PN 56124 7.242

PROGRESS REPORT NO. 1

BIOLOGICAL CONTROL OF BACTERIAL WILT OF GINGER BY COUPLING RESISTANT CULTIVARS WITH GENETIC TRANSFORMANT BACTERIA

A RESEARCH PROJECT

USAID/PSTC PROGRAM
Grant No. 936-5542-G-00-7032-00

Submitted by

MR. NIPHONE THAVEECHAI, Ph.D.
Project Leader

KASETSART UNIVERSITY

Department of Plant Pathology
Faculty of Agriculture
Bangkhen, Bangkok 10903

THAILAND

PROJECT PROFILE

Country	: Thailand
Grant No.	: 936-5542-G-00-7032-00
Program	: Program on Science and Technology Cooperation
Project Title	: Biological Control of Bacterial wilt of Ginger by Coupling Resistant Cultivars with Genetic Transformant Bacteria
Project Leader	: Mr. Niphone Thaveechai, Ph.D. Assistant Professor
Organization	: Kasetsart University
Co-investigators	: Mr. N. W. Schaad, Ph.D. Professor
	: Mr. Wichai Kositratana, Ph.D. Lecturer
	: Mrs. Sasithorn Wuthiwanich, M.S. Lecturer
Project Consultants	: -
Authorized Officer	: Mr. Thira Sutabutra, Ph.D. Associate Professor Director of K.U. Research and Development Institute
Total Project Budget	: US$ 150,000
Project Duration	: 3 Years
Reporting Period	: August-December, 1987
Budget Allocation for This Period	: US$ 33,931.50 (Baht 882,220) 1 US$ = 26 Baht

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1. Background/Introductions:

Ginger (Zingiber officinale Rosc.), a monocotyledon of the family Zingiberaceae, is an economic crop for local and export markets of several countries around the world namely: Nigeria, Sierra Leone, Jamica, Bangladesh, India, Indonesia and Thailand. Ginger has been used for many purposes such as vegetables, spices, and medicinal plants. In industrialized countries of Europe, North America, Australia and Asia, ginger is being used as raw material for preserved food, candy, cosmetic, beverage and medicine (Burkill, 1966; Hill, 1976). Because of multiple utilization as agricultural and indrustrial crop plants, ginger is potentially a valuable export comodities in many LDCs including Thailand. However, cultivation of ginger in these countries frequently faces a disease problem that causes a significant yield reduction.

Several plant pathogens such as fungi (Tripathi and Grover, 1977, Verma and Vyas, 1981), virus (Mayee, 1974), nematodes (Shah and Raju, 1977) and bacteria (Hayward, 1983) have been reported to infect ginger. Among these pathogens the bacterium Pseudomonas solanacearum, a soilborne causal agent of wilt disease of crop plants, is considered the most serious pathogen of ginger in Thailand (Chantaraotan, 1982) and other countries (Hayward et al, 1967; Ishii and Aragaki, 1963; Quinon et al, 1964). In 1978, this bacterium caused total losses greater than 40% of ginger production in Thailand. At present, bacterial wilt still be a serious disease of ginger in Thailand. Ginger infecting with this bacterium usually develops wilting and yellowing symptoms on the lower leaves then expands upward until all leaves are affected. At the late stage leaves show upward rolling with brown necrosis and entile plant die. Generally, water soak lesion can be seen easily on young rhizome and consequently the affected tissues disintegrate then becoming soft rot-like symptom. The rot lesion and vascular tissues of stem usually associate with milky exudate (Chantaraotan, 1982; Hayward, 1983).

Although substantial losses of ginger due to P. solanacearum, few works have been done for development of appropriate control measures. Most control strategies have been employed to combat P. solanacearum on other crops

using crop rotation, cultural practices, chemical and disease resistant cultivars. However, information on the control of bacterial wilt of ginger is less available. Furthermore, several attempts using copper fungicides, antibiotics, and sodium chloride solutions treated rhizome seed-pieces before planting in artificial infested soils were not success (Chantaraotan, 1982). The failure of these experiments probably may be due to inappropriate selection of sample, inoculum concentrations and method of treating samples. Therefore, new approaches for an effective and reliable control method are urgently needed.

