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PROGRESS REPORT NO. 2

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RI-PLASMID INLUCED ROOT CULTURE AS A POTENTIAL SUBSTRATE
FOR IN-VITRO PROPAGATION OF MYCORRHIZAL FUNGI

2. Hyperparasitism of New Species VA mycorrhizal Fungi

A RESEARCH PROJECT:

USAID/PSTC PROGRAM

Grant No. 936-5542-6-00-3077-00

Submitted by

Malee suwana-adth
Project Leader

Kasetsart University Research and Development Institute
Kasetsart University
Bangkhen, Bangkok

Project Profile

Country : Thailand

Grant No. : 936-5542-6-00-5077-00

Program : Program on Science and Technology Cooperation

Project Title : Ri-Plasmid Induced Root Culture as a potential Substrate for In-Vitro Propagation of Mycorrhizal Fungi

Project Leader : Malee Suwana-adth, Ph.D.

Organization : Kasetsart University Research and Development Institute Kasetsart University, Bangkok.

Co-investigators : Ms. Poonpilai Suwanarit
Dr. Wiwut Daengsubha
Ms. Yupa Mongkolsook

Project Consultants : Professor Dr. Chua Nam Hai,
The Rockefeller University
Professor Dr. John Monge,
University of California (Riverside)

Authorized Officer : Associate Professor Dr. Thira Sutabutr
Director, Kasetsart University Research and Development Institute

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INTRODUCTION

The present study was emphasized on the morphological characteristics of tropical VA mycorrhizal fungi which can be reproduced in pot culture. Identification of those species were made New species were described.

MATERIAL AND METHOD

Spores of VA mycorrhizal fungi were separated from corn planting soil by wet sieving and decanting method described by Gerdermann (1963). The separated spores were then morphologically studied and grouped under stereomicroscope. Each type of spores were separately surface-sterilized by using 0.5% Sodium hypochlorite for 3-5 minutes. Sterilized spores were inoculated into the pot which host plant had already been germinated. Distilled water was used for watering the plants. After 2-3 months, the soil in the pot was checked for the reproduction of VA mycorrhizal fungi.

RESULT AND DISCUSSION

In the process of isolation of VA mycorrhizal fungi from the soils of corn fields it was found that certain fungal isolates showed unique characteristics not reported elsewhere. Four such strains were discovered and were tentatively classified as new species—three belong to genus *Entrophospora* and one to genus *Gigaspora*.

Characteristics of four new species of VA mycorrhizal fungi are described as follow :

Description of Four New Species of VA Mycorrhizal Fungi from Thailand.

Entrophospora sp. no. 1

Zygospores formed singly in the soil, 100-130 μ in diameter, globose with hyaline when young and become light brown at maturity. One Azygospore was developed in a hyaline hypha which subtended a hyaline vesicle. Azygospore was about 70 μ apart from vesicle forming drum-bell looking structure. Subtending hypha between spore and vesicle was 20 μ in diameter, subtending hypha and vesicle collapsed and disintegrated at maturity. Spore wall was readily separated into three layers. The outer layer (2-3 μ) was thicker than the middle layer (1 μ) the outer layer had pitted wall when young and smooth when mature while the most inner layer (2-3 μ) was rough. (Figure 1.)

