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

Principal Investigator : Mr. Niphone Thaveechai, Ph.D.

Grantee Institution : Kasetsart University, Thailand.

Collaborator : Mr N.W. Schaad, Ph.D.

Institution : University of Idaho, U.S.A.

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Executive Summary

Several research activities including many supporting topics were employed to meet the ultimate goal. For the first objective on manipulation of genetic improvement for production of resistant ginger, somaclonal variation and induction mutation by chemical (M,N-nitrosoguanidine, MNG) and radiation (Cs^{137}) at various concentrations and doses, respectively, were carried out through tissue culture technology. Ginger tissue culture (TC) plantlets were successfully produced only by multiple shoot induction from ginger bud tissue either on Murashige and Skoog (MS) medium amended with 0.5 $mg.l^{-1}$ NAA and 1.5 $mg.l^{-1}$ BA (modified MS) or Vacin and Went (OR) medium. An average of 5-8 new plantlets/flask was obtained from both media within 45 days of cultivation or it generated for at least $4x10^5$ plants/year. However, modified MS medium provided slightly higher number of plantlets than OR medium but they were less growth and vigor. Therefore, modified MS medium was suitable for clonal propagation and OR medium was chosen to produce TC plantlets for potting in soil. The plantlets were tested for disease-free and maintained for further *in vitro* resistant screening. The disease-free stock also served for producing disease-free rhizomes for immediate use in farmer's field to reduce disease dissemination and infestation into new ginger growing areas. *In vitro* resistant screening which was the first development for bacterial wilt was done for more than 700 plantlets from somaclonal, radiation and chemical treatments. The result showed resistant plant was obtained at 0.73 %, 3.53 % and 2.46 % respectively. Total of ten resistant clones were 4,4 and 2 clones from somaclonal, radiation and MNG treatments, respectively. However, degree of disease resistance of these clones has not yet comparatively evaluated either under greenhouse or field conditions. Therefore, confirmative test of diseasse resistance in the greenhouse and fields should be done before releasing to farmers.

Since plantlets derived from callus culture usually produced a high degree of genetic variation than multiple shoot in several crop species, ginger explant sources and culture media were tested for callus induction. Callus was obtained when grew either basal bud tissues or root tip on Schenk and Hildebrandt (SH) medium+2, 4-D 0.5 $mg.l^{-1}$+kinetin 0.1 $mg.l^{-1}$ and CPA 2.0 $mg.l^{-1}$. It was the first record on callus production of ginger from root tip and basal bud tissue. Callus was also obtained from liquid culture. However, both calli from liquid and solid cultures were difficult and inconsistant to regenerate into complete plantlets. Eventhough a great effort was done by using several modifications and formulations of media, the calli usually produced tremendous rootlets whereas only green spot-like shoot primodia was observed. Only one callus was regenerated into plantlet when SH medium amended with ginger extract. Therefore, plantlets from bud tissues were substantially used in mutagenesis treatments for disease resistant screening.

In regard to the second objective on development of a rapid and reliable method for screening bacterial wilt resistant ginger plantlets and cell cultures by using hypersensitive reaction (HR) and significant metabolic products of disease resistant reaction were carried out. The HR experiment was employed both intact plants and tissue and cell cultures. Information on host-pathogen interactions

