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UNITED STATES OF AMERICA
AGENCY FOR INTERNATIONAL DEVELOPMENT
REGIONAL DEVELOPMENT OFFICE/CARIBBEAN

P. O. BOX 302
BRIDGETOWN
BARBADOS

May 8, 1989

Dr. Keith D. Hunte
Principal of the Cave Hill Campus
University of the West Indies
P.O. Box 64
Bridgetown
BARBADOS

SUBJECT: Grant No: 534-0936-G-00-9118
The Development of DNA Techniques for a
Vesicular-Arbuscular Mycorrhizal (VAM) System

Dear Dr. Hunte:

Pursuant to the authority contained in the Foreign Assistance Act of 1961, as amended, the Agency for International Development (hereinafter referred to as "A.I.D." or "Grantor") hereby grants to The University of the West Indies (UWI) (hereby referred to as the "Grantee"), the sum of $150,000 to develop protoplast isolation and regeneration technology, to isolate and analyse total DNA and to isolate benomyl resistant and orotidine decarboxylase mutants of the VAM fungus Gigaspora gigantea, as described in the Schedule of this grant and the Attachment II, entitled "Program Description."

This grant is effective June 1, 1989, and shall apply to commitments made by the Grantee in furtherance of program objectives during the period beginning with the effective date and ending December 31, 1992.

This grant is made to the Grantee, on condition that the funds will be administered in accordance with the terms and conditions as set forth in Attachment I, entitled "Schedule"; Attachment II, entitled "Program Description;" and Attachment III, entitled "Standard Provisions," which have been agreed to by your organization.

Please sign the original and two (2) copies of this grant to acknowledge your receipt, and return the original and one (1) copy to the Regional Development Office/Caribbean.

Sincerely,

Stanley D. Heishman

Stanley D. Heishman
Regional Contracting Officer

Attachments:

I. Schedule
II. Program Description
III. Standard Provisions

ACKNOWLEDGED: THE UNIVERSITY OF THE WEST INDIES (UWI)

By: Keith D. Hunte

Title: Principal - UWI. Cave Hill

Date: 1989-05-19

Fiscal Data

Appropriation No:	72-1191021.6
Budget Plan Code:	DDSA-89-29534-KG[illegible]
Allowance:	946-51-534-00-19-[illegible]1
Project No:	936-5542.19
Total Estimated Amount:	$150,000
Total Obligated Amount:	$150,000
Funding Sources:	AID/W

cc. Dr. Richard Dales, U.W.I.
Dr. Louis Chinnery, U.W.I.
Prof. William Timberlake, Univ. of Georgia
Mr. Stanley Michelini, FRESCA

Attachment I

SCHEDULE

A. Purpose of Grant

The purpose of this Grant is to provide support for The University of the West Indies' research proposal, "The Development of Recombinant DNA Techniques for a Vesicular-Arbuscular Mycorrhizal (VAM) System" as described in Attachment Two to this Grant, entitled "Program Description." The principal investigator is Dr. Richard B.G. Dales, Lecturer, Department of Biology, University of the West Indies (UWI), Cave Hill Campus, Barbados. The principal collaborator is Professor Timberlake, Department of Genetics, University of Georgia, Athens, Georgia, U.S.A. Dr. Louis Chinnery, Department of Biology, UWI, Cave Hill Campus, Barbados and Mr. Stanley Michelini, Director, Fruit Experiment Station for the Caribbean (FRESCA), St. James, Barbados are co-investigators for the research.

B. Period of Grant

The effective date of this Grant is June 1, 1989. The expiration date of this Grant is December 31, 1992.

C. Amount of Grant and Payment

1. AID hereby obligates the amount of $150,000 for the purposes of this Grant. This is the maximum obligation of funds for the specific research objectives supported under this Grant.

2. Payment shall be made in U.S. dollars to the Grantee, by the RDO/C Controller, in accordance with procedures set forth in Implementation Letter No. 1 under this Grant.