Species No. 1

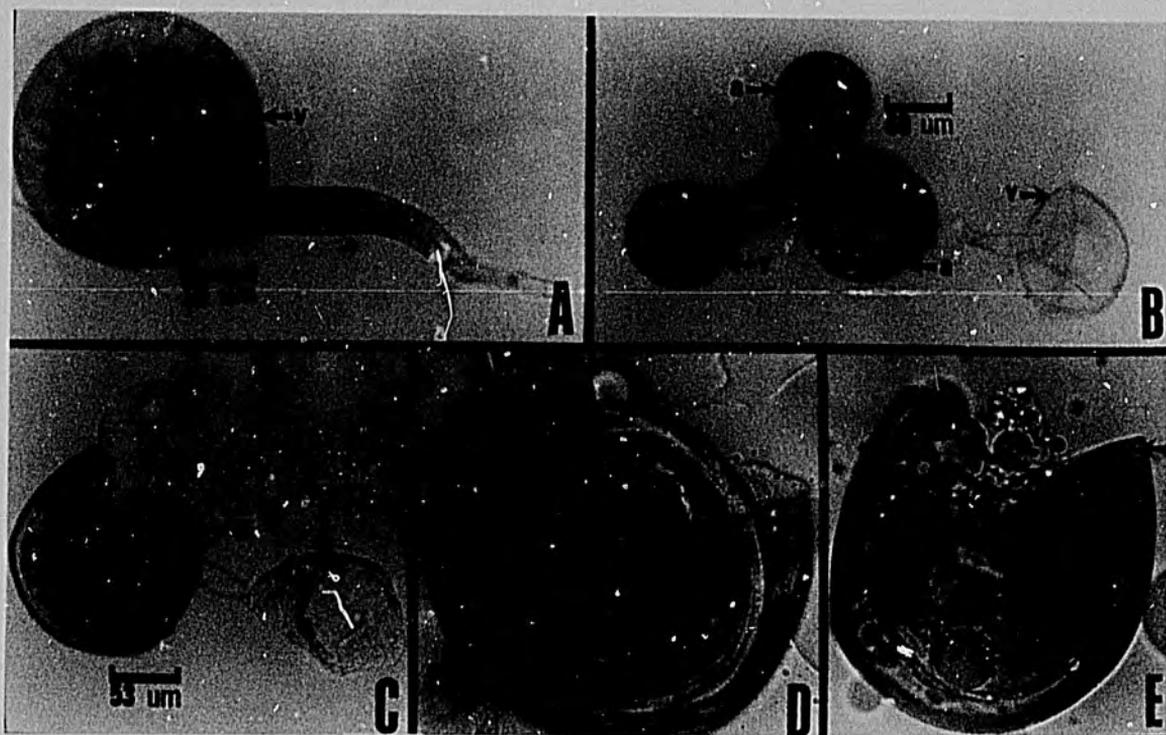


Figure 1. *Entrophospora* sp. no 1. A. Young vesicle(v) B. Azygospore (a) forming inside subtending hypha of vesical (v) C. Mature azygospore (a) with collapsed vesicle (v) D. Pitted outer layer of young azygospore (arrow) E. Three layers of spore wall (arrows) with the most inner wall being rough.

Entrophospora sp. no 2.

Azygospore formed singly in the soil, globose to subglobose, 100-150 μ in diameter. One azygospore was developed in a hyaline hypha (30 μ in diameter) which subtended a hyaline vesicle. Azygospore was about 150 μ apart from vesicle, forming drum bell looking structure, Azygospore still had a long hypha attached at the opposite side to the vesicle, Spore was light brown at maturity, subtending hypha and vesicle collapsed and disintegrated at maturity. Spore wall was not easily seperated, three layers, the outer layer not smooth, 1-4 μ , yellow color. Middle and inner layers were about 1 μ . (Figure 2)

Species no. 2



Figure 2. Entrophospora sp. no 2. A Young vesicle (v) B. Young azygospore (a) formed inside subtending hypha of vesicle (v) C. Developing azygospore (a) showing hypha at both sides of spore (arrow). D. Three layers spore wall not easily seperated (arrows).

Entrophospora sp. no 3

Azygospore formed singly in the soil, globose to subglobose. $130 \times 130 - 158 \times 188 \mu$ in diameter. One azygospore was developed in a hyaline hypha (22 U) when subtended a hyaline vesicle. Azygospore was 64μ apart from vesicle, forming drum bell looking structure. Spore was hyaline and smooth when young. When mature the spore was cover with interwoven branch byphae, vesicle was then collapsed. (Figure 3)

Species no. 3

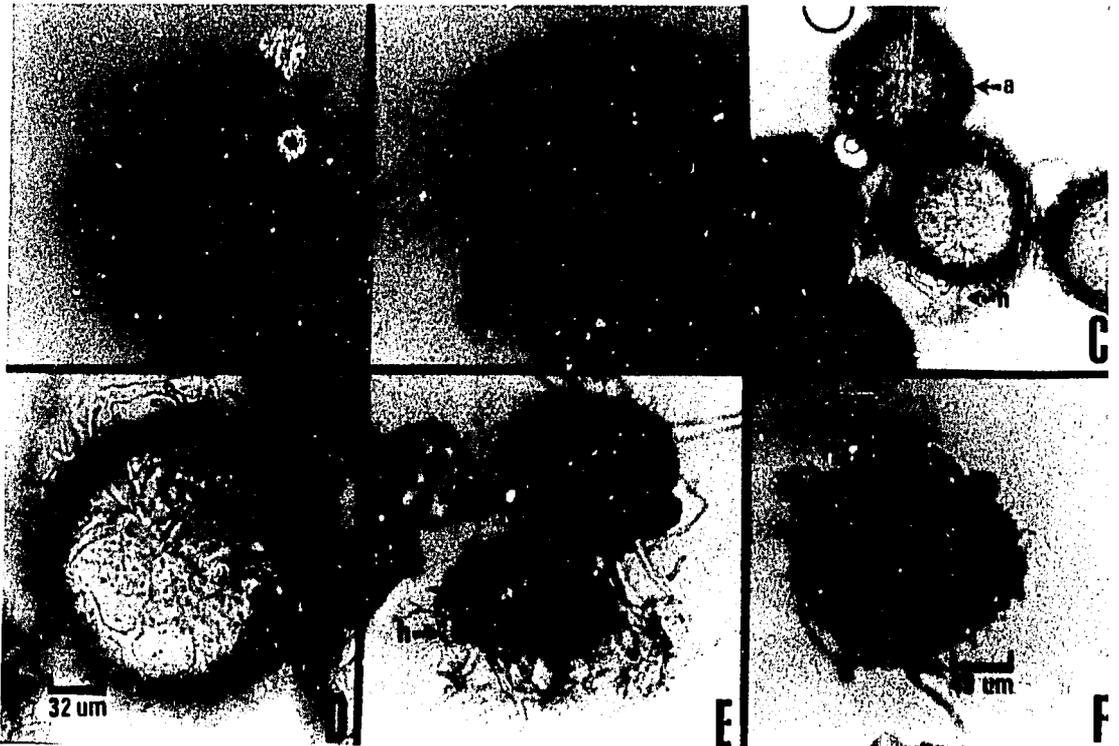


Figure 3. Entrophospora sp. no 3 A. Young vesicle (v) B. Young azygospore (a) formed inside subtending hypha of vesicle (v) C. Mature azygospore (a) covered with interwoven hypha (h) D. Mature azygospore (a) E. The characteristics of interwoven hypha around the spore (h) F. Spore wall (arrow).