was investigated emphasizing on compatible (disease development) and incompatible (resistance or HR) reactions. Although some difficulties were involved such as technique for HR infiltration, collection of HR sample and lack of a highly resistant ginger cultivar, several parameters for detection of HR were successfully obtained by using electrolyte loss, biological staining and scanning electronmicroscopy to compare HR reactions of moderate resistant of cultivars (cv) Phed (GH) or TaDang (GT) with susceptible cultivar Yuak (GY). However,these HR reactions were not adequate for direct using as a rapid or mass screening for disease resistance. Further study in details of mechanism of electrolyte leakage, elicitor for induction of HR and detection and isolation of metabolic products during HR development may be valuable to develop the resistant screening method. In particular, enzymes and secondary metabolites associated with resistant or HR expression of the host to the pathogen infection were reported. Polyacrylamide gel electrophoresis of peroxidase and esterase isozymes was done in cv GH and cv GY inoculated with virulent and avirulent strains of *P. solanacearum* at different postinoculation periods. It showed no significant difference of these isozymes pattern between two gingers. However, isozymes pattern of the two enzymes was different among members of the Zingiberaceae that can be valuably used for taxonomic identification and classification of related species. In addition, phenylalanine ammonia-lyase (PAL), the key enzyme of shikimic acid pathway involving phytoalexin or HR productions during fungus or virus infection, markedly increased its activity in incompatible reaction at 3-6 hr postinoculation as in fungus or virus disease. This was the first record in bacterial disease especially bacterial wilt caused by *P. solanacearum*. However,the coincide increasing PAL activity in resistant cultivar may need to reconfirm since we found that in incompatible reaction of tomato resistant cultivars aganist *P. solanacearum* was not always higher than in compatible reaction. In accordance with instability of PAL activity during spectrophotometry measurement at 290 nm, further modification or establishment of PAL assay and isolation with highly sensitive tool is a key for successful development a rapid resistant screening method. Preliminary detection of PAL enzyme in incompatible reaction of tomato resistant cultivars with *Xanthomonas campestris* pv. *vesicatoria* by HPLC at Harris Moran Seed Co with Dr. N.W. Schaad, reseach collaborator, indicated that HPLC was a sensitive tool to detect PAL enzyme and can be used for purification also. However, using of HPLC for detection and purification of PAL activity and products were not successful because of several constraints such as lack of highly resistant ginger to provide a strong incompatible singnal, standard protocol to obtain reliable results and technical support. An alternative method for detection PAL activity by hybridization cDNA probe of PAL or chalcone synthase (CHS), the related enzyme of HR reaction was proposed. Plasmids pPAL and pCHS1 obtained from Dr.C.J. Lamb of Salk Institute for Biological Studies,San Diego, U.S.A. were used for cDNA probe by nick translation labeling with non-radioisotope. However, there was no hybridization in incompatible reaction of ginger and tomato. Therefore, eluciation of PAL activity or mechanism involving incompatible reaction is necessary required as a key factor for development of a rapid and reliable resistant screening using serological detection of HR products.

Research Objectives

Ginger, one of the earliest important spices, is a native of Southeast Asia. Presently, ginger is cultivated worldwide for multipurpose uses on a commercial scale in India, Jamica, Australia, The United States, Sierra Leone, Nigeria, Brazil, China, Japan, Indonesia and Thailand (Annonemous, 1980). However, cultivation of ginger in these countries frequently faces a disease problem especially bacterial wilt caused by *Pseudomonas solanacearum* which is considered as the most serious disease of ginger because of a great yield reduction and a wide dissemination (Chantaraotan, 1982; Hayward *et al*, 1967; Quinon *et al*, 1964). Although substantial losses of ginger due to *P. solanacearum*, development of appropriate control measures is less available. Therefore, new approaches of an effective and reliable control method are proposed in this study. The specific goals of the project are emphasized on manipulation of genetic improvement of ginger for disease resistance through plant and cell culture techniques and development of a rapid and reliable method for screening disease resistant of ginger either from intact or *in vitro* cultured plants.

Breeding for disease resistance using conventional method and pathogenicity test for screening is time and labor consuming. Plant cell culture technology offers an alternative aspect to overcome the problems (Evans *et al*, 1983). However, little information on ginger plant cell cultures is available except clonal propagation from ginger buds has been reported (Hosoki and Sugawa, 1977). Somaclonal variation (Larkin and Scowcroft, 1981) has capability to produce disease resistance in several crops such as sugarcane (Heinz *et al*, 1977) and potato (Secor and Sharp, 1981). In addition, genetic variation in treated cultures of callus, cell suspension and protoplast with chemicals and radiation has become a new and useful source for new genetic traits such as disease resistance (Reisch, 1983). Therefore, plant cell cultures as proposed will not only resolving breeding for resistance problems but also offer a rapid method for propagating disease-free ginger.