3. Grantee agrees to submit documentation required by RDO/C to support expenditures made with Grant funds.

4. Pursuant to Optional Standard Provision 16, the provisional overhead rate for reimbursement of indirect costs is 10%, and the final overhead rate to be negotiated will not exceed 10%.

D. Financial Plan

The following is the Budget for the Grant. All expenditures under this Grant are expected to be made during years one to three of the Grant period; the final seven months of the Grant period shall be used for finalizing research results and no expenditures are expected or planned for this portion of the Grant period. Revisions to this Budget shall be made in accordance with Standard Provision of this Grant, entitled "Revision of Grant Budget". The Grantee shall not exceed the total obligated amount of $150,000. Following are the approved cost elements amounts.

GRANT BUDGET

		US$ 6/89-5/90	US$ 6/90-5/91	US$ 6/91-5/92	US$ Total
1.	Personnel	9,034	9,907	10,876	29,817
2.	Equipment	41,289	-0-	-0-	41,289
3.	Supplies	12,460	8,475	10,500	31,435
4.	Training	-0-	-0-	2,000	2,000
5.	Travel	5,500	-0-	8,500	14,000
6.	Other Costs	13,322	2,000	2,500	17,822
7.	Overhead Costs	8,161	2,038	3,438	13,637
	TOTAL:	89,766	22,420	37,814	150,000

E. Reporting and Evaluation

1. Progress Reports

Interim progress reports are required every six months, except the last six month period, which will be pre-empted by a final report. Progress reports are due within thirty days following each six month period. The first report should contain any revisions in key dates projected for the research schedule.

2. Final Report

The principal investigator should submit a final report no later than December 31, 1992, the completion date of the grant. This report should be sufficiently detailed to substantiate findings and to permit scientific evaluation of research.

3. Distribution of Progress Reports

The principal investigator shall forward one copy of each interim progress report to the following addresses:

a) Mr. Howard F. Batson
RDO/C Project Officer
United States Agency for International Development
P. O. Box 302
Bridgetown, Barbados

b) Lloyd Frederick
Technical Backstop Office
AID/ST/AGR
Room 406, SA-18
Department of State
Washington, D.C. 20520
U. S. A.

c) Ms. Wendy White
BOSTID
National Academy of Sciences
2101 Constitution Avenue, N.W.
Washington D.C. 20418
U. S. A.

d) AID/SCI
Room 320, SA-18
Department of State
Washington, D.C. 20520
U. S. A.

e) AID Reference Center
PPC/CDIE/DI
Room 105, SA-18
Department of State
Washington, D.C. 20520
U. S. A.

4. Distribution of Final Report

The principal investigator should submit three copies of the final report to AID/SCI, one copy to the AID Reference Center, one copy to the Technical Backstop Office, one copy to BOSTID and one to the RDO/C Project Officer.

F. **Special Provisions**

1. Standard Provisions applicable to this grant are the Mandatory Standard Provisions for Non-U.S., Non-Governmental Grantees. The following optional Standard Provisions for Non-U.S., Non -Governmental Grantees, are not applicable to this grant; 2, 7, 9, 11, 12, 14, 15, 17, 19, 20, 21.

2. International Travel between Barbados and the U.S. is hereby approved subject to the limitations of Standard Provision 3 "Air Travel and Transportation".

G. **Overhead Rate**

See Article C.4 of this Attachment.

H. **Title to Property**

Title to all property financed under this grant shall vest in the grantee subject to the conditions specified in Standard Provision No. 18, "Title To and Use of Property".

I. **Authorized Geographic Code**

The authorized geographic code for procurement of goods and services under this grant is Code 935, "Special Free World", as stipulated in optional Standard Provision 6, "Ineligible and Restricted Goods and Services".

