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PROGRESS REPORT NO.3

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

A RESEARCH PROJECT

USAID/PSTC PROGRAM

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## PROJECT PROFILE

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| <u>Project Title</u>                     | : Biological Control of Bacterial Wilt of<br>Ginger by Coupling Resistant Cultivars<br>with Genetic Transformant Bacteria                                   |
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### 1. Background/Introductions:

Production of ginger (Zingiber officinale Rosc.) in Thailand and other ginger growing countries still faces a serious problem because of bacterial wilt disease caused by a soilborne Pseudomonas solanacearum E.F.Smith. In all ginger growing areas of Thailand, yield losses attributed to the wilt disease were steadily increased each year. Severe disease incidence usually found from the repeated growing fields more often than from the rotated fields. However, the disease frequently observed in the new ginger growing fields, also. This disease evidence suggested that the important primary sources of inoculum were the infected ginger planting rhizomes and infested growing field soils. In addition, previous studies (Chantaraotun, 1982; Thaveechai et al, 1988) demonstrated that the bacterial wilt pathogen is considered a complex and variable organism and also has a cross infection to crop plants other than species of Zingiberaceae. Tentative characterization and identification of ginger strains collected from ginger growing areas of Thailand in 1988 (Thaveechai et al, 1988) using the standard phylobacteriological methods of Schaad (1988) and biovars determination method of Haward (1964) revealed that the pathogen is Pseudomonas solanacearum biovar 3 and 4 as the same reported from other countries (Haward, 1986). Virulence of ginger wilt bacterium was different among the strains but there was no correlation with geographical areas and biovars (Thaveechai, 1988).

Several control strategies have been developed mostly to combat P. solanacearum of crops other than ginger especially solanaceous and musaceous plants. The present information of ginger wilt in Thailand is very little and incipient stage of establishment of disease control. Basic and applied research programs of ginger wilt bacterium concerning with the reliable and

effective control measures still open and urgently require. Therefore, an alternative approach for development and screening resistant ginger cultivars to the wilt bacterium is proposed in this research project.

Recently, several advanced biotechnologies and molecular recombinant technologies have been established and provided a promise alternative approach to overcome the wilt disease problem. This research project, therefore, initiates application of plant cell culture techniques to produce ginger resistant cultivars and using hypersensitive reaction (HR) as a new disease resistant screening method. Immunological techniques of enzyme-linked immunosorbent assay (ELISA) or dot immunobinding assay (DIBA) for detection of related disease resistant metabolites were also proposed to screen disease resistant ginger both in vitro cell cultures and intact regenerated plantlets. By using these modern technologies, ginger resistant to bacterial wilt probably obtain easily. Thus, the results will definitely contributed to resolve the bacterial wilt problem of ginger.

## 2. Objectives:

The major purpose of this research project is to develop an effective in vitro manipulation and screening disease resistant technique for production of ginger cultivars resistant to bacterial wilt. Several approaches for production of resistant ginger are somatoclonal variation and improvement of resistant traits by induced mutation. To develop a sensitive and reliable disease resistant screening method, HR and detection of resistant products by either ELISA/DIBA or PAGE (polyacrylamide gel electrophoresis) are proposed.

The objectives of this reporting period as proposed in the workplan are to collect and characterize the bacterial wilt pathogen, to study host and pathogen interactions, to regenerate callus and cell cultures and to develop an in vitro disease resistant screening technique.

### 3. Materials and Methods:

#### A. Characterization of Bacterial Wilt Pathogen:

Characteristics of the previous and new isolated strains of P. solanacearum were carried out to determine their authentic and biovars by the standard phytobacteriological procedures as described by Schaad (1988) and Hayward (1964), respectively. In addition, antiserum against membrane protein complex (MPC) of both avirulent and virulent strains of P. solanacearum was produced by followed the method of Thaveechai and Schaad (1986) in New Zealand white rabbit. Antiserum titers were determined by microprecipitation plate method and serological relationship of each strain was analyzed by either Ouchterlony double diffusion (ODD), indirect immunofluorescent staining (IF) or ELISA/DIBA techniques. Specificity of antiserum and authentic of suspected strains were also evaluated before using in the further experiment.