The hypersensitive reaction is an incompatible reaction occurred when plant respond to infection by fungal (Stakman, 1915), viral (Holmes, 1929) and bacterial (Klement and Goodman, 1967) pathogens. The HR symptom induced by pathogens is known to be one of disease resistant reaction of crop plants and usually associated with localizing pathogens near the entry port and developing necrosis of the affected plant tissues within 24-48 hr. The HR technique is now accepted for screening disease resistant to pathogen in callus culture (Miller and Maxwell, 1983; Tschen and Chang, 1982). In intact plant, HR has been used in bacterial disease much less than fungal and viral diseases. Therefore, the approach of using HR for a rapid screening of bacterial disease resistance of intact and cell culture has not exploited and remains to be proven.

Based on the facts and due to the urgent need to develop an effective and simplified method for control of bacterial wilt of ginger caused by *P. solanacearum*, disease-free ginger for immediate use and disease resistant ginger for long-term use might be considered

as a promising control measure. Therefore, if this proposes success, it will be easily employed by LDC people and may be implicated to other crops having the same pathogen with less additional input. In addition, the technique may be logically applied for the control of other soilborne bacteria such as soft rot pathogens at the commercial level. Furthermore, it would certainly be useful to LDCs and Thailand for increasing crop production while reducing pesticide utilization which cause less damage to the environment and mankind.

Methods and Results

1. Characterization of bacterial wilt pathogen and resistant ginger

Surveying and collecting of bacterial wilt pathogen, ginger and ginger related species were carried out in major ginger growing areas of Thailand. Isolation and identification of *P. solanacearum* were performed according to Schaad (1988) and biovar was determined by method of Hayward (1964) based on utilization of lactose, maltose, cellobiose, mannitol, sorbitol and dulcitol. Micropipette tip inoculation technique of Chantaraotan (1983) was used for pathogenicity and virulence tests of the strain whereas hypersensitive response (HR) test was followed the method of Klement (1963) by infiltration with a high concentration of bacterial suspension (>10^8 cfu/ml) into leaves of ginger and other hosts before observed for necrotic symptom development. Antiserum and serological characterization of the pathogen was employed according to Thaveechai and Schaad (1986). Evaluation of ginger and ginger related species for disease resistance was carried out using average disease indices of Winstead and Kelman (1952) having scale of resistance as susceptible (S=1-29 % surviral), moderate susceptible (MS=30-59 % survival), moderate resistant (MR=60-79 % survival) and resistant (R=80-100 % survival).

A total of 24 strains isolating from infected ginger in Thailand was *P. solanacearum* which was consisted of 15 strains of biovar 3 and 9 strains of biovar 4 (Table 1). Virulence of strains based on pathogenic reaction on ginger cv Yuak (GY) and tomato cv Sida showed a different degree of virulence varied from high (H), moderate (M), low (L) and avirulent (O) after inoculation 7-10 days (Table 1). These strains caused a severe wilting disease on ginger more than tomato. Virulence has no correlation with biovar and geographical distribution of strains (Table 1). However, all strains were serologically homologous or same serovar by CDD and DIBA test with antiserum to membrane protein complex (MPC) of strain LL60. Sensitivity of DIBA for detection of *P. solanacearum* was 300 cfu/3ul. However, there was some cross reaction with *P. syringae* pv. *tomato* whereas no cross reaction with *X. campestris* pv. *oryzae* and *P. fluorescens* was observed (Fig 1).