Attachment II

PROGRAM DESCRIPTION

A. Purpose of Grant

The overall objective is to develop protoplast isolation and regeneration technology, to isolate and analyse total DNA and to isolate benomyl resistant and orotidine decarboxylase mutants of the Vesicular-Arbuscular Mycorrhizal (VAM) fungus Gigaspora gigantea (Nicol. & Gerd.) Gerd. & Trappe. Ultimately, a transformation system for G. gigantea will be developed so that the molecular genetics of a VAM fungus can be rapidly investigated and strategies developed for improving crop production by genetically engineering the fungal symbiont.

B. Specific Objectives

1. To develop methods for the routine production of non-contaminated VAM fungal hyphae.

2. To establish enzyme systems and conditions for efficient protoplast release from VAM fungal hyphae.

3. To isolate genomic DNA from VAM fungal hyphae or protoplast suspensions.

4. To analyse VAM fungal DNA using restriction endonuclease enzymes and to construct gene libraries in bacteriophage λ .

5. To develop a transformation system for a VAM fungus.

C. Implementation

1. Technical Work Plan

The project consists of several discreet tasks, as indicated by the various sub-headings below, but which may be conveniently divided into three phases. The first phase consists of spore germination studies (section a) which should be finalized within the first three months of funding. Phase two consists of sections b), c) and possibly d) which will run concurrently and is expected to take two years to complete. The commencement of phase three (sections e and f) depends on the successful completion of section b) of phase two section b, but is expected to run during the third year of the project.

a) Spore germination: Spores of G. gigantea will be produced in pot cultures of sterile sand containing either water melon (Citrullus lanatus) or onion (Allium cepa) seedlings as these rapidly form VAM. Onions also have an added advantage in that mycorrhizal roots develop a yellow pigmentation. The pot cultures incoulated with 5 or 10 G. gigantea spores will be maintained at 25°C in an air-conditioned greenhouse and watered with a low phosphate "Long Ashton" formula fertilizer. The spores will be harvested by wet-sieving, surface sterilized and germinated either on cellophane sheets laid over agar or in liquid culture.

In a final year undergraduate research project, supervised by Dr. Dales between November 1986 and May 1987 a wet-sieving technique was adapted for the rapid isolation of VAM fungal spores from relatively large amounts (c. 800g.) of pot-culture sand; a proportion of these spores (c. 20%) were successfully induced to germinate on cellophane sheets laid over petri plates of 2% agar (Difco Bacto).

Several laboratories have recently reported new techniques for the production of VAM fungal mycelia (Dr. S. Nemec, USDA, ARS, Orlando, Florida pers. comm.). These involve the formation of regrowth hyphae from surface sterilized mycorrihizal root fragments in solidified or liquid media. These techniques would be ideal for the production of mycelia for protoplasting and DNA extraction and are currently being worked up in our laboratory.

It is intended that this program of spore germination studies will be largely completed prior to the commencement of USAID funding and should be finalized within the first three months of funding.

b) Protoplast technology: Protoplasts will be isolated from germinated spores or regrowth hyphae using a range of enzyme systems. Enzymes such as "NovoZym 234" (Novo Biolabs, Denmark), freeze-dried Heliz pomatia gastric juice (kindly supplied by Prof. L. Ferenczy, Department of Microbiology, Attila Joszef University, Szeged, Hungary) and an autolytic enzyme prepared from Aspergillus nidulans will be used individually in combinations or in complex cocktails with protease, chitinase and cellulase enzymes. Osmotic stabilizer (KCl, NaCl) concentrations, pH and temperature will be optimised so that an overall system can be obtained which yields large numbers ($> 10^6$ cm^{-3}) of protoplasts.

Protoplasts will be regenerated in osmotically stabilized soft agar overlays or shake-flask cultures. It will be of interest to see whether regenerating protoplasts cease growth at the extended germ-tube stage in the absence of a host plant as is the case for germinating spores. Regenerating protoplasts can also be used to multiply the amount of available mycelium for DNA extraction purposes as has been successfully employed for the extremely poor-growing interspecific allodiploid hybrids produced by the protoplast fusion of Aspergillus spp.