#### B. Host-Parasite Interaction:

Interaction of ginger and P. solanacearum, the wilt bacterial pathogen, especially hypersensitive reaction (HR) of host response to pathogen infection was investigated using cell suspension culture derived from ginger callus as described by Adkinson et al (1985). A 50 ml of cell suspension culture obtained from callus of ginger cultivar Yuak in liquid SH medium (Schenk and Hildebrandt, 1972) was placed in 125 ml Erlenmeyer flask and incubated at 25 C on rotary shaker at the speed of 130 rpm for one month. One month old suspension culture containing about 0.4 gm of cell/ml was inoculated with 1 ml each of 48-hr bacterial culture adjusted to 0.2 O.D. (optical density). Bacteria used in this experiment were consisted of avirulent and virulent strains of P. solanacearum, P. fluorescens from soil and Xanthomonas

campestris pv. citri from citrus. After incubating the inoculated suspension culture for 2,4,6,8,10,12,24, and 30 hr, each culture was washed with assay medium containing 0.175 M mannitol, 1 mM MES buffer adjusted to pH 6.0 with Tris. The washed cells in assay medium were shaken for 30 min at 25 C and then frozen. Net electrolyte loss of inoculated cells was determined from frozen cells by thawing in 10 ml of 1% Triton X-100 and then stirred for at least 2 hr. Uninoculated suspension culture was served as a control. Before frozen, cells were observed under light microscope to evaluate cytological and structural changes. Population dynamics of bacteria in cell suspension were also determined by dilution plate count on KB medium (King et al, 1954).

Soluble proteins especially enzymes from healthy and disease ginger cultivars were comparatively studied by SDS-PAGE of Laemmli (1970). Leaf tissues of each ginger were ground in a mortar containing cold extraction buffer then clarified the supernatant by low speed centrifugation at room temperature (about 25-27 C). The supernatant samples were mixed with sample buffer of Laemmli and boiled for 3-5 min then subjected into the gel and electrophoresed for 3.5 hr at a constant current of 20 mA/gel. Gel was removed from electrophoresis apparatus and stained over night in 0.05% Coomassie Brilliant Blue R 250 and destained for visualization of peptide profiles of each sample.

### C. Development of Callus/Cell Culture and Regeneration:

Callus culture obtained from the previous report were used for either direct or indirect regeneration into complete plantlets. In the direct regeneration, callus cultures were raised on MS medium (Murashige and Skoog, 1962) plus 0-5 ppm BA and maintained at 25-27 C in tissue culture room with a 16 hr photoperiod from cool white fluorescent tubes. For indirect regeneration, callus cultures were transferred into liquid SH medium for callus formation before regenerated into plantlets. The fresh liquid medium

was replaced the old one for every 2-4 weeks depending on the density of cells in the cultures.

#### D. In Vitro Disease Resistant Screening Technique:

In vitro disease resistant screening technique was developed to evaluate ginger plantlets from somatoclonal multiplication by drenched and clipped inoculations of plantlets either in tissue culture vessels or aquarium chamber. Two concentrations of the pathogenic strain (PB21) were used to compare the disease reaction for 20 days after inoculation whereas water inoculation was served as the control. Ginger plantlets cv. Yuak were used for screening disease resistance in this experiment. Plantlets showed wilt symptom were reisolated to confirm the pathogen on the selective medium for P. solanacearum, SM-1 medium (Granada and Sequeira, 1983).

#### 4. Results/Discussion/Tables:

##### Characterization of bacterial wilt pathogen:

All of suspected strains of the new isolation from ginger wilt samples and the previous isolation showing typical colony characters on TZC and SM-1 media were P. solanacearum, the bacterial wilt pathogen of ginger. These strains infected ginger and tomato plants causing typical bacterial wilt symptom when inoculated with 0.05 ml of bacterial suspension containing  $10^4$ - $10^6$  cfu/ml by micropipette inoculation technique. Plants inoculated with the heavy bacterial cells ( $10^6$ - $10^9$  cfu/ml) induced rapid development of wilt symptom which was approximately 5-7 days after inoculation. However, the low concentration of cells was preferred to use in this experiment to avoid giving too much disease pressure which may lead to produce uncertain or atypical

disease symptoms. Characterization into races and biovars of these ginger strains were homogeneous of race 1 and biovar 3 or 4.