Gigaspora sp. no. 1

Azygospore formed singly in the soil, globose 260-300 μ in diameter, brown, suspensor like cell 43 x 70 μ broad, bulbous, yellow to light brown hypha extending from suspensor cell to the spore wall. Soil borne vesicles borne in cluster (4-12 spores), light brown, spore wall knobby, irregular. Azygospore have a warty projection around the spore when young and turned to become rough spore wall when mature. Three layers of spore wall, outer layer 1 μ , middle 3 μ and inner layer 1 μ , outer layer wall was rough. (Figure 4)

Species no 4.

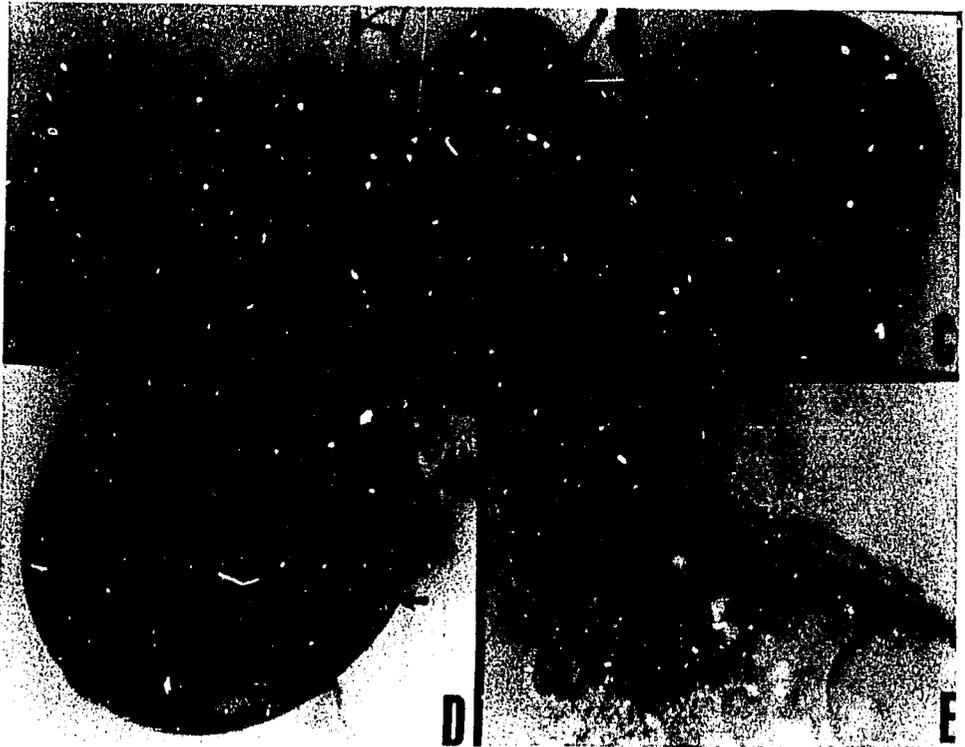


Figure 4. Gigaspora sp. A. Soil borne vesicle(v) B. Young azygospore (a) with warty projection (wp) wall. C. Mature azygospore (a) with rough wall and bulbous suspensor - like cell (s) D. Three layers of spore wall (arrows) E. High magnification of the three layers of wall (arrows) showing the rough outer layer.

OCCURRENCE OF HYPERPARASITISM ON SPORES OF ENTROPHOSPORA

Occurrence of hyperparasitism of Entrophospora no 1. and Entrophospora no 3. were observed (Figure 5). Hyperparasitic fungi that infected those two species of Entrophospora were the same and identified as Glomus. This species of Glomus was similar to Glomus YS. described by Koske (1984)

