phase IV

Insert cloned gene(s) into candidate microorganism.

A. Make the recipient microorganism tolerant to the gene that is to be introduced and to that gene's expressed function. The following are required:

   (1) PBR322 sequences - sequences of DNA that can easily be added to and reintroduced.

   (2) A selectable gene (for either auxotrophy or drug resistance) - a detectable trait for confirming the presence of the new gene.

   (3) A promoter to permit expression of the inserted gene.

   (4) A centromere (to make the inserted DNA behave like a chromosome). (optional)

   (5) A gene for origin of replication. (optional)

B. Develop a gene transfer system including a shuttle vector system and transformation protocol.

   (1) Prepare protoplast.

   (2) Make the mechanisms efficient.

C. Provide for biological containment of the engineered microorganism by building in conditional lethal characteristics (e.g. a specific nutrient dependency).

Phase V

Develop an assay for expression of the cloned gene(s) and fine tune the engineered system.

Phase VI

Scale up the production of the engineered microorganism and determine its efficacy against the host plant.

A. Test tube to aquarium level assays.

B. Quarantine testing considerations.

C. Approval for field testing.

D. Field efficacy.

Phase VII

Fermentation development and commercial scale up.

A. Field tests.

B. Formulation.

C. Registration.

Session 4: Practicality

Table 1 was developed at the meeting to address the questions of time frame, costs, and decision points.

Session 5: Conclusions

Q: Have all meeting objectives been met? If not, what options are available to complete objectives?

A: Yes, all objectives were met.

Q: Is a full-scale workshop justified?

A: No.

Q: Where may further information be obtained, if needed?

A: Each visiting participant will provide as a part of their written report a short list of people whose expertise they feel is especially applicable to this project.

Table 1

Project Time and Cost Flow Sheet

Phase		Task	Year: Feasibility 1	2	3	Development 4	5	6	7	8	9	10	11	12	Estimated Cost* Per Year (thousands)	Total (millions)
I	A	Isolate as many culturable microorganisms as possible that are specifically associated with the target plant.	X	X	X										300	0.90
I	B	Screen substances or mechanisms for injurious effects on the target plant including fungi, bacteria, chemicals, etc. (optional)				X	X	X	X	X					100	0.50
II	A	Study the biology of microorganism/target plant interactions to define mechanisms amenable to bioengineering.				X	X	X	X	X	X				200	1.20
	B	Obtain shuttle vectors, i.e., genes for producing and/or regulating the effect(s) desired.						X	X	X	X	X			100	0.50
III		Develop assays to:														
	A	Determine the effects of the added genetic traits on the target plant.				X	X	X							50	0.15
	B	Determine the effects of the added genetic traits on the recipient microorganism.				X	X	X							100	0.30
	C	Measure the efficiency with which the added genetic trait is expressed by the microorganism.				X	X	X	X	X	X	X			100	0.70
IV	A	Make the recipient microorganism tolerant to the introduced gene and its expressed function(s).							X	X	X				200	0.60
	B	Make a gene delivery system.							X	X	X				200	0.60
	C	Provide for biological containment of the engineered microorganism by building in conditional lethal characteristics.							X	X	X	X			300	1.20
V		Develop an assay for expression of the cloned gene(s) and fine tune the engineered system.									X	X	X	X	150	0.60
VI		Scale up.											X	X	150	0.30
Total cost by year (thousands)			300	300	300	550	550	650	1,200	1,200	1,250	650	300	300		7.55

* 1983 dollars.

Summary

Existing technology in bioengineering is sufficiently advanced to produce a microorganism effective in biological control of submersed aquatic plants. The tools are available and the necessary steps have been performed with microorganisms for other purposes. Each new system subjected to genetic engineering has its own unique set of difficulties to be overcome; however, the basic engineering technology is virtually the same in all cases and is well developed.

To develop a microorganism for the control of submersed aquatic plants, two steps must be taken prior to initiation of genetic engineering. The first and potentially most limiting step is the identification of at least one, but preferably several, microorganisms specifically associated with the target plant. The microorganisms need not be pathogenic, but specificity is essential. Because specificity usually involves several genes or sets of genes that may be widely separated on the chromosome(s), difficulties in finding, isolating, and manipulating the genes are increased greatly. Engineering host specificity is impractical at this time.