Samples of regenerated G. gigantea protoplasts will be screened for full regeneration potential and infectivity by individually inoculating into sterile sand supporting non-mycorrhizal water melon or onion seedlings. Similar work has recently been reported for the ectomycorrhizal basidiomycete Laccaria bicolor and as the principal investigator has a great deal of experience in protoplast technology no difficulties are envisaged which could prevent the successful completion of this section of the workplan.

c) Isolation of DNA: Total DNA will be isolated from lyophilized mycelium obtained from germinated spores, regrowth hyphae and regenerated protoplasts or alternatively from freshly prepared protoplast pellets. Each system will be investigated to see which gives the best DNA yield. The procedure will involve buffer extraction, RNase and proteinase K treatments, phenol and chloroform extractions and precipitation of DNA with ethanol.

It is estimated that the total DNA may contain a number of distinct DNA species, other than the nuclear and mitochondrial components, as VAM fungi have been shown to have "bacteria-like" and gram negative bacterial inclusions which are expected to possess DNA. The DNA species will be separated using caesium chloride, density-gradient centrifugation when the ultracentrifuge is in place. However, if there is a delay prior to installation of the ultracentrifuge then chromatographic separation techniques will be attempted using sepharose and NACS-52 (BRL). The DNA types will be visualized in agarose gels and restriction enzyme analysis will be performed on the mitochondrial DNA and any extrachromosomal DNA species encountered.

DNA isolation is a routine procedure and again the principal investigator has had a lot of experience in isolating and analysing DNA from filamentous fungi. A second, final-year undergraduate research project, supervised by Dr. Dales, has successfully isolated total and mitochondrial DNA from small amounts (c.0.015g) of lyophilized A. nidulans mycelium. This means that the protocol is available to isolate DNA species from small quantities of G. gigantea material and it is considered that this programme should prove straightforward.

d) Isolation of mutants: Resistance mutants will be isolated to the fungicide benomyl and to 5-fluoro orotic acid. It is envisaged that this section will be the most problematic. Large amounts of, preferably uninucleate, material would be required for successful mutant isolation. The number of nuclei in G. gigantea spores is not known at present and it would not be feasible to produce large samples (c. 5×10^6 spores cm^{-3}) of these spores. Consequently, it is proposed to use dense protoplast suspensions. Far UV light and N-methly-N-nitro-N-nitrosoguanidine (MNNG) will be both used as mutagens at between 1 - 5% survival in conjunction with the strong selection pressure that can be exerted for the isolation of resistance mutants. Any surviving protoplasts which regenerate in liquid culture at benomyl or 5-fluoro orotic acid levels greater than the previously determined tolerance levels for untreated G. gigantea protoplasts will be retained.

These will be individually incoluated onto agar slopes supporting water melon or onion seedlings. When infection is observed an agar plug containing the mycorrhizal seedling will be carefully transplanted into sterile sand from which spores will be recovered after 2 - 3 months. A sample of these spores will be screened for elevated resistance. Alternatively, regenerating surviving protoplasts will be re-protoplasted. A sample of these protoplasts will be screened for increased tolerance and if so the regenerated remainder would be used to infect water melon or onion seedlings in sterile sand for the production of spores. In either case the end product would be asexually-produced spore samples which are resistant to increased levels of benomyl or 5-fluoro orotic acid.

e) Preparation of DNA libraries: Total DNA from resistant clones will be partially restricted with Sau 3A and cloned into the λ vector EMBL 3.

f) Transformation: If a naturally-occurring plasmid is encountered in the programme outlined in section c) it will be utilized for transformation purposes, otherwise pBR322 or a derivative will be employed. DNA fragment pools from a DNA library will be "shotgun" cloned into the plasmid at a single, natural or manufactured, Sal 1 site. Recombinant plasmid preparations will then be induced by polythylene glycol treatment to transform protoplasts from the wild-type strain to benomyl resistance. The DNA fragment encoding resistance will be recovered either by direct plasmid recovery from transformants or by subsampling the DNA fragment pools until a single clone from the library is identified.