Several ginger cultivars and ginger related species were almost susceptible to bacterial wilt. Only cv GT and cv GH were moderately resistant in which they were small rhizome-type and contained more fiber which was not suitable for salted and pickle ginger factories for export. For ginger related species, most of them were susceptible showing typical wilt symptom as in ginger after

artificial inoculation with these strains. Turmaric (*Curcuma domestica*) and Krachaai (*Boesenbergia pandurata*) were the most susceptible whereas the moderate susceptible were wild ginger (*Z. zerumbet*) and Plai (*Z. cassumunar*). Only some strains can induce HR on ginger and Plai whereas the other hosts were no HR development. Recently, natural infection of *P. solanacearum* in addition to ginger was observed on Krachaai, Turmaric and Patumma (*Curcuma alismatifolia*), a new crop for cutting flower.

2. Investigation on host-pathogen interactions

Interaction of ginger and *P. solanacearum* especially HR was investigated in intact plant, cell and callus cultures. The HR development of intact plant was carried out according to Klement (1963) using virulent and avirulent strains of *P. solanacearum* and ginger cultivars GY, GT, GH and GA (Australian ginger) including ginger related species whereas tobacco was used as control. Restricted necrotic symptom at inoculation site was recorded after 24-48 hr of inoculation. Enzymatic changes during HR development in cv GY and cv GT or cv GH was followed the method of Zimmermann and Hahlbrock (1975) for phenylalanine ammonia-lyase (PAL), esterase and peroxidase isozymes were detected by staining polyacrylamide gel (PAGE) of Laemmli (1970) with substrate solution as deseribed by Thom and Meretzki (1970). The HR reaction in cell and callus cultures was used the method of Atkinson *et al* (1985) to compare the reaction of incompatible and compatible by inoculating susceptible (cv GY) and moderate resistant (cv GH or cv GT) with virulent and avirulent strains of *P. solanacearum* including bacterial plant pathogen (*X. campestris* pv. *oryzae*) and soil saprophyte (*P. fluorescens*). Scanning electron microscope (SEM) was used to study the interaction in callus culture after inoculated with 5 ul of 2 x 10^8 cfu/ml of bacterial suspension for 60 hr.

Electrolyte leakage and cell death were compared between compatible and incompatible reaction in cell culture of cv GY (susceptible) and cv GT or GH (moderate resistant) by inoculated each culture with 2 x 10^8 cfu/ml of bacterial suspension at 5 ml/50 ml cell culture. The reaction was determined at 1.50 hr interval until 12 hr after inoculation. Vital stain with 0.05 % trypan blue was used for detecting cell death showing blue color in cytoplasm after staining for 5-10 min.

Using *P. solanacearum* strain PB71 to infiltrate tested plant leaves showed HR symptom only in tobacco, but HR-like symptom was observed in ginger cv GT and cv GH by showing restricted chlorosis at infiltration site 4-10 days after inoculation and following plant dead within 15-20 days. In ginger cv GY and cv GA, chlorosis symptom developed more faster and plant dead was observed within 15 days (Fig 2A). It indicated that cv GH and cv GT had higher degree of resistance than cv GY and GA and slow development of leaf chlorosis may be used for evaluation of resistance. In addition, bacterial growth on cv GH and cv GT was lower than on cv GY and cv GA either in leaf or callus tissues. Therefore, it may support the conclusion that ginger cv GH and cv GT was more resistant than cv GY and cv GA. However,degree of resistance of both cultivars was still at a moderate level.

Study HR in callus demonstrated that incompatible (AV and Pf) reaction induced more intense necrosis of callus tissue than in compatible (V) but it was no different in cv GT and cv GY (Fig 2B). For SEM investigation, there was more fibrous material and cell attachment in incompatible (avirulent) reaction (Fig 3D) than compatible (virulent) reaction (Fig 3C) and it was greater in cv GH than cv GY. Cell death of cv GY and cv GT was also higher in incompatible than in compatible reaction at 6 hr after inoculation (Fig 4A) whereas conductivity was different in cv GY but not in cv GT (Fig 4B).