The second preliminary step involves examination of the physiology of the target plant so that its susceptibility to various disease mechanisms can be defined. Once susceptibility mechanisms have been defined, the trait that the candidate microorganism must be given to make it an effective plant pathogen can be selected. At this point genetic engineering technology can be applied to the host-specific candidate.

Basic steps in the genetic engineering process include (1) obtaining a gene, or genes, capable of giving the desired trait to the candidate microorganisms; (2) cloning (making many copies of) the gene(s); (3) developing a mechanism for inserting the desired gene into the candidate microorganism; and (4) testing the engineered microorganism for expression of the desired trait. Once these steps have been accomplished, the engineered microorganism must be subjected to quarantine testing, host-specificity testing, scale-up procedures, and large-scale field tests.

Guests' Letter Reports

Status of Genetic Engineering Technology for Development of Bacterial Plant Pathogens

a report by

George H. Lacy, Ph.D.
Associate Professor
Plant Pathology

Department of Plant Pathology, Physiology and Weed Science
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061

FEASIBILITY: Bacteria that cause disease of submersed weeds would constitute the best system available presently for developing biological control agents for these plants since all the necessary engineering technology and techniques are realities at this moment. In contrast, fungi that are pathogens of these weeds have many more barriers will slow their genetic engineering. The reason for this difference in adaptability to genetic engineering techniques is two-fold: 1) bacteria are simple biological systems with essentially only a cell wall and single membrane barring access by engineered genetic elements to the single chromosome while fungi process a thicker cell wall and at least two membranes as well as more complex genetic organization consisting of several chromosomes, and 2) the bulk of genetic engineering research has been completed with bacteria and more is understood about these systems.

The essential question remains: Is there a host-specific pathogen or plant-associated bacterium available that would serve as a host for genetically-engineered elements? This bacterium would have to be dependent solely on the target weed species for survival or capable demonstrably of only causing disease in that specific host. Herein is the weakest part of the proposal to use a bacterium for biological control. Extrapolation from bacteria that are pathogens of terrestrial plants, none would be host-specific enough to qualify. In this area, some fungi and viruses that are pathogens of plants demonstrate much more host specificity than bacteria.

The success of this program will hinge on discovery of a host specific bacterium to be engineered.

TECHNIQUES: The techniques for genetically altering bacteria are available. Sufficient experience with a wide range of plant pathogenic bacteria (Agrobacterium, Erwinia, Pseudomonas, and Xanthomonas species) has been accumulated to predict success with genetic alteration of these organisms. Basically, to genetically alter an organism requires the following steps:

1. Isolation of the organism's DNA
2. Creation of a library of the organism's DNA in a molecular vector such as plasmids, cosmids, or bacteriophage.
3. Modification of the DNA using transposons or in vitro DNA manipulations.
4. Reinsertion of the modified DNA by transformation, transfection, conjugation, or fusion of cells.
5. Expression of the modified DNA by manipulations of controlling elements (promoters, operators) or modification of the genetic code to accommodate the bacterium's protein synthesis apparatus.

All of these techniques are available with bacteria.

APPROACH:

Senario for Development of a Bacterial Pathogen

1. Assumption: That a host specific, plant pathogenic bacteria is available.

2. Basic biology of the parasitic interaction with the target weed: The biology and the mechanisms for the interaction of the pathogen with its host must be studied. This will allow development of a strategy to genetically modify the pathogen to make it a more efficient agent for biological control of weeds. Some genetic elements involved with pathogenesis are available presently and from other plant pathogens and might be exploited to enhance the biological control ability of the bacterium. For instance, genetic insertion elements from Agrobacterium tumefaciens are available on plasmids, genes involved in plant growth regulation are available on plasmids from Agrobacterium tumefaciens and Pseudomonas syringae pv. savastanoi, and genes for tissue necrosis and maceration are available on plasmids and cosmids from soft-rotting Erwinia species. Two general tactics for genetic manipulation may be chosen at this point: a) To enhance the native ability of the pathogen to damage the target plant and b) to introduce genes such as those described above to enhance pathogenicity.

   For this senario, insertion of more efficient genes for tissue maceration (that produce pectolytic enzymes) will be the tactic chosen as an example and since most plant pathogenic bacteria have this capability.

3. Discovery of native genes for pectolytic enzymes (PL genes): A library of the weed pathogen's DNA in cosmids will be probed by DNA hybridization with Erwinia PL genes to discover the location of indigenous gene(s). Once discovered, it will be isolated from the large fragments of the weed pathogen in the cosmid library and re-cloned in smaller plasmids.