Species no. 5

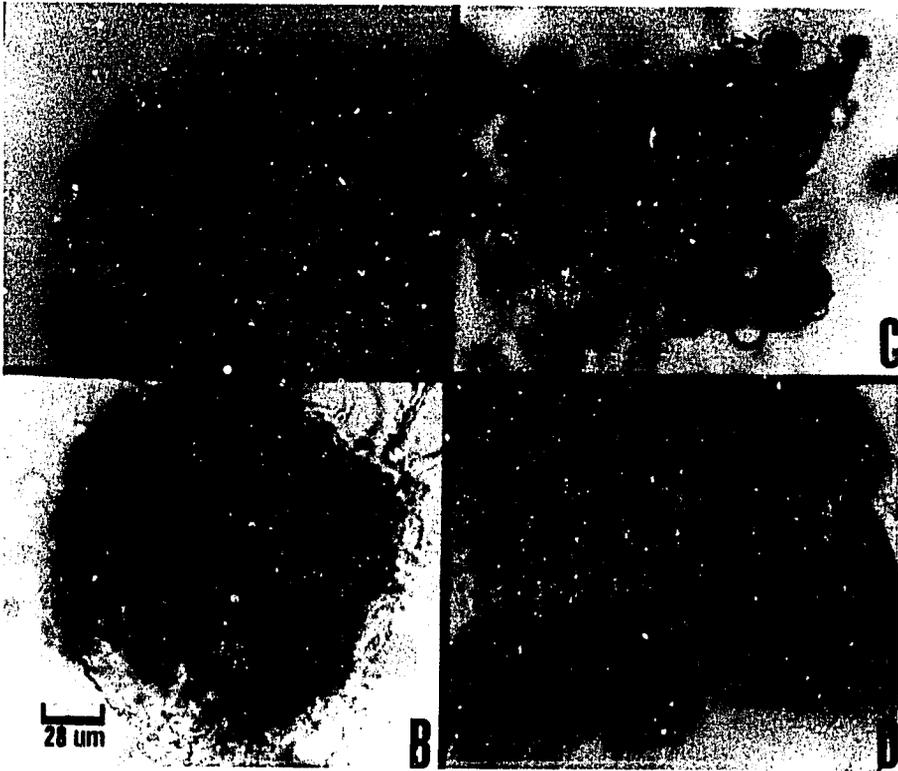


Figure 5. Occurrence of hyperparasitism, A. Mature azygospore of Entrophospora no 1. with chlamydospores (c) of Glomus sp. inside B. Mature azygospore with Entrophospora no 3. with chlamydospores (c) of Glomus sp. inside. C. Chlamydospores (c) of Glomus sp. were formed around the spore of Entrophospora no 3. D. High magnification of chlamydospores (c) of Glomus sp. with subtending hypha (sh).

The discovery of hyperparasitism of VA mycorrhizal fungi is considered to be of significant because its potential implication in future inoculant production. Hyperparasitism will make it even more difficult to prepare VA mycorrhizal fungi inoculant of high (pure) quality. The fact that the hyperparasitism was shown to be involved with non-fastidious species such as these belong to genus Glomus poses another major constraint to the development of in vitro production of VA mycorrhizal fungi.

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PROGRESS REPORT NO. 3

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RI-PLASMID INDUCED ROOT EXCULTURE AS A POTENTIAL SUBSTRATE
FOR IN-VITRO PROPAGATION OF MYCORRHIZAL FUNGI

3. VA Mycorrhizal Fungi and Host Plant Infection Specificity

A RESEARCH PROJECT

USAID/PSTC PROGRAM

Grant No. 776-5542-G-00-5077-00

Submitted by

Maiee suwana-adth
Project Leader

Kasetsart University Research and Development Institute
Kasetsart University
Bangkhien, Bangkok

Project Profile

Country : Thailand

Grant No. : 936-5542-G-00-5077-00

Program : Program on Science and Technology Cooperation

Project Title : Ri-Plasmid Induced Root Culture as a potential Substrate for In-Vitro Propagation of Mycorrhizal Fungi

Project Leader : Malee Suwana-adth, Ph.D.

Organization : Kasetsart University Research and Development Institute Kasetsart University, Bangkok.

Co-investigators : Ms. Poonpilai Suwanarit
Dr. Wiwut Daengsubha
Ms. Yupe Mongkolseok

Project Consultants : Professor Dr. Chua Nan Hai,
The Rockefeller University
Professor Dr. John Monge,
University of California (Riverside)

Authorized Officer : Associate Professor Dr. Thira Sutabutr
Director, Kasetsart University Research and Development Institute

Total Project Budget : 2,585,250.- bahts

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INTRODUCTION

VA mycorrhizal fungi are species of fungi that intimately associate with plant roots forming a symbiotic relationship with the plant. They are normally maintained in pot culture with their host plants.

Although mycorrhizae, symbiotic associations between roots and fungi, are widespread, they differ considerably in their specificity depending on the host plant and fungus involved (Gianinazzi - Pearson 1984.) Attempts were therefore made to establish such relationship between the isolated VA mycorrhizal fungi and certain economic crops.

The present report covers the studies on fungi - plants relationship conducted during the period between August 1986 - January 1987.

OBJECTIVE

The objective of the study during this period is to establish relationship between the mycorrhizal fungi, isolated from various soil samples throughout Thailand, and certain economic plants. The information will assist in the selection of plant roots for further in vitro cultivation improvement.

Material and Methods

VA mycorrhizal fungi. A total of 21 strains of VA mycorrhizal fungi were studied. Eleven strains were isolated from mung-bean growing areas throughout Thailand and ten strains were from the collection of the Department of Microbiology, Kasetsart University.

Host Plants Four different types of host plants were used in the growth relationship study, namely, mung bean, tomato, potato and carrot.