Detection of enzymatic activity of esterase and peroxidase in cv GT and cv GY on PAGE revealed no differences between avirulent and virulent treatments at each time interval after inoculation except some sightly different of esterase in cv TaDang (Fig 5). However, these isozyme patterns among ginger and ginger related species were greatly different (data not show) in which it may be useful for taxonomy and classification of plants in Zingiberaceae. Incompatible (inoculated with avirulent) reaction provided maximum PAL activity greater than in compatible (inoculated with virulent) reaction for both cv GY and cv GT at 3-6 hr after inoculation (Fig 6). However, PAL activity was greater in cv GT than in cv GY at 6 hr post inoculation. Therefore, PAL activity may possibly involve in resistant reaction and may useful for screening disease resistance.

3. Development of ginger cultures and regeneration

The major activity was to develop *in vitro* production and screening ginger tissue culture (TC) plantlets for bacterial wilt resistant ginger. The schematic work plan for *in vitro* production resistant TC was summarized in Fig 7.

a. Production of disease-free ginger plantlets

Somaclonal propagation of disease-free ginger was performed by modified method of Hosoki and Sagawa (1977), Vacin and Went (1949), and Murashige and Skoog (1962). Various plant growth regulators were amended solely or together with others in basal medium such as 6-benzylaminopurine (BA), 1-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2,4-D), p-chlorophenoxyacetic acid (CPA) 6-furfurylamino purine (kinetin), 6-(4 hydroxy-3 methylbut-2-enylamino) purine.

Healthy rhizomes cultivars GY, GT, GH and GA showing no sign of pathogens infection were selected for further treatment with dry heat at 38 C for 4-8 weeks to eliminate virus especially ginger chlorotic fleck virus (Buchen-Osmond *et al*, 1988) that may latent associated with rhizome. After bud germination, the bud tissues were excised and surface sterilized with 0.5 % sodium hypochlorite at 10 % plus 0.1 % Tween 20 for 15-20 min then washed twice with sterile distilled water (SDW). Outer bud scales were removed and placed on the tested media. Other ginger explant tissues of disease-free clones such as root, leaf and pseudostem were also included in the experiment. Ginger cultures were incubated in tissue culture room equiped with fluorescent light. Numbers and growth performance of plantlets were recorded and compared for efficiency of the media. All TC plantlets were visual observed for incidence of pathogens infection and

plantlets from healthy clones were intensively checked for bacterial wilt infection by DIBA or ELISA, virus by visual and electron microscope observations and nematode and fungus by specific staining for light microscope observation. Disease-free clones were routinely propagated as sources for production of disease-free rhizomes and *in vitro* induction mutation and screening for disease resistance.

b. Production of ginger callus and cell cultures and regeneration

Callus culture of ginger cultivars was established by using MS and SH media plus 3 % sucrose as basal medium and amended with growth regulators such as NAA, BA, 2, 4-D, CPA, zeatin and kinetin for comparision of callus development and regeneration into complete plantlets. For production of cell culture, friable callus was cultured in liquid SH plus 3 % sucrose and 0.5 mg. l^{-1} 2,4-D, 2.0 mg. l^{-1} CPA and 0.1 mg.l^{-1} kinetin for 2-3 months before regeneration. In regard to regeneration, compact callus tissues were grow on SH amended either with several plant extracts such as ginger, tomato and coconut water or growth requlators under dark or light conditions whereas cell culture was performed in liquid SH plus 3 % sucrose amened with NAA and zeatin at two combinations for 2 weeks before subcultured to regenerate in liquid SH plus 3 % sucrose for 1-2 months.