Conventional breeding for disease resistant cultivars with pathogenicity test screening is time and labor consuming. Furthermore, several factors are involved in limiting of breeding program such as source of genetic traits, inter or intraspecific cross, and time for offspring production. Presently, many advanced biotechnologies offer an alternative aspect to overcome these problems. To solve bacterial wilt of ginger, a new aspect on using plant cells and tissues cultures for production of disease resistance incorporated with the use of hypersensitive reaction (HR) for disease resistant screening has been proposed and carried out in this research project.

2. Objectives:

The specific objectives of this project include development of a rapid and reliable method for screening ginger resistance from either intact or cultured plants and manipulation of genetic improvement of ginger for disease resistance through plant and cell cultures technique.

As proposed in the research activities plan, major works of this reporting period were performed on preparation of research facilities such as procurements of necessary equipments and renovation of laboratory and greenhouse. Surveying and collecting of ginger and ginger related plants including bacterial wilt pathogen were investigated. Improvement of somaclonal propagation technique for pathogen-free ginger through tissue culture was also initiated.

3. Materials and Methods:

A. Renovation of laboratory and greenhouse:

Renovation of laboratories and greenhouse were done at the Department of Plant Pathology, Kamphaengsean campus, Nakhon Pathom. Laboratories and greenhouse were modified to be temperature control laboratories and greenhouse in order to maintain temperature of about 28 C and to reduce

contamination of cultures. One laboratory was used for tissue and cell cultures production and others were bacteriology and general analysis laboratories. For greenhouse, it was repaired and equipped with a temperature control unit to grow tissue culture regenerated plantlets, and multiplication of ginger and ginger related plants for production of resistant cultivars including production of pathogen free plants.

B. Procurement of equipments:

All essential equipments as proposed in the project proposal were procured and purchesed from either local or abroad. The obtained equipments were allocated mainly at Kamphaengsaen campus while the less were at Bangkhen campus. They are incubators, refrigerator, freezer, circulating bath, heating bath, gel electrophoresis apparatus including power supply, UV illuminator and transblot chamber. There are other requested equipments which will be purchesed in the next period such as microfuge, environmental shaker and shaking bath.

C. Collection of bacterial pathogen, ginger and ginger related plants:

Surveying and collecting of bacterial pathogen, ginger and ginger related crops including other hosts infected with this bacterium were carried out in the major ginger growing areas of north, northeast, central and south of Thailand between August to December, 1987. Ten provinces were surveyed for the collection of bacteria and ginger plants. They were Chiang Mai, Chiang Rai, Phetchabun, Loei, Bangkok, Nonthaburi, Chanthaburi, Nakhon Pathom, Prachuap Khiri Khan and Chumphon. Samples of ginger and related plants with and without bacterial wilt symptoms (Chantaraotan, 1982) were collected at least 5-10 samples per field. All samples were placed in polyethylene bags for transportation and isolated for the bacterial pathogens either at Kamphaengsaen or Bangkhen campus. Intensive surveys for field resistant ginger to bacterial wilt were also done and suspected gingers were collected for further testing of disease escape in the greenhouse at Kamphaengsaen cumpus.

Isolations of bacterial wilt pathogen were performed by using samples from infected stems and rhizomes showing typical wilt symptom. Three replications were done for each sample. The samples were washed under running tap water for 5 min and blotted on paper towels. Small tissue segments (approximately 1-2x3 mm) were excised from margin of lesions, surface-sterilized with a fresh preparation of 0.5% NaOCl (Clorox) in water (1:9, v/v) for 2-3 min and washed in sterile distilled water (SDW). The tissue was

comminuted in a drop of SDW on a sterile petri dish and a loopful of the suspension streak onto SM-1 (Granada and Squeira, 1983), KB (King et al, 1954) and TZC (Kelman, 1954) media each of two plates. Plates were incubated at 27-30 C for 24-72 hr depending on the medium. Colonies tentatively identified as P. solanacearum on TZC and SM-1 showing fluidal, milky with pink at center were purified by streaking on TZC and kept in KB slant at 4-5 C in refrigerator for further tests and uses.