Preparation of inoculum Soils with VA mycorrhizal fungi were processed to isolate fungal spores according to the method of Gerdemann and Nicolson (1963). Clean and surface sterilize the spors with 0.5 per cent sodium hypochlorite for 1-3 min. Wash with sterile distilled water three times before maintaining then in sterilized soil tubes for use as inoculum. Each soil sample was treated separately.

Host plant Infection Low fertility soils from Pakthongchai District of Nakornratchasima were used in the infection experiments. Soil were treated with methylbromide gas for 72 hours and left overnight for the gas to evaporate before use. Soils were placed in the 4.5 cm. dia. x 10 cm height wide - month jars for use with mung beans and tomato plants and in the 7 dia. x 10 cm. height clay pots for use with potato and carrot plants.

Seeds were treated with 10 percent chlorox solution for 5-10 min. and then washed 4 to 5 times with sterile water before planting. Each jar or pot was inoculate with soil - spore inoculum of each strain of VA mycorrhizal fungi at approximately 150 spores per plant. Watering was carried out daily using distilled water.

Infectivity was assessed after 2-3 months at maturity of the plants. Assessment was made by screening the soil for the presence of new spores and by microscopic root examination. (Phillip and Hayman, 1970) Infection of root cells by fungal hyphae and the presence of vesicle and/or arbuscules indicated positive relationship.

Results and Discussions

1. VA mycorrhizal fungi from the Department's collection were 2 species of Acaulospora, 3 species of Entrophospora, 4 species of Gigaspora and one species of Glomus, as follow :

- (1) Acaulospora spinosa,
- (2) A. scrobiculata,
- (3-5) Entrophospora sp. 3 strains (No. 1, 2 and 3),
- (6-9) Gigaspora gigantea,
G. heterodama,
G. margarita,
G. sp. No. 1, and
- (10) Glomus monosporus

Fungi isolated from field and tested for host plant infectivity were 6 species of Glomus (KUMD 8601, 8602, 8603, 8604, 8605 and 8606) 2 species of Gigaspora (brown and orange strains), one species of Sclerocystis (KUMD 8607) and two unidentified species.

Host plant infections and specificity

Most fungi tested showed certain degree of specificity to host plants. On the other hand, certain plant such as carrots appeared to be susceptible to most fungi as compared to mung beans. Glomus species showed preference toward mung bean host plant while Acaulospora showed preference toward carrot host plant. Table 1 shows host plant specificity of mycorrhizal fungi

Table 1. Infection specificity of VA mycorrhizal fungi

<u>VA mycorrhizal fungi</u>	<u>Host plant infection</u>			
	<u>mung bean</u>	<u>tomato</u>	<u>potato</u>	<u>carrot</u>
<u>Acaulospora spinosa</u>	-	-	-	+
<u>A. scrobiculata</u>	-	-	-	+
<u>Entrophospora sp. No. 1</u>	-	-	-	+
<u>Entrophospora sp. No. 2</u>	-	-	-	-
<u>Entrophospora sp. No. 3</u>	-	-	-	-
<u>Gigaspora gigantea</u>	-	-	+	+
<u>Gi. heterogama</u>	-	-	-	-
<u>Gi margarita</u>	-	+	+	+
<u>Gigaspora sp. No. 1</u>	-	-	-	+
<u>Glomus monosporus</u>	+	-	+	+
<u>Glomus sp. KUMD 8601</u>	+	0	-	+
<u>Glomus sp. KUMD 8602</u>	+	0	L	-
<u>Glomus sp. KUMD 8603</u>	+	0	-	-
<u>Glomus sp. KUMD 8604</u>	+	0	-	-
<u>G. fasciculatus (KUMD 8605)</u>	+	0	+	-
<u>G. manihotis (KUMD 8606)</u>	+	0	-	+
<u>Sclerocystis sp.</u>	+	0	-	-

Results obtained indicated that it may not be possible to establish a multifungal substrate of root culture for in vitro production of mycorrhizal fungi. Therefore, it will be necessary to maintain a number of root culture lines.

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PROGRESS REPORT NO. 4

RI-PLASMID INDUCED ROOT CULTURE AS A POTENTIAL SUBSTRATE
FOR IN-VITRO PROPAGATION OF MYCORRHIZAL FUNGI

4. The Development of the Root VA Mycorrhizal Fungus Culture System

A RESEARCH PROJECT

USAID/PSTC PROGRAM

Grant No. 936-5542-G-00-5077-00

Submitted by

Malee suwana-adth
Project Leader

Kasetsart University Research and Development Institute
Kasetsart University
Banghen, Bangkok

Project Profile

Country : Thailand

Grant No. : 936-5542-G-00-5077-00

Program : Program on Science and Technology Cooperation

Project Title : Ri-Plasmid Induced Root Culture as a potential Substrate for In-Vitro Propagation of Mycorrhizal Fungi

Project Leader : Malee Suwana-adth, Ph.D.

Organization : Kasetsart University Research and Development Institute Kasetsart University, Bangkok.