4. Reinsertion of the modified DNA: The modified DNA may be reinserted into the weed pathogen directly by transformation (plasmids) or transduction (cosmids), or it may be inserted indirectly by conjugation (plasmids and some cosmids).

5. Expression of the Erwinia PL gene in the weed pathogen: Expression of the PL gene inserted in the weed pathogen may need to be optimized. For instance, the gene may be controlled by the pathogen to produce only low levels of enzyme by the indigenous control system, or the genetic code used by Erwinia may be sufficiently different that the protein produced by the weed pathogen from the inserted gene may be less efficient. To remedy this, it may be necessary to introduce Erwinia-controlling elements or modify the Erwinia genetic code.

6. Recombination of the Erwinia PL gene with the weed pathogen's DNA: Chromosomally-inserted genetic elements may be more stable than those located extrachromosomally on plasmids. Therefore, in vivo recombination selection techniques using suicide plasmids and the homology of indigenous DNA sequences flanking the inserted PL gene may be used to accomplish this purpose.

7. Pathogenicity of the genetically-engineered weed pathogen: The altered pathogen must be more efficient than the wild type pathogen at causing disease in the target weed. It may be that introduction of foreign genes may cause a resistant reaction rather than a susceptible reaction in the interaction of the pathogen and the weed or that the additional energy drain of enhanced PL enzyme production may slow the growth rate and, hence, the aggressiveness of the pathogen. In both cases, additional genetic alterations may be necessary. The inserted DNA may be altered to prevent the resistant reaction or the DNA of the pathogen may be "tailored" to re-balance its use of biological energy.

8. Field testing and release of genetically-altered weed pathogens for biological control: At this point, the genetically-altered organism will be available for testing, and evaluation in the same manner as any exotic imported organism.

PRACTICALITY: The cost and time for research leading to genetic alteration of a bacterium pathogenic on submused weeds, as described above, to produce more pectolytic enzymes (PL genes). Note that this estimate does not include estimates for quarantine studies and product development. Provide that a pathogenicity gene not presently in the library is used the total cost below would be increased about $0.75 million.

Objective or Study	Time Required (yr) 1 2 3 4 5 6 7 8 9	Cost (k$) Year	Total
1. Discovery of a host-specific pathogen of submused weeds	* * *	500	1,500
2. Biology and mechanisms for pathogenicity	* * * * *	150	750
3. Assay development - pathogen vs. weed	* * *	100	300
4. Characterization of control mechanisms and genes for PL production from Erwinia spp.	* * * *	150	600
5. Location of PL genes in weed pathogens as site for genetic alteration	* * *	200	600
6. Insertion of PL genes in site in weed pathogen genome	* * *	200	600
7. Study of effect of inserted PL genes on pathogenesis	* * * * *	150	750
8. Fine tuning of PL production in weed pathogen	* * * *	200	800
			5,900

CONCLUSIONS:

1. Biological engineering of bacteria pathogenic on submersed is practical and less expensive ($5,900,000-6,650,000) than a comparable program for fungi (results of cost-time studies, 29 September 1983: $9,500,000) because the technology is presently available and less development would be necessary.

2. It is less likely that bacteria will be as host-specific as fungi and viruses.

3. A workshop is not justified; however, a panel of scientists should be instituted to review the pre-proposal, the proposal itself, contract awards, and project progress at regular intervals. Persons that I believe would be useful to you in this capacity are:

General pathology of submersed aquatic weeds:

Dr. John Andrews
Department of Plant Pathology
University of Wisconsin
Madison, WI 53706
608/262-1410

General biological control strategies:

Dr. Ann K. Vidaver
Department of Plant Pathology
University of Nebraska
Lincoln, NE 68583
402/472-3164

Plant pathology and molecular biology:

Dr. T. Kosuge
Department of Plant Pathology
University of California
Davis, CA 95616
916/752-0301

General research excellence (and experience with viruses of green algae):

Dr. J. Allan Dodds
Department of Plant Pathology
University of California
Riverside, CA 92521
714/787-4491

Genetics Institute

October 18, 1983

Mrs. Judy Pennington
Biologist
Environmental Laboratory
Waterways Experiment Station
P.O. Box 631
Vicksburg, Mississippi 39180

Dear Judy:

Thank you for your hospitality and excellent organization during my visit to the Waterways Experiment Station. Please extend my gratitude and best wishes to Drs. Sanders and Harrison and Messrs. Decell and Rushing. I enclose two items: my bill for services and expenses, and a sample proposal outline for you.