c. Production of genetic mutant ginger for disease resistance

Genetic mutant gingers were obtained from somaclonal variation and induce mutation by radiation and chemical. In case of radiation, basal bud tissue (3x3x3 mm^3) and basal stalk of plantlet were treated with Cs^{137} at 500, 1,000, 1,500, 2,000, 2,500 and 3,000 rad. Basal bud tissues were cultured on SH amended with 0, 0.1, 0.5 and 1 mg.l^{-1} 2,4-D whereas basal stalks of plantlet were grown on OR medium for 2 months for differentiation. Each treatment consisted of 3 pieces of each explant. In regard to chemical induction, basal bud tissue was soaked in solution of N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNG) at 0, 100 and 200 mg.l^{-1} for 1, 3 and 6 hr then washed in SDW before cultured on SH plus 0.1 mg.l^{-1} kinetin. Each treatment consisted of 10 pieces of explant of ginger cv GY and cv GH.

The TC plantlets of each treatment were clonal propagated and *in vitro* screening for disease resistance by leaf-clipping inoculation technique. A sterile scissors was dipped in a suspension of *P. solanacearum* strain PB21 at A_{600} = 0.12 containing approximately 10^8 cfu/ml and cut ginger leaf blade at about 30-45 degree angle inward until closing to the midrib. The inoculated TC plantlets in the vessel were observed for wilt symptom development for 5 days after inoculation. Most susceptible plantlets died or showed yellowing on uninoculated leaves within 5 days whereas the resistant plantlets showed only yellowing area of 4-5 cm from inoculated site. The resistant plantlets were removed and excised stalk at 1 cm above the collar for pathogen isolation on selective SM-1 medium (Granada and Sequeira, 1983). Plantlets showed no pathogen infection were propagated for further greenhouse and field tests to confirm degree of disease resistance.

Disease-free TC plantlets were successfully obtained from bud tissues of ginger cv GY, cv GA and cv GT grown on OR medium or

modified MS medium which was better than on Hosoki and Sakawa medium (Fig 8A). In addition, SH medium was also good for clonal propagation of ginger. However OR medium provided the most preference plantlets with rapid growth, high vigor and shoot proliferation (Table 7). Callus culture was successful produced from bud, basal bud tissue and root tip of ginger on SH and modified SH (Fig 8B) whereas other explants were died after cultured for 8 weeks (Table 3). Regeneration of callus on solid medium into complete plantlet was obtained only one plantlet from SH medium amended with ginger extract (Fig 9A) whereas vigorus roots were found from most of testing media. Cell culture was sucessful induced into callus in liquid medium (Fig 9B,2) but regeneration into plantlet was not obtained. However, rootlets and dome-like leaf primodia were observed in the liquid liquid culture (Fig 9B,3).

Development of *in vitro* screening for bacterial wilt resistant ginger by leaf-clipping was the first establishment. The method was rapid and reliable for screening intact ginger plantlets. For a rapid mass screening, inoculation of ginger plantlets growing in an aquarium chamber containing 50-60 plantlets is more convenient than in tissue culture vessel. Genetic variation according to somaclonal propagation, radiation and chemical was found in ginger. Total of 10 resistant clones obtained from 740 plantlets were 4, 4, 2 clones from somaclonal, radiation and chemical treatments, respectively (Table 4). However, these clones were relatively low to moderate resistant. Therefore, improvement of resistant of these clones by retreated with those methods may be possible. Chemical treament provided the most highest percentage of resistance in which the optimum treatment was at 200 $mg.l^{-1}$ MNG for 1 hr or 100 $mg.l^{-1}$ MNG for 3 hr. The optimum dose of radiation treatment to obtain good resistant was 2000-2500 rad.