Co-investigators : Ms. Poonpilai Suwanarit
Dr. Wiwut Daengsubha
Ms. Yupa Nongkolsook

Project Consultants : Professor Dr. Chua Nam Hai,
The Rockefeller University
Professor Dr. John Monge,
University of California (Riverside)

Authorized Officer : Associate Professor Dr. Thira Sutabutr
Director, Kasetsart University Research and Development Institute

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Introduction

VA mycorrhizal fungi, which form symbiotic association with plants, cannot be propagated on artificial media but has been shown to infect root cultures of tomato (Miller - Wide man and Watrud, 1984). Infection was, however, constrained by root thickening and lignification as well as by limited formation of lateral roots. Furthermore, the cultivation of roots depended on plant hormones in the culture medium.

On the other hand, Agrobacterium is known as an oncogenic agent and can neoplastically transform cells of many kinds of plants into tumour cells. Agrobacterium - induced tumour can be removed and cultured in vitro where they can continue to grow indefinitely (Hooykaas and Schilperoort, 1986).

It is therefore proposed to use Agrobacterium to induce and accelerate root formation in selected plants and to develop bacteria - free, fast-growing (tumour) root cell lines suitable for mycorrhizal association with useful VA mycorrhizal fungi. The two-member root-mycorrhizal fungus culture system could subsequently be used to propagate, aseptically, VA mycorrhizal fungi inocula.

Material and Method

Bacterial Culture

Agrobacterium tumefaciens ATCC 15955 and Agrobacterium rhizogenes ATCC 15834 were obtained from the American Type Culture Collection, U.S.A. through the Bangkok Microbiological Resource Center for SE Asia at Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. The culture were maintained on a nutrient agar medium under refrigeration. Inoculum was prepared by suspending cells from a 48-hour slant culture in a small amount of distilled water to obtain approximately 10^9 cells per ml.

VA mycorrhizal fungi

Glomus fasciculatus KUMD 8605 was isolated from soils in the legume field and shown to infect mung bean and potato in pot culture (Progress Report No. 3).

Plant materials

Model plants used were carrot and potato. Others tested plants were five different leguminous plants, i.e. soybean, mung bean, peanut, winged bean and sugar pea. All tested plant materials were obtained as seed from local markets. Carrots and potatoes were obtained as fresh materials.

Tumour induction

Fresh carrots and potatoes were peeled and then washed clean and surface-sterilized according to the procedures of Lippincott and Lippincott (1969). They were then aseptically cut into disks 0.5 cm. thick and 1.0 cm. diameter. Legume seeds were similarly treated and allowed to germinate on Murashige and Skoog (1962) medium. Wounds were inflicted on the stem of seedlings. Inoculation was performed by dropping two drop of 10^9 cells suspension to the cut tissue (disc) surface or the cut wound of plant materials maintained on the same medium at 23-25° c under light.

Nutrient media for root cultures and tumour root cultivation.

The growth media for tumour root cultures was according to Linsmaier and Skoog (1965) with the addition of 0.5 percent carbenicillin but without the addition of plant hormones. Tumour roots were removed from plant seedlings and repeatedly transferred to fresh plates every 1-2 weeks to remove the agrobacteria after decontamination, root cultures were maintained on the same medium with no carbenicillin.

Results and Discussion

Pathogenicity of Agrobacterium

Both A. tumefaciens and A. rhizogenes caused tumour growth formation when tested with the model tissue system of potato. A. tumefaciens produced unorganized compact clumps of tissue growth which were hard and brittle in texture while A. rhizogenes produced white tender lateral roots (Figure 1). A. rhizogenes was chosen for further studies with other test plants.

Susceptibility of test plants to *A. rhizogenes* infection

Infection experiments with test plants revealed that soybean seedlings showed no response while winged bean seedling showed slight abnormal fluffy cell differentiation at the wound sites. Mung bean, peanut and sugar pea showed positive response with varying degrees of tumour root formation. Figure 2 shows varying degrees of susceptibility of test plants to *A. rhizogenes* infection.

Mass Propagation of Ri-induced root cultures

Serial transplantation of Ri-induced roots of carrot, potato and peanut could be successfully made while that of mung bean and sugar pea showed degeneration of growth.

Transplanting of tumour roots, after bacterial decontamination by successive cultivation on a carbenicillin-containing medium, was most successful with only peanut and with the two model systems of potato and carrot. Root tumor cell lines of peanut, potato and carrot could be and are being maintained through periodic transplantation.

Table 1-3 show growth responses of Ri-induced root cultures transplantation of mungbean, peanut and sugar pea respectively. Figure 3. shows series of transplanted Ri-induced root culture of peanut.