I have delayed writing to you because I felt it was important to put some distance between this letter and the exuberance and enthusiasm which accompanied our conference.

I have some general comments about the project we discussed:

- In general, I am optimistic that a genetic engineering project is technically achieveable. I am seriously concerned, however, whether you will be able to achieve the goals of this project in the time frame we discussed.

- I fear that I was not forceful enough in stressing the potential difficulties involved in this project to you. The engineering of toxicity into a microorganism will be a challenging task, and this is the least difficult of all problems. One must further ensure that the engineered toxicity of the organism does not adversely affect its environmental fitness (specificity and competitiveness). Finally, one must ensure that there will be significant genetic barriers to prevent the spread of the engineered toxicity to otherwise beneficial microorganisms. Some of these tasks can be performed simultaneously, but others, especially ensuring environmental fitness, must be performed sequentially.

225 Longwood Avenue
Boston, Massachusetts 02115
Telephone 617 232-6886
Telex 948219

° I fear that I was not forceful enough in stressing to you the difficulties involved in each genetic engineering step. It was, for example, scientifically naive for my colleague to propose that a gene from one organism will function appropriately in another organism without considerable expenditure of time and effort. Theoretically, it is possible you will be fortunate and lucky. However, the alternative is equally possible.

I would like to revise my recommendation to you, along the following lines:

Phase I (3 years)

Spend as much money and manpower possible in identifying as many microorganisms as possible which closely associate with hydrilla or milfoil. Pathogenicity is desirable, but not essential. [$1.5 million]

Phase II (3 years)

a) Initiate biological studies of microbes to identify:

- key features of association
- key features of successful competitiveness

[$1.5 million]

(b) Procure as many potential "toxin" genes as possible from university laboratories [no cost].

Phase III (5 years)

a) Issue contracts for getting specific toxin genes inserted into and expressed by specific microbes. Specify the key association features and competitiveness features the engineered microbes must satisfy.

[$3.0 million]

Phase IV [7-10 years]

Scale-up, formulation, registration, commercial development, etc.

I favor this project flow because each phase represents a critical decision point. For example, if the goals of Phase I are not achieved, then there is no sense in continuing the project. [The same can be stated for each phase.]

I hope these comments are of use to you. As I've indicated before, this project is not the sort of thing in which it is appropriate for a company like Genetics Institute to be involved. Nevertheless, I am keenly interested in biological control programs and would be happy to give you whatever informal assistance that I can. Should you have any questions about this letter, please do not hesitate to call me.

Sincerely yours,

Gerard E. Riedel, Ph.D.
Senior Scientist
Agricultural Applications

GER/mbe

Attachments

PROJECT TIME FRAME (ORIGINAL)

	Feasibility			Research					
Functions:	1	2	3	4	5	6	7	8	9	10
A. Isolate Host specific Microorganisms (M.O.) which all culturable	X	X	X							
B. Develop Assays										
A toxin vs. weed	X	X	X							
B toxin vs. M.O.			X	X	X					
C tox. prod. by M.O.				X	X	X	X	X	X	X
C. Use Assays		X	X	X	X	X				
D. Isolate genes* involved in phyto toxin production	X	X	X	X	X	X				
E. Biology study of M.O.-host interaction				X	X	X	X	X	X	X
F. Make RDNA gene delivery system to M.O.				X	X	X				
G. Make M.O. tolerant to toxin(s)				X	X	X				
H. Deliver tox genes to M.O. and assay						X	X	X	X	X
I. Fine tune expression of tox genes and assay						X	X	X	X	X
J. Large scale test, field trials										
K. Quarantine protocols										
L. Commercial prod. dev. (Fer) (Form)								start once experimental success is achieved		
M. Registration										
N. Release								+5 yrs.		