Pathogenicity test was performed with standard method as described (Schaad, 1980). Inocula were prepared by inoculating a loopful of a 24 hr culture from KB plate into liquid medium 523 (Kado and Heskett, 1970) and incubating for 24 hr on a rotary shaker at room temperature (27-30 C). Suspension was adjusted to an optical density (O.D.) of 0.1 at 640 nm and diluted to 10^{-4}. Ginger and tomato plants were used for testing pathogenicity of the suspected bacteria by injecting approximately 0.1 ml of bacterial suspension into rhizome or stem using micropipette tip technique. Plants were placed in greenhouse for observing symptom development.

D. Tissue and cell cultures of ginger:

a. Improvement of somaclonal propagation of pathogen free ginger through tissue culture:

Somaclonal propagation of pathogen-free ginger were performed by modified the procedure of Hosoki and Sagawa (1977), Vacin and Went (1949) and Murashige and Skoog (1962). Various plant growth regulators at different concentrati ns were added solely to the basal medium or in combinations with others. Plant growth regulators incorporated in the experiments were 6-Benzylaminopurine (BA), 1-Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D), and Kinetin (KIN) for a rapid stimulating root and shoot developments. Several types of ginger tissues such as bud, root, leaf, rhizome and stem were included in the experiments. Plant cell culture technique of Hu and Wong (1983) were followed to obtaine a good performance. Explant tissues of ginger from various parts were excised, surface sterilize with 0.5% sodium hypochlorite (Clorox) at 10% plus 0.1% Tween 20 for 15-20 min then rinsed twice with SDW. The tissues were dissected into 2-5mm small pieces under laminar flow hood and placed onto the test media. Exception for bud that the whole bud was used by removed only the outer budscales. Ginger cultures were incubated for growth and development under continuous light of about 2.1 klux in tissue culture room at temperature of 24-27 C. Numbers of plantlet obtained from one piece of tissue were recorded and compared to evaluate the efficiency of the media. At about 10% of

plantlets from the same clone were randomly tested for bacterial wilt infection by serological method of enzyme-linked immunosorbent assay (ELISA) and for viruses by visual and electronmicroscope examinations.

b. Callus induction:

Various tissues of ginger were used to induce callus formation by modified from the method of Murashige and Skoog (1962) and Schenk and Hildebrandt (1972). Surface-sterilized ginger tissues were raised on the media incorporated with different concentrations of plant growth regulators. The cultures were kept in tissue culture room with the same environmental condition as of somaclonal propagation. Callus formation was observed and recorded.

4. Results/Disscussion/Tables:

Renovation of laboratory and greenhouse

After grant approval, laboratories and greenhouse were immediately renovated and the works were finished within 30 days. Laboratories were used for tissue culture investigations, general bacteriology, biochemistry analysis and installation of equipments whereas greenhouse was used for growing tissue culture plantlets and vegetative propagating of ginger (Plate 1). Although the limitation of spaces, laboratory conditions were generally good and doing the research experiments in these laboratories was suitable and efficient. The renovated greenhouse showed a good performance on temperature control, however, the greenhouse still need to be equipped with light and humidifying unit for a good plant growth conditions since our first two lots of ginger plantlets from tissue culture of about 400 plants were died according to insufficient humidity and light. Therefore, installation of automatic mist sprayer or humidity chamber and artificial light sources will probably solved these problems.

Procurement of equipments:

During this reporting period, two procurements were carried out to obtain the requested equipments. The first set of equipments was consisted of incubators, refrigerator, freezer and renovation of laboratories and greenhouse. All of these equipments were installed and equipped at Kamphaengsaen campus except one incubator was placed at Bangkhen campus. These equipments were routinely used to support the research experiments such as storage of bacterial cultures, chemicals, enzymes, antisera and plant samples. Gel electrophoresis apparatus and necessary accessories were obtained from the second procurement including horizontal and vertical gel electrophoresis

chambers for agarose and acrylamide gels, power supply, UV illuminator, transblot chamber, refrigerated circulating bath and heating bath. These equipments will be used for characterization and detection of metabolites produced in infected susceptible and resistant cultivars. These equipments are being checked and adjusted for a good operation. Some equipments were shown in Plate 2.