VA mycorrhizal infection of *A. rhizogenes*-induced root cultures

Potato tumour root cultures, with extensive branching and root hair formation were used, initially, to develop the two-member (root-VA mycorrhizal fungus) culture system. It was found that *A. rhizogenes* induced roots were susceptible, as hosts, to mycorrhizal association with the test fungus, *Glomus fasciculatus* (Figure 4)

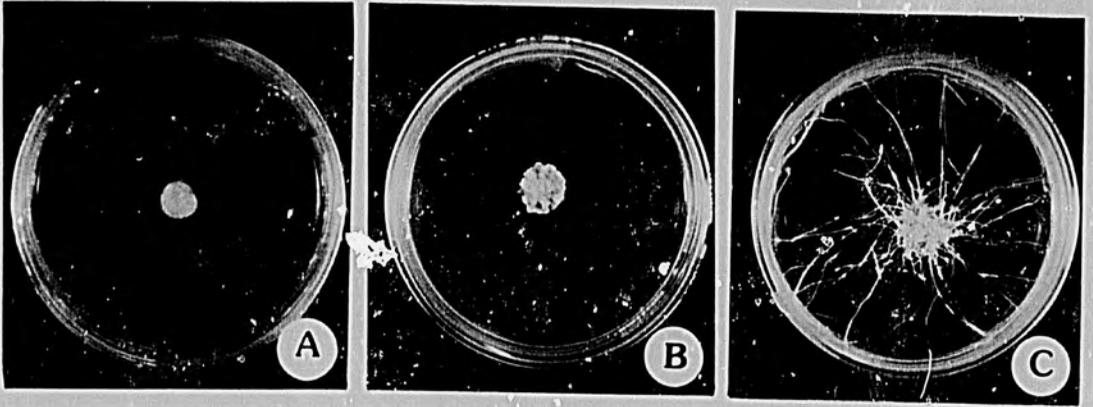


Figure 1. Tumor formation on potato disc
(A) control (B) infected with *B. tumefaciens*
(C) with *B. rhizogenes*

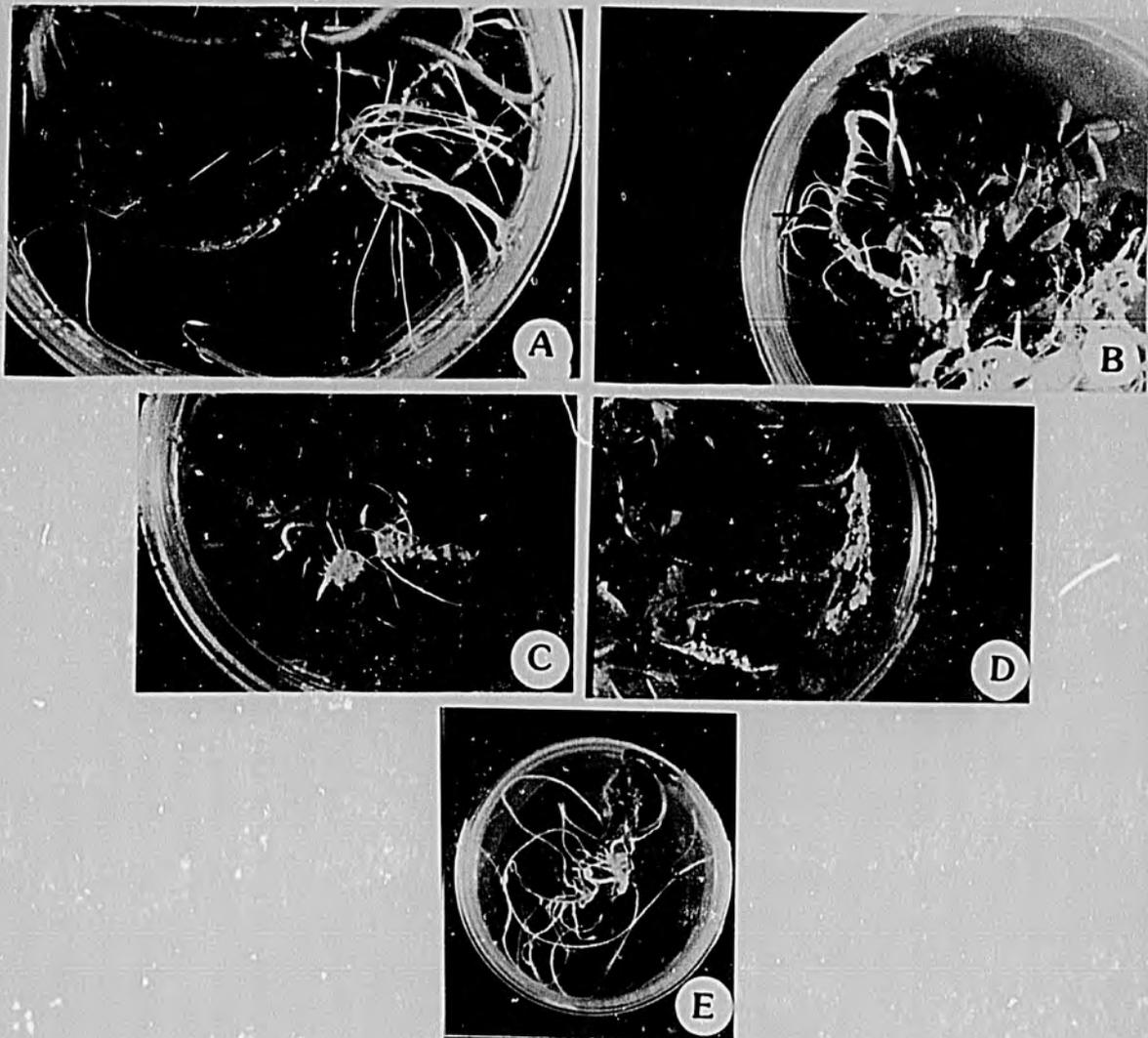


Figure 2. Variation in susceptibility of test plants to *A. rhizogenes* infection.