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A Department of the New York State College of Agriculture and Life Sciences, a Statutory College of the State University at Cornell

Telephone: 607-256-3245 Telex: 937478

October 24, 1983

Judy Pennington
Biologist
Department of the Army
Waterways Experiment Station
Corps of Engineers
Environmental Laboratory
P.O. Box 631
Vicksburg, Mississippi 39180

Dear Judy:

This letter constitutes my report on "Status of Genetic Engineering Technology", which was the topic under discussion at the meeting held September 28-29, 1983 at WES. Included are: a summary of the broad 10-year scenario aimed at construction and development of a biocontrol agent for aquatic weeds, a scheme for development of tools needed for genetic engineering of a fungus, a statement on feasibility, names of potential consultants to the project, and a bill for services.

The project is conceived in several phases.

Phase I

Part A. - Survey as many microorganisms as possible that have been recovered from aquatic weeds. The desired characteristics to be sought include culturability, specificity for the target weed, and either pathogenicity toward or ability to intimately associate with the target weed. A sexual stage is important but not essential.

Part B. - Simultaneously with Part A, a set of shuttle vectors will be constructed that will be suitable for isolation of genes from the chosen microorganism and for reinserting genes into it, as well as for the cloning of isolated genes in E. coli. The vectors will be based on the E. coli plasmid pBR322 and will contain a gene that is selectable in the chosen microorganism (a gene either for drug resistance or for correction of auxotrophy) and a promoter for that gene. Certain versions of the vector will have an origin of DNA replication that functions in the chosen microorganism so that autonomous maintenance of the vector will be possible; other versions will lack an origin so that they can be used to insert cloned genes stably into the genome of the chosen microorganism. If the microorganism is eukaryotic a centromere will be used when appropriate to maintain the copy number of autonomously replicating vectors at one per haploid genome and to ensure predictable inheritance in mitosis. A part of the vector development program will involve determination of conditions necessary for transformation of the chosen microorganism by the vector.

Since the chosen microorganism will be relatively undeveloped at this stage, and therefore not yet amenable for these studies, other more developed microorganisms will be used as model systems.

Phase II

Part A. - The biology of the association between the chosen microorganism and the target weed will be studied to gain clues about the molecular nature of the interaction. If the microorganism is pathogenic, what molecules does it produce which lead to deleterious effects on the target weed? What plant parts are colonized? What is the spatial relationship between cells of plant and microorganism? Answers to such questions could determine the kinds of traits to be genetically engineered.

Part B. - Tools for genetic manipulation of the microorganism will be developed. A conventional recombination system, either sexual or asexual, will be sought. Auxotrophic mutants will be induced. Procedures will be found for preparation of high quality DNA and, if a eukaryote, for isolation and regeneration of protoplasts. A biological containment system will be devised so that the genetically engineered microorganism can be tested for efficacy without danger to the environment. Conditionally lethal mutants, such as the auxotrophs mentioned above or temperature sensitive mutants, will be tested under appropriate physical containment using both permissive and nonpermissive conditions.

Phase III

The microorganism will be engineered for a satisfactory level of virulence toward the target weed. This will be done by acquiring from various laboratories all of the cloned pathogenicity genes that are available at the time. These will be inserted singly and in combination into the microorganism and its virulence and host-specificity then assayed. If the pathogenicity gene controls production of toxic materials, a means will be found to protect the microorganism itself from them. Once a gene or set of genes is found which determines the desired set of qualitative characteristics, the system will be fine-tuned by adding regulatory sequences which will ensure that the genes are expressed at the right time and that the proper amount of gene product is produced.

Phase IV

The testing of the microorganism will move from physically contained aquarium tests to similarly contained but larger scale facilities such as tanks or ponds.

Phase V

Full scale field tests will be performed under appropriate biological containment. Formulations will be developed. The machinery for eventual registration will be set in motion.

Feasibility

The foregoing plan is visualized within a ten-year time frame. Successful completion of the program depends on Phase I, Part A, the acquisition of a suitable microorganism. Prospects for this are good, since virtually all plants that have been observed carefully have been found to sustain an associated microflora, at least some of which are host-specific. It would be very surprising if a candidate microorganism is not found quickly. Indeed, it is likely that several microorganisms fitting the criteria in Phase I, Part A will be found within the three-year limit designated for this portion of the program. However, success is not guaranteed. In the event that appropriate candidates cannot be identified within three years, it would seem futile to go on. If that happens, the project will have been successful anyway because of the activities described in Phase I, Part B, the construction of vectors. This part of the plan is low risk because all of the required technology is now available with E. coli and yeast. The most exciting aspect of this proposal is that it lies at the leading edge of research on the molecular biology of economically important microorganisms. If successful, it will be a first. But even if the project does not result in release of a genetically engineered biocontrol agent, it will have contributed substantially to the development of technology for the genetic manipulation of undeveloped microorganisms generally.