Using antiserum of formaldehyde fixed-cells for serological determination of the ginger strains either by ODD or IF techniques showed that no serological different was observed among them. The ginger strains also were not differentiated from tomato strains by these serological tests but they were serologically different from other plant pathogenic bacteria, saprophytic pseudomonads and other bacteria (Table 1). Only one strain of P. florescens showed slight cross reaction by IF test. Therefore, it was indicated that antiserum to formaldehyde fixed-cells was high specific to the pathogen.

The MPC antiserum against P. solanacearum was just obtained , therefore, the titer and specificity of antiserum were not tested in this research period. The achieved results will report in the next progress report.

#### Host-Parasite Interaction:

Interaction of ginger and P. solanacearum especially HR and pathogenicity was emphasized and carried out in intact plants and cell culture. Ginger plants obtained from rhizome and tissue culture of two commercial cultivars, Yuak and Phed, were not developed HR reaction which it was indicated that either the tested gingers were no resistant characters or environmental conditions were not suitable to produce HR. Disease symptom was observed in both cultivars. Yuak cultivar from the two sources was no difference in disease severity. However, the Phed cultivar did show different of disease severity that the ginger plants from rhizome were lesser severity than the gingers from tissue culture (Table 2).

In cell culture experiment, only Yuak cultivar was investigated for electrolyte loss, cytological and structural changes, and population dynamics of bacteria at periodical times from 2-30 hr after inoculation into the cell culture. Avirulent strain induced a great difference of electrolyte loss from

virulent, other bacteria such as plant pathogenic and saprophytic bacteria at 4 hr after inoculation (Table 3). Electrolyte loss in the treatment of avirulent strain was two and three times greater than virulent and plant pathogenic or saprophytic strains. Noninoculated control treatment showed no change of electrolyte leakage. Cytological observation of cell suspension found that avirulent treatment showed the most darkening and clumping of cytosol after inoculation for 2 hr. Virulent treatment induced clumping of cell organelles and dark cytosol more than the other treatments whereas the control was still normal. The longer incubation the greater numbers of abnormal and empty cells were observed. After incubation for 6-8 hr other treatments started showing cell lysis and no cytoplasm except plant pathogenic and control treatments were normal. Cell numbers of avirulent strain were significantly increased greater than virulent strain and other bacteria at 10-12 hr after inoculation and gradually declined to nearly equal numbers. The virulent strain and other bacteria starting increased their population numbers after 24 hr (Table 4).

Preliminary investigation on metabolic changes especially enzymes related to disease resistant was carried out using ginger Yuak cultivar inoculated with pathogen and noninoculated served as control. SDS-PAGE of soluble protein extracted from infected and noninfected ginger leaf tissues showed similar polypeptide profiles for all samples (Plate D). Loading plant extracted sample to the gel at 10 or 15 microliter provided an optimum intense peptide bands. However, the gel system will be improved by using other system or modified from the existing methodology.

#### Development of Callus/Cell Cultures and Regeneration:

From the previous reporting period, callus of ginger cv. Yuak was successfully obtained. The callus cultures were consecutively used to

regenerate into complete plantlets on MS medium plus 0-5 ppm BA, however, all of these formulations were not stimulated callus differentiation into plantlet. But, only rooted callus was observed after raised on MS plus 5 ppm BA for 2-3 months. The successful establishment of cell culture of ginger was done by shaking mother callus in liquid SH medium under 16 hr photoperiod. After showing slightly turbidity of cellculture, the mother callus was removed from the culture which was about 3-4 weeks after cultivation. Initial clumping of cells and microcallus formation were observed in the 2-month old culture and the callus gradually enlarged to diameter of 0.4-0.6 mm within 2 months. Some calli were rooted whereas the other developed a dome-like structure similiary to form shoot (Plate 2).