Collection of bacterial pathogen, ginger and ginger related plants:

Bacterial wilt pathogen of ginger were surveyed and collected from ten provinces of ginger growing areas of north, northeast, central and south of Thailand from August to December, 1987 (Table 1). The diseased and healthy samples in infected fields were collected for isolation and propagated for testing field resistance. Field resistant testing was not performed in this reporting period since there were lack of large numbers of suitable growing stage plantlets.

Bacterial wilt symptoms of ginger were observed in 22 of 31 fields surveyed. A total of 18 fields was demonstrated positive isolation of *P. solanacearum*. Strains of pathogen isolated from these fields were proved to be pathogen on both ginger and tomato. However, virulence of these strains was different. For severity of symptom and disease development, the virulent strains caused a pronounced wilting and plant death within a short period after infection whereas the less virulent strains induced a slow wilting symptom and some case plant death was not occured. These strains are being characterized for races, biovars and serotypes.

Ginger and ginger related plants were collected for further investigations on development of disease resistant cultivars (Table 2). These plants were vegetatively propagated in greenhouse or somaclonally propagated through tissue culture in the laboratory. Five cultivars of ginger (*Zingiber officinale*) were obtained from different provinces for disease resistant screening. The collected ginger related plants were Roe (*Alpinia allughas*), Galanga (*A. galanga*), Krachaai (*Boesenbergia pandurata*), Turmaric (*Curcuma domestica*), Plai (*Z. cassumunar*) and Wild ginger (*Z. zerumbet*). These plants will be tested for the expression of disease resistant reactions by hypersensitive reaction test.

Tissue and cell cultures of ginger:

The technique for somaclonal propagation of pathogen-free ginger was successfully developed by using bud tissues. Three ginger cultivars of Yuak, Ta Dang and

Australia were propagated by this technique and obtained several hundred of tissue culture plantlets within a month. Modified media of this experiment provided a rapid formation of root and shoot better than Hosoki and Sakawa's medium. The media contained the same concentrations of micro an macronutrients of Hosoki and Sakawa medium but there was different in type and concentration of plant growth regulators. The medium was amended with a combination of NAA, BA, and 2,4-D at concentration of 0.5 to 2 ppm. Statistic analysis of this experiment will be done after complete data are obtained that expected to be in the next reporting period. For developing the medium suitable for callus formation, the medium of Schenk and Hildebrandt (1972) was modified by adding 2,4-D, CPA and kinetins. Ginger tissues from bud developed into callus after growing in the medium 10-15 days. A modified medium of Murashige and Skoog (1962) by adding 2,4-D, NAA and BA at concentration ranging from 0.1 to 0.5 ppm showed a good callus development especially when the medium contained 0.5 ppm of 2,4-D. However, this experiment still need to be continued in oder to obtain a complete data and results.

Table 1. Presence of bacterial wilt of ginger in the growing areas of north, northeast, central and south of Thailand in 1987

Growing area	Province and District	Fields observation		
		Total	Wilt symptom	Positive isolation
North	Chiang Mai, Saraphi	2	2	2
	Chiang Rai, Mae Sai	4	4	4
	Mae Suai	3	3	3
	Wiang Papao	3	2	2
Northeast	Loei, Dan Sai	2	2	1
Central	Bangkok, Taling Chan	1	1	1
	Chanthaburi, Tha Mai	2	0	0
	Nakhon Pathom, Bang Len	2	0	0
	Nonthaburi, Pak Kret	2	1	0
	Phetchabun, Kao Khaw	2	2	2
	Prachuab Khiri Khan, Bang Saphan	2	1	0
	Thap Sakae	1	1	0
South	Chumphon, Tha Sae	5	3	3
	Total	31	22	18

Table 2. Ginger cultivars and ginger related plants collected in Thailand during August to December, 1987