4. <u>Production of disease-free ginger rhizomes under greenhouse and concrete block conditions</u>

Ginger TC plantlets that proved to be disease-free and resistance to bacterial wilt were clonal propagated on SH medium (Schenk and Hildebrandt, 1972) and subcultured on OR medium (Vacin and Went, 1949) before transplanting TC plantlets into soil for potting in "plastic bag" under greenhouse. After 10-12 months TC gingers were harvested for disease-free rhizomes which was designated as TCR-1. The TCR-1 rhizomes were checked for bacteria and virus infections as described previously before growing in methyl bromide fumigated soil in 8-10" clay pot for 10 months to produce TCR-2 rhizomes for yield evaluation in concrete block. Ginger TCR-3 rhizomes rhizomes derived from the block were collected and statistically analysed their yields. After checking for the infections, selected clones of disease-free TCR-3 rhizomes were kept as stock to produce disease-free rhizomes either for commercial production or for field evaluation of growth performance and disease incidence by farmers. If possible, the farmer rhizomes (FR) may need to test for 3 consecutive years to observe percentage of reinfection of the wilt disease which will valuable support for establishment of effective integration disease control.

Survival of TC plantlets in greenhouse (Fig 10A) was about 70-80 % and its TCR-1 rhizome yield was approximately 200-300 g/pot

(4-6" diameter) (Fig 10B) whereas TCR-1 rhizomes showed 100 % survival and provided an average fresh weight of TCR-2 rhizome at about 800-1000 g/pot (8-10" diameter) (Fig 10C). All of TCR_3 rhizomes were free from bacteria and virus infections. Production of disease-free TCR-3 rhizomes of cv GY and cv GA in concrete block (Fig 11A) in 1994 was not significantly different (Table 5). An average net yield of each plant was 1.11 kg for cv GY and 1.22 kg for cv GA (Fig 11B). However, the conversion ratio of rhizome of cv GA and cv GY was 44.14 and 26.56 times, respectively in which cv GA was approximately 1.66 times higher than cv GY. The TCR-3 rhizomes of some selected clones (Fig 11C) were multiplied for further growth performance test in farmer field next year.

Table 1. Biological characteristics of *Pseudomonas solanacearum*, the causal agent of bacterial wilt of ginger (*Zingiber officinale*) in Thailand.

Location	Strain No.	Biovar[a/]	Virulence[b/]	
			Ginger	Tomato
Central				
Bangkok	PT-11	3	M	L
North				
Chiang Mai	CM-31, CM-51	4	H	M
	CM-52	3	L	L
	CM-61	3	M	M
Chiang Rai	CR-02, CR-11	3	H	H
	CR-10	3	M	M
Phetchabun	PB-23, PB-72	4	H	M
	PB-21	4	M	L
	PB-24	4	L	L
	PB-70,PB-71	3	H	H
	PB-73	3	H	L
	PB-22	3	M	L
	PB-26	3	L	L
Northeast				
Loei	LL-60	3	H	H
	LL-61	3	H	M
	LL-62	3	M	M
South				
Chumphon	CP-40	4	H	H
	CP-60	4	H	M
	CP-80	3	M	L
	CP-81	3	L	O

a/ Biovar was determined according to Hayward (1964) on the utilization of lactose, maltose, cellobiose, manitol, sorbitol and dulcitol.

b/ Virulence was categorized by average disease indices of ginger or tomato plant after 7-10 days postinoculation. H=high, M=moderate, L=low and O=avirulent.

Table 2. *In vitro* production of disease-free ginger plantlets on various culture media amended with plant hormones

Medium1/	Hormone (mg.l-1) NAA	BA	No. of plantlets2/	Growth3/	Plantlets' performance
MS	0.5	1.5	10-15	+++	Small, succulent, prolific shoot
MS	1.0	1.0	10-14	+++	Small, succulent, prolific shoot
OR	-	-	10-12	+++	Large, high vigor, prolific shoot
SH	-	-	10-15	+++	Large, succulent, prolific shoot

1/ MS = Murashige and Skoog (1962); OR = Vacin and Went (1949) and SH = Schenk and Hildebrandt (1972)
2/ average from 5 replications
3/ - = no growth, + = poor, ++ = moderate and +++ = good performance