If Phase I is successfully completed, the remainder of the project will be routine. It will only be a matter of adapting existing technologies to work in this particular system.

Specific Requirements for Development of a Fungal Biocontrol Agent

The scenario for a fungus would be just what is outlined in Phase I, Part B and Phase II, Part B. The parts of the approach for a fungus that would differ from that for a bacterium include: vectors carrying centromeres and the isolation and regeneration of protoplasts. Otherwise all of the steps for bacteria and fungi would be similar, at least in principle, although there would be technical variations.

Suggested Consultants for the Project

Dr. Alan Collmer
Department of Botany
University of Maryland
College Park, MD 20742

Dr. Tsune Kosuge
Department of Plant Pathology
University of California
Davis, CA 95616

Both Alan and Tsune are trained plant pathologists who have become molecular biologists within the last few years. Thus, both of them have knowledge of and a "feel" for agriculture and applied biology and at the same time are competent in molecular biology and recombinant DNA technology. Recent accomplishments: Tsune has cloned and examined in vitro two genes required for indole acetic acid (IAA) production, a virulence factor in the bacterium Pseudomonas savastanoi. Alan has cloned from Erwinia chrysanthemi a gene for pectic enzyme production, another virulence factor. These two individuals not only have expertise particularly well suited for this project, but in addition each has a commendable personality as well, i.e., amiable, self-effacing, effective in discussions, and respected by colleagues.

A third possibility is Dr. Bill Timberlake, at Kosuge's address. Bill is a molecular biologist who specializes in filamentous fungi. Just recently he has successfully developed a transformation system for Aspergillus nidulans.

Sincerely yours,

O. C. Yoder
Associate Professor

OCY:bjm
28479/w1

Conclusions

The conclusions of the Bioengineering Technology Meeting of September 28-29, 1983, were as follows:

(a) Existing technology in bioengineering is sufficiently advanced and complete to produce a microorganism effective in the biological control of submersed aquatic plants. Applicable tools are described in "Answers to Questions Addressed in Each Session."

(b) Two prerequisites are required prior to initiation of genetic engineering for development of a biocontrol agent for Eurasian watermilfoil and hydrilla. They are:

(1) Isolation of one or more microorganisms that are specifically associated with the target plant.

(2) Examination of the physiology of the target plant so that its susceptibility to various disease mechanisms can be defined.

An approach for the research effort is outlined in "Answers to Questions Addressed in Each Session."

(c) Production of an engineered biocontrol microorganism will require a minimum of twelve years and $7.25M.

(d) No full-scale workshop is warranted because sufficient information was obtained in the meeting to determine feasibility of using genetic engineering technology for the development of microorganisms as biological agents for aquatic plant control.

(e) Specific information on genetic engineering may be obtained from any of the scientists recommended by the guests in their letter reports.

Proposed New Aquatic Plant Control Study

Title: Development of Genetically Engineered Microorganisms for Control of Hydrilla and Eurasian Watermilfoil.

Objective: To develop genetically engineered microorganisms that provide effective control of hydrilla and Eurasian watermilfoil.

Problem. To date, no microorganisms have been found through conventional approaches that have all the desired characteristics of a plant pathogen for control of hydrilla or Eurasian watermilfoil. Species found thus far are ineffective because they are insufficiently virulent or lack host specificity.

Value to Aquatic Plant Control: Genetic engineering can provide aquatic plant control with strong, host-specific pathogens for the effective control of hydrilla and Eurasian watermilfoil.

Overall Approach: The effort will consist of the following phases: (1) isolation of host-specific microorganisms; (2) modification of their genes to produce the desired virulence; and (3) laboratory and field evaluation.

Work Planned First FY: Extensive searches for host-specific microorganisms associated with the target plants will be conducted.

Funds Required by FY:

FY 85 - $ 300K	FY 91 - $ 1,200K
FY 86 - $ 300K	FY 92 - $ 1,200K
FY 87 - $ 300K	FY 93 - $ 1,250K
FY 88 - $ 550K	FY 94 - $ 650K
FY 89 - $ 550K	FY 95 - $ 300K
FY 90 - $ 650K	FY 96 - $ 300K

Total = $7.55M