Botanical name and cultivar	Common or Thai name	Place of collection (Province)
Alpinia allughas Rosc.	Roe	Chanthaburi
A. galanga Sw.	Galanga	Bangkok, Chumphon Prachuap Khiri Khan
Boseuberrgia pandurata Hol.	Krachaai	Nakhon Pathom
Curcuma domestica Val.	Turmaric	Bangkok
Zingiber cassumunar Roxb.	Plai	Nonthaburi
Z. officinale Rosc.	Ginger	
cv. Yuak		Bangkok, Chiang Mai, Chiang Rai, Chumphon Nonthaburi, Pathum Thani, Phetchabun
cv. Ta Dang		Nonthaburi
cv. Australia		Bangkok
cv. Phet		Chumphon
cv. Phatthalung		Chumphon
Z. zerumbet Smith	Wild Ginger	Nonthaburi

5. Conclusion/Remarks:

Administration of the research project and research experiments faces a minor problem, however, after several consultations with experienced persons, the problem was solved without difficulty. All equipments obtained were as requested and still operated well. As mentioned previously, the renovated greenhouse need to be installed with an automatic mist sprayer or a humidity chamber including artificial light source in oder to obtain a better growing condition that will lead to increase a successful propagation of tissue culture plantlets.

Results from the surveyed of bacterial wilt of ginger throughout Thailand in 1987 demonstrated that the wilt disease caused by P. solanacearum still be a serious problem of ginger. In rainy season with hot and humid conditions, the wilt disease usually occurs and spread very rapidly. The disease cause a heavy loss of rhizome either in the fields or during transportation and storage. For dissemination of the disease, infected rhizomes or infested field soils is considered to be a significant primary source of inoculum. Using pathogen-free transplants in the new planting areas is suggested for an immediated control measure of bacterial wilt of ginger.

Somaclonal propagation was successful only from bud tissues whereas the others were not. However, some tissues such as leaf sheath showed a promise sign of root and shoot development. Tissue culture plantlets of three ginger cultivars, Yuak, Ta Dang, and Australia obtained from a modified medium of Vacin and Went (1949) were better root and shoot developments and stronger plantlets than obtaining from the media of Hosoki and Sagawa (1977) and Murashige and Skoog (1962).

For induction of callus formation, only bud tissues showed a successful callus development. Several media were found to be used for inducing callus formation such as modified media of Schenk and Hildebrandt (1972) and Murashige and Skoog (1962). However, a modified medium of Schenk and Hildebrandt showed a good callus formation than a modified medium of Murashige and Skoog. This is the first report on the successful induction of callus formation of ginger.

6. Workplan for the next period:

In the next period, research activities of this project will be performed as proposed in the activity plan that has been submitted to the USAID. Several research activities of the next period are as followed:

a. Collection and screening of field resistant ginger and ginger related species.

b. Studies on host-parasite interactions and hypersensitivity reaction.

c. Development of disease resistant screening technique.

d. Development of plant cell culture and regeneration of ginger.

e. Development of disease resistance through plant cell culture.

In addition to the research activities mentioned above, some research experiments of the previous preiod will be continued to complete the experiments. In general our previous research experiments are on schedule and need not to adjust or modify in the activity plan.

7. References:

Burkill, I. H. 1966. A dictionary of the economic products of the Malay Peninsula. Ministry of Agriculture and Cooperatives, Kuala Lumper. Vol. 2 (I-Z).

Chantarnotan, S. 1982. M.S. Thesis, Graduate School, Kasetsart University, Bangkok, Thailand.

Granada, G. A., and Sequeira, L. 1985. Plant Disease 67: 1034-1038.

Hayward, A. C. 1983. Pages 108-140 in: Plant bacterial disease: A diagnostic guide. P. C. Fahy, and G. J. Persley, eds. Academic Press, New York.

Hayward, A. C., Moffett, M. L., and Pegg, K. G. 1967. Queensland J. Agr. Anim. Sci. 24: 1-5.

Hill, A. F. 1976. Economic botany. A textbook of useful plant products, Tata McGraw Hill Co. Ltd., New Delhi.