#### Development of Disease Resistant Screening Technique:

Ginger plantlets obtained from tissue culture were used to test for their somatoclonal variation to resist the pathogen at the early stage either in tissue culture vessel or in transplanting aquarium chamber (Plate 3). Leaf clipping technique using two bacterial concentrations of 0.5 and 0.15 O.D. produced wilt symptom at 10 and 20 days after leaf inoculation while the control treatment inoculated with sterile distilled water showed no symptom (Table 5). Inoculated leaf developed initial wilt symptom then extended to other leaves and finally the whole plantlet died (Plate 3).

Table 1. Immunofluorescent staining (IF) reaction of ginger with strains and other bacteria using antiserum to formaldehyde fixed-cells of P. solanacearum strain LL60

| Bacteria               | Strain | Host/Source    | IF Reaction |
|------------------------|--------|----------------|-------------|
| <u>P. solanacearum</u> | LL60   | Ginger         | ++++        |
|                        | PB21   | Ginger         | ++++        |
|                        | PB71   | Ginger         | ++++        |
|                        | CR02   | Ginger         | ++++        |
|                        | DR11   | Ginger         | ++++        |
|                        | CP40   | Ginger         | ++++        |
|                        | CP61   | Ginger         | ++++        |
|                        | CM31   | Ginger         | ++++        |
|                        | PS-A   | Tomato         | ++++        |
|                        | W5/1   | Potato         | ++          |
|                        | W5/2   | Potato         | +++         |
| <u>P. cepacia</u>      | -      | -              | -           |
| <u>P. fluorescens</u>  | B4     | Sugarcane Soil | +           |
| <u>A. tumefaciens</u>  | -      | -              | -           |
| <u>Bacillus</u> sp.    | -      | -              | -           |
| <u>E. coli</u>         | -      | -              | -           |
| <u>C. michiganense</u> | -      | -              | -           |
| <u>X. campestris</u>   | BR     | Kale           | -           |
| Unknown                | P-10   | Pepper         | -           |

++++ = strong fluorescent, + = weak fluorescent, - = no fluorescent

antiserum dilution of strain LL60 = 1/64,

FITC conjugated antiserum dilution = 1/64

Table 2. Hypersensitive reaction and Pathogenicity of ginger obtained from rhizome and tissue culture of cv. Yuak and Phed inoculated with P. solanacearum strain LL60

| Type           | Cultivar | Pathogenicity <sup>a</sup> | Hypersensitive reaction <sup>b</sup> |
|----------------|----------|----------------------------|--------------------------------------|
| Tissue culture | Yuak     | 1.7                        | Neg                                  |
|                | Phed     | 3.0                        | Neg                                  |
| Rhizome        | Yuak     | 1.7                        | Neg                                  |
|                | Phed     | 0.3                        | Neg                                  |

a = Pathogenicity was conducted by injecting a 100 microliter (mcl) of the pathogen at  $1.6 \times 10^6$  cfu/ml into basal stem by micropipette technique. Pathogenicity was recorded using disease severity scales from 0-3 : 0 = no disease, 1 = wilt of 1-2 lower leaves, 2 = wilt of half plant, 3 = whole plant wilt.

b = HR reaction was carried out by infiltrating a 40 mcl of bacteria at  $1.6 \times 10^{10}$  cfu/ml into the 3 rd and 4 th leaves from the top. The HR reaction was recorded as negative (Neg) and positive (Pos) upto 10 days after inoculation.