Alternatively, if mutants resistant to 5-fluoro orotic acid are recovered in section d) then these will be screened for deficiency in orotidine decarboxylase. Such auxotrophs may be complemented by the Neurospora crassa pyr 4 gene. The Principal Investigator already possesses the Aspergillus nidulans transformation vector which contains this gene and can therefore attempt transformation of orotidine decarboxylase auxotrophs of G. gigantea directly with this plasmid system.

Benomyl resistance has been successfully employed as a selectable marker in the development of a transformation system for Aspergillus niger and, besides A. nidulans, the pyr 4 gene has been used for transformation systems in A. awamori, A. niger and Trichoderma reesei. Consequently, both approaches have a strong potential for success with G. gigantea.

Sections (e) and (f) of the workplan will start as soon as suitable resistance mutants have been isolated and are expected to run during the third year of funding.

2) Scientific Collaboration:

The principal investigator, Dr. Richard B.G. Dales, Lecturer, Department of Biology, UWI, Cave Hill Campus, Barbados and co-investigators, Dr. Louis Chinnery, Department of Biology, UWI, Cave Hill Campus, Barbados and Mr. Stanley Michelini, Director, FRESCA, St. James, Barbados will be

collaborating with Professor William Timberlake, Department of Genetics, University of Georgia, Athens, Georgia, U.S.A. Professor Timberlake is a leading authority on transformation procedures with filamentous fungi and will be making a strong contribution to the molecular genetic aspects of the project.

It is proposed that Professor Timberlake and his research associate visit Barbados for one week to oversee the commencement of the research programs. Collaboration will continue by post, phone and at meetings of learned societies until the recombinant DNA program is about to commence. At such a time Professor Timberlake and his research associate will return to Barbados for one week for consultations regarding this final major stage of the project and to confirm that all requirements necessary for this research are being met with regard to th National Institute of Health (USA) DNA guidelines. Provision has also been made in the budget for the possibility of some of the final year work being conducted in Professor Timberlake's laboratory.

Dr. Richard B.G. Dales, the principal investigator, will supervise the whole of the research and will be heavily committed to conducting benchwork in every program of the project in association with a graduate student.

The co-investigators: Dr. Louis Chinnery and Mr. Stanley Michelini have experience in the cultivation of VAM systems and in the taxonomy of the endogonaceae. Mr. Michelini will provide the project with a continuous supply of G. gigantea spores harvested from large pot cultures at FRESCA, Lascelles, St. James, Barbados. Dr. Chinnery will be concerned with the germination studies of VAM fungal spores and with the micropropagation of VAM systems in the laboratory.

3. Grant Budget

	US$ Year 1	US$ Year 2	US$ Year 3	US$ Totals
Personnel				
Graduate Student	8,574	9,432	10,376	
FRESCA	460	475	500	
Sub-total	9,034	9,907	10,876	29,817
Equipment				
Beckman TL-100 Ultra-centrifuge and accessories	41,289	-0-	-0-	41,289
Supplies				
Chemicals and laboratory Supplies	12,000	8,000	10,000	
Greenhouse supplies	460	475	500	
Sub-total	12,460	8,475	10,500	31,435
Training				
Graduate Student	-0-	-0-	2,000	2,000
Travel				
Collaborators visits	4,000	-0-	4,000	
Conferences	1,500	-0-	-0-	
Graduate Student to visit University of Georgia	-0-	-0-	4,500	
Sub-total	5,500	-0-	8,500	14,000
Other Costs				
Postage & Packing	13,322	2,000	2,500	17,822
Overhead Costs (10%)	8,161	2,038	3,438	13,637
Totals	89,766	22,420	37,814	150,000

GRAND TOTAL $150,000