Hosoki, T., and Sagawa, Y. 1977. Hort. Science 12:

451-452.

Ishii, M., and Aragaki, M. 1963. Plant Dis. Reptr. 47: 710-713.

Kado, C. I., and Heskett, M. G. 1970. Phytopathology 60: 969-975.

Kelman, A. 1954. Phytopathology 44: 693-695.

King, E. O., Ward, M. K., and Raney, D. R. 1954. J. Lab. Clin. Med. 44: 301-307.

Mayee, C. D. 1974. Indian Phytopathol. 27: 609-610.

Murashige, T., and Skoog, F. 1962. Physiol. Plant. 15: 437-497.

Quinon, V. L., Aragaki, M., and Ishii, M. 1964. Phytopathology 54: 1096-1099.

Schaad, N. W. (ed.) 1980. Laboratory guide for identification of plant pathogenic bacteria. The American Phytopathological Society, St Paul, Minnesota.

Schenk, R. U., and Hildebrandt, A. C. 1972. Can. J. Bot. 50: 199.

Shah, J. J., and Raju, E. C. 1977. Phyton (HORN) 18 (1/2): 79-84.

Tripathi, N. N., and Grover, R. K. 1977. Indian Phytopathol. 30: 222-223.

Vacin, E., and Went, F. 1949. Bot. Gaz. 110: 605-613.

Verma, R. K., and Vyas, S. C. 1981. Indian J. Mycol. Plant Pathol. 11: 14-16.

8. <u>Annex/Pictures</u>:

Plate 1. Renovation of laboratories and greenhouse at Kamphaengsaen campus, Nakhon Pathom, Thailand.

Plate 2. Some equipments and installation in laboratories at Kamphaengsaen campus.

Plate 3. Ginger growing field, occurance of bacterial wilt in the field and infected rhizomes.

Plate 4. Somaclonal propagation of ginger bud tissues through tissue culture and callus formation.

Plate 1. Renovation of laboratories and greenhouse at Kamphaengsaen campus, Nakhon Pathom, Thailand

a. Renovated laboratories of KU-USAID ginger research project containing general utility area (foreground) for instrallation of refrigerator, freezer, enviromental shaker and centrifuges. Bacteriology and tissue culture laboratories were on the far left and right, respectively.

b. A renovated greenhouse equipped with temperature control unit for growing tissue culture plantlets of ginger and other related species.

a
b

Plate 2. Some equipments and installation in laboratories at Kamphaengsaen campus

a. Gel electrophoresis apparatus and accessaries included (from left to right) refrigerated circulating bath, vertical and horizontal gel electrophoresis chambers, power supply unit, transblot chamber and heating bath.

b. Two 5.5 cu. ft. incubators (center) installation in bacteriology laboratory for incubating bacterial cultures at temperature ranging from 15-50 C.

c. Tissue culture shelves containing fluorescent lights and timer placing in tissue culture room equipped with air conditioning unit.

a
b
c

Plate 3. Ginger growing field, occurance of bacterial wilt in the field and infected rhizomes

a. New ginger growing area of northeast at Dan Sai, Loei showing healthy ginger field.

b. Heavy infected ginger field of north at Mae Suai, Chiang Rai demonstrated wilting and yellowing of stems and death gingers.

c. A culled pile of infected ginger rhizomes, as a source of inoculum for dissemination of bacterial wilt, showed rotten tissues and discarded near the growing field.

a

b
c

Plate 4. Sonaclonal propagation of ginger bud tissues through tissue culture and callus formation.