Table 3. Electrolyte loss of ginger cell suspension after treated with avirulent and virulent strains of P. solanacearum and other bacteria

| Bacteria | Electrolyte loss (umhos) at |      |      |      |       |       |       |       |
|----------|-----------------------------|------|------|------|-------|-------|-------|-------|
|          | 2 hr                        | 4 hr | 6 hr | 8 hr | 10 hr | 12 hr | 24 hr | 30 hr |
| A        | ND                          | 220  | 110  | 110  | 190   | 150   | 100   | 150   |
| V        | 90                          | 110  | 80   | 50   | 100   | 90    | 70    | 100   |
| F        | 20                          | 70   | 10   | 40   | 100   | 60    | 70    | 70    |
| X        | -130                        | 70   | 50   | 50   | 90    | 50    | 60    | 50    |

A = P. solanacearum (avirulent strain), V = P. solanacearum (virulent strain), F = P. fluorescens, X = X. campestris pv. citri, ND = not determine

Table 4. Population dynamics of bacteria in cell suspension at 10, 12, 24 and 30 hr after inoculation

| Bacteria | Bacterial colony on NGA medium ( $\times 10^7$ cfu/ml) |      |       |       |
|----------|--|------|-------|-------|
|          | 10 hr  | 12 h | 24 hr | 30 hr |
| A        | 1.99   | 2.25 | 1.17  | 1.25  |
| V        | 1.35   | 1.10 | 1.50  | TNC   |
| F        | 0.33   | 0.19 | 1.20  | TNC   |
| X        | 0.18   | 0.15 | 0.59  | 2.10  |

Bacteria were the same as described in Table 3. TNC = too numerous to count.

Table 5. In vitro disease resistant screening of ginger tissue culture plantlets by clipping technique with two bacterial concentrations

| Treatment                              | <u>Disease development after inoculation</u> |       |                |        |                |
|--|--|-------|----------------|--------|----------------|
|  | 0 day  | 5 day | 10 day         | 15 day | 20 day         |
| <u>P. solanacearum</u> PB21, 0.50 O.D. | -  | -     | 1 <sup>a</sup> | 1      | 1              |
| 0.15 O.D.                              | -  | -     | -              | -      | 1 <sup>a</sup> |
| Control (water) 0.00 O.D.              | -  | -     | -              | -      | -              |

a = all treatments showed wilt symptom and P. solanacearum was recovered from the wilt ginger. Each treatment contained 5 replications.

## 6. Conclusion/Remarks:

Biological and serological characterizations of ginger wilt strains collected from ginger growing areas in Thailand demonstrated that the wilt bacteria were P. solanacearum and serological homology. The ginger wilt strains were slightly different in their virulence and biovar. Degrees of virulence varied from mild to severe whereas two biovars of 3 and 4 were found. Antiserum to formaldehyde fixed-cells was high specificity to the pathogen.

Although no HR reaction in intact plantlets, cell culture suspension may be used for HR investigation because electrolyte loss and darkening cytoplasm of cells treated with avirulent strain was significantly high at 4 hr after inoculation. This possibly indicated that incompatible reaction was occurred between avirulent strain and host. If resistant ginger cell culture existed, incompatible reaction of virulent strain and resistant host may be observed by this technique.

In vitro disease resistant screening by leaf clipping of intact plantlets is required for further improvement. Somatic clonal propagation of ginger by tissue culture may naturally induce genetic change from susceptible to resistant. Thus the method may provide a rapid method for mass disease resistant screening. Preliminary study of polypeptide profiles using SDS-PAGE of ginger cultivars inoculated and noninoculated with the pathogen was not significantly different among them. However, several modifications will be done in the next period which may possibly success to detect metabolites relating resistant reaction.

Regeneration of callus cultures from cells culture or bud culture were not achieved at this period, but callus from both sources was rooted on MS plus 5 ppm BA or Liquid SH media after growing for 2-3 months. In liquid SH medium some callus differentiated to form organ similar to shoot. Further

modification of medium recipes will certainly concentrate for production of complete plantlets which may be possible in the near future.

#### 6. Workplan for the Next Period:

Research workplan for the next period will be carried out as in the submitted research activities. The activities of the next research period are summarized as follows:

- a. Collect and screen for field resistant ginger and related species.
- b. Study on host-parasite interactions and hypersensitive reaction.
- c. Develop technique for screening disease resistant ginger.
- d. Manipulate and regenerate resistant ginger plant/cell cultures.