(A) soybean

(B) peanut

(C) Mung bean

(D) winged bean

(E) Sugar pea

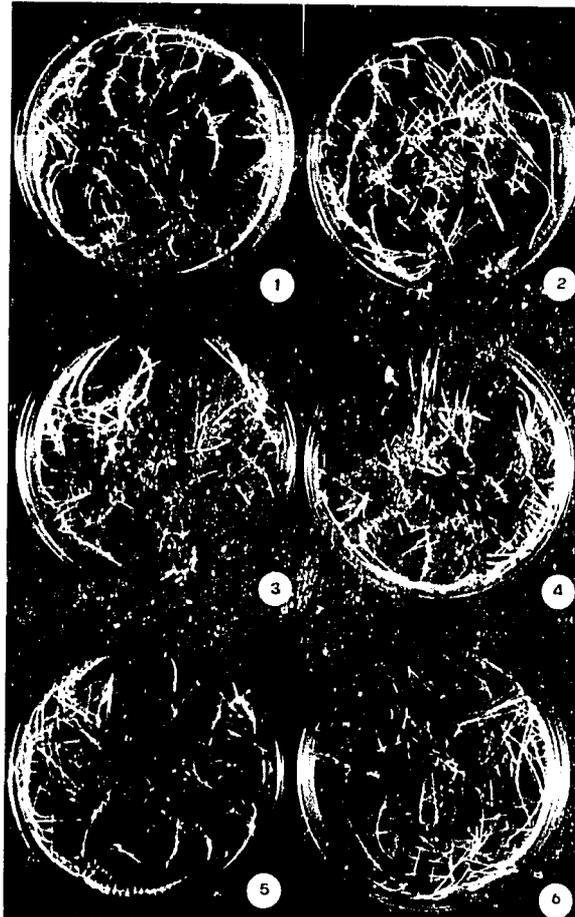


Figure 3. Series of transplanted Ri-induced root culture of peanut

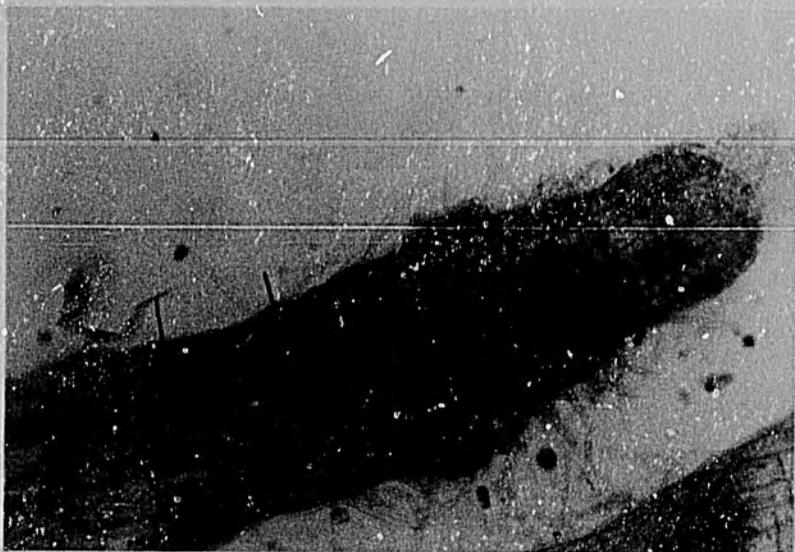


Figure 4. The root-VA mycorrhizal fungus system of potato and *Glomus fasciculatus* showing vesicle formation (arrows)

Table 1. Growth response of R₁-induced root culture of mung bean after serial transplantation.

culture No.	initial weigh (gram)	change in wet weight per gram after 30 days of transplantation.
1	0.08	2.88
2	0.17	1.88
3	0.04	1.0
4	0.02	5.5
5	0.10	1.4
		average = 2.53

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Table 2. Growth response of Ri-induced root culture of peanut after serial transplantation

Culture no.	initial weight (gram)	Change in wet weight per gram after 50 days of transplantation
1	0.02	199
2	2.23	28.65
3	0.08	13.50
		average = 80.38

Table 3. Growth response of Ri-induced root culture of sugar pea after serial transplantation.

Culture No.	initial weight (gram)	Change in wet weight per gram after 50 days of transplantation.
1	0.40	0.28
2	0.42	1.60
3	0.51	0.96
4	0.76	0.05
5	0.45	0.38
		average = 0.65

Conclusions :

1. Bacteria - free hairy-root tumour cell lines of carrot, potato and peanut have been successfully developed and maintained.
2. Root cultures with extensive lateral root formation could be successively transplanted and propagated to obtain effective in vitro substrate for cultivation of mycorrhizal fungi based on the two-member culture system of potato tumour root and Glomus fasciculatus.

Future Research plan :

1. Studies on the effectiveness of the mycorrhizal root inocula.
2. Induction of sporulation of mycorrhizal fungi

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