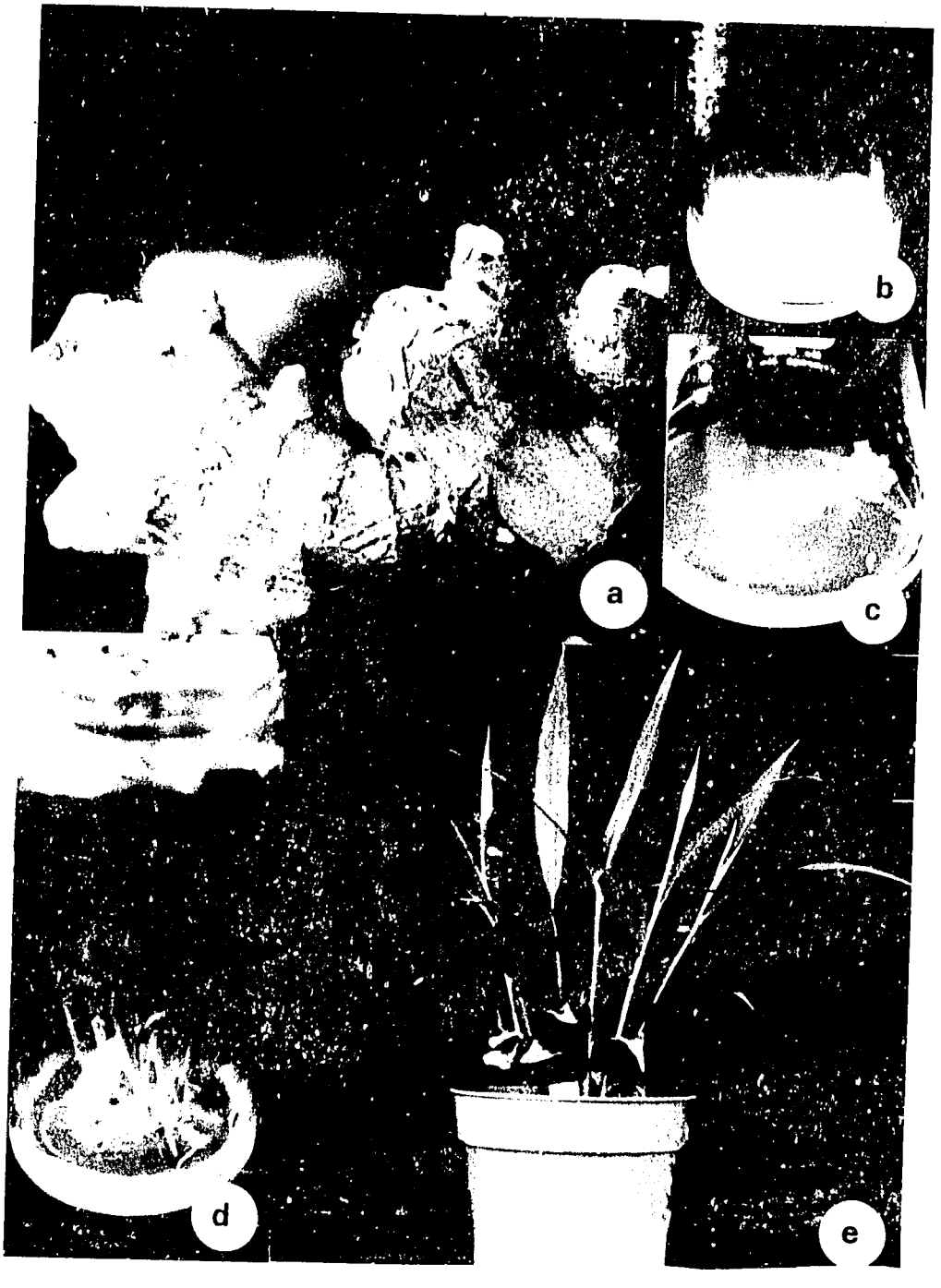
a. Germinated ginger buds from rhizome after incubating at room temperature for 2-3 weeks readily for tissue culture propagation

b. Bud tissues culturing on a modified Murashige and Skoog medium developing roots and shoots.

c. Callus of ginger tissues from bud inducing by culturing on a modified medium of Schenk and Hildebrandt.

d. Tissue culture plantlets of ginger obtaining by growing a single bud on a modifed medium of Vacin and Went for 4-6 weeks in tissue culture room having 12 hr lighting a day.

e. Ginger plantlets obtained from tissue culture of bud growing in 4 in. plastic pot containing a mixture of sterilized soil, sand and coconut husk dust at 1:1:1 ratio for 4-5 weeks in greenhouse at temperature of 24-27 C.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