In general, the next period will have research topics similar to this reporting period which are our central research activities. The experiment will be emphasized on regeneration of callus into complete plantlets, manipulation disease resistant callus/cell culture and development of screening technique. Although there were some difficulties concerning regeneration callus into plantlets, the promising results should be obtained in the near future by some modifications. One of the major limiting factors is a slow growth of callus and cell cultures that affect the development of resistant ginger. However, several basic information to help solving problems were carried out parallelly. In conclusion, the research work is slightly behind the schedule especially development of resistant cultivar and screening technique.

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#### 8. Annex/Pictures:

- Plate 1. SDS-PAGE of proteins from infected and noninfected ginger cultivars.
- Plate 2. Cell suspension culture and callus regeneration.
- Plate 3. In vitro screening for disease resistant.

Plate 1. SDS-PAGE of proteins from infected and noninfected ginger cultivars

Lanes from left to right: lane 1, standard molecular weight proteins of 66, 45, 36, 29, 24, 20.1 and 14,2 Kd (from top to bottom); lane 2 and 3, coat protein of papaya ringspot virus (PRV) contained 10 and 15 microliter (mcl) of sample as references, lane 4 to 6, noninoculated ginger cv. Phed contained 5, 10 and 15 mcl of samples, respectively; lane 7 to 9, noninoculated ginger cv. Yuak contained 5, 10, and 15 mcl of samples, respectively; lane 10, inoculated ginger cv. Yuak contained 10 mcl of sample.

160

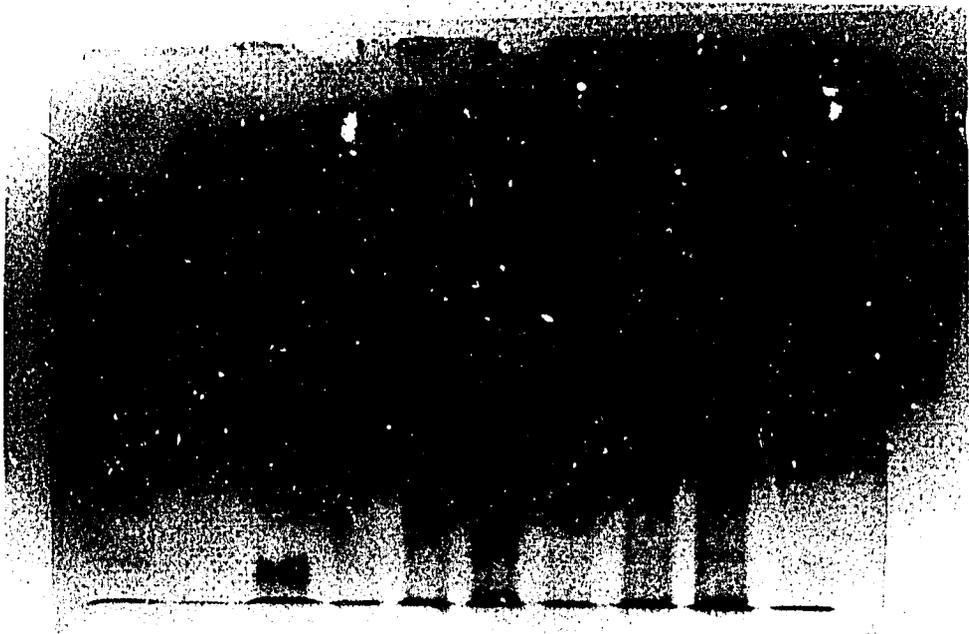


Plate 2. Cell suspension culture and regeneration

- a. Calli development from cell suspension culture after cultivated for 3 months
- b. Enlargement of callus from cell suspension culture showing dome-like structure of shoot
- c. Enlargement of callus from cell suspension culture showing root formation

17a

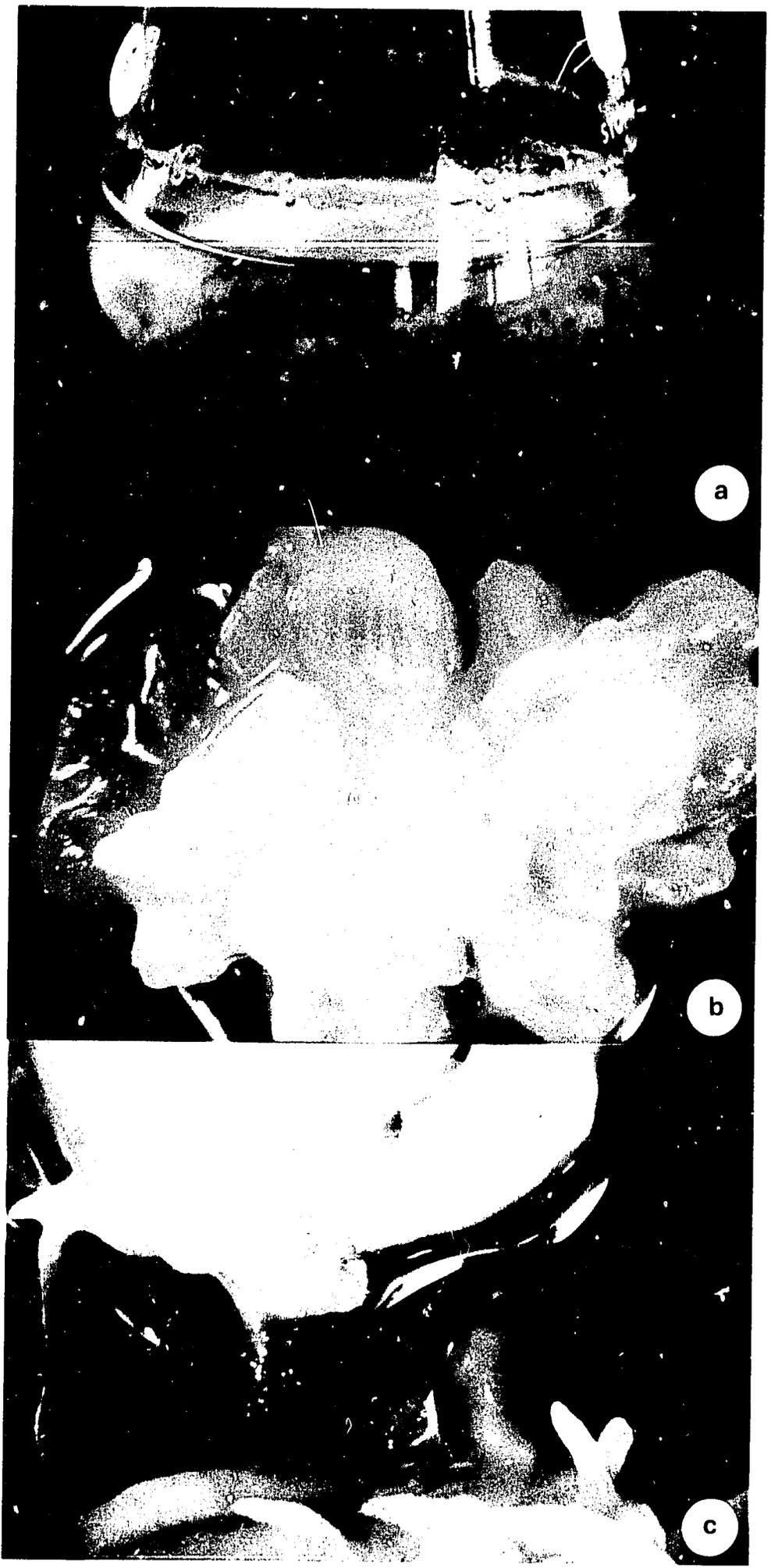


Plate 3. In vitro screening for disease resistant

- a. Aquarium chamber used for transplanting of ginger plantlets before inoculation either by drenching or clipping techniques
- b. Clipping inoculation of ginger plantlet cv. Yuak in tissue culture vessel. Noninoculated showing healthy (right) and wilt disease (left) after 15 days of incubation

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