Table 3. Effect of culture media on callus formation of ginger explant sources

Explant sources	Number of callus formation a on			
	MS	OR	SH	modified SH
Bud	0 b	2	20	20
Basal bud tissue	0	0	20	20
Root tip	ND c	ND	ND	20
Inner leaf sheath	Die	Die	Die	Die
Outer leaf sheath	Die	Die	Die	Die
Leaf blade	Die	Die	Die	Die

a/ Twenty pieces of each explant source were tested in each medium; SH = Schenk and Hildebrandt (1972), modified SH = SH+0.5 mg.l-1 2,4-D, 2.0 mg.l-1 CPA and 0.1 mg.l-1 kinetin, MS = Murashige and Skoog (1962), OR = Vacin and Went (1949)
b/ No callus produced but tissue developed either into root, shoot or complete plantlets.
c/ Not determine

Table 4. *In vitro* screening of genetic mutant ginger for bacterial wilt resistant in ginger cultivar Yuak

Induction method	Tested clones	Resistant clones (%)
Somaclonal variation	546	4 (0.73)
Chemical (Nitrosoguanidine)	113	4 (3.53)
Radiation (Cesium 137)	81	2 (2.46)
Total	740	10

Table 5. Comparative yield production of TCR-3 rhizome of ginger cultivars Yuak (GY) and Australia (GA) in concrete block for 10 months at Kamphaengsaen campus. Kasetsart University, Nakhon Pathom.

Ginger	Seed rhizome(kg)		Net yeild1/ of TCR rhizome(kg)						Conversion
	One piece	Total Use	Rep1	Rep2	Rep3	Rep4	Total	Average	ratio
GY	0.04	3.35	26.0	20.0	20.0	23.0	89.0a2/	1.11	26.56
GA	0.02	2.22	32.0	18.0	12.0	20.0	98.0a	1.22	44.14

1/ Yield of rhizome was obtained from 20 plants/replication and one plant was seeded with 0.02 kg and 0.04 kg of seed rhizome of cv GA and cv GY, respectively.

2/ Yields followed by the same letter are not significantly different ($P=0.05$) by DMRT

Fig. 1. Specificity and sensitivity of MPC antiserum of *Pseudomonas solanacearum* strain LL60 for detection the pathogen strains and other bacteria by dot-immunobinding assay. Bacterial tested were *P. solanacearum* strains from Phetchabun (PB), Loei (LL), Chiang Rai (CR) and Chumphon (CP) with virulent (V) and avilulent (av)characters. The other plant pathogenic bacteria were *P.syringae* pv.*tomato* (Ps. tomato) and *X. campestris* pv.*oryzae* (Xco) whereas soil saprophyte was *P. fluorescens* (Pf).

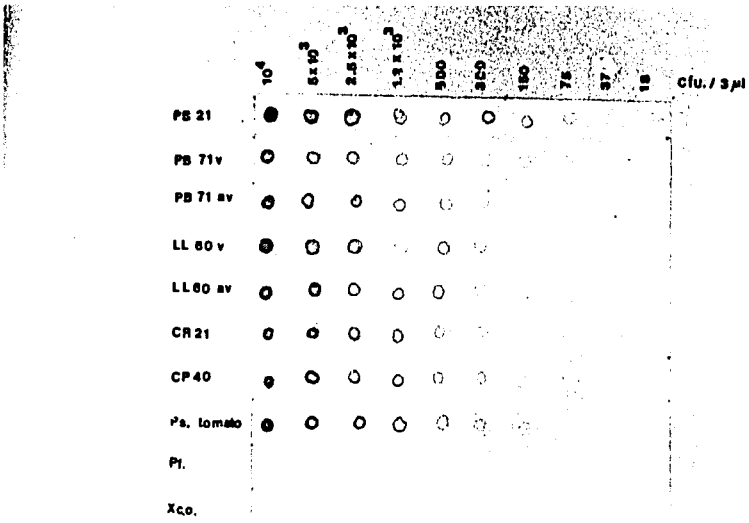

10^4
5×10^3
2.5×10^3
1.3×10^3
500
250
150
75
37
18
cfu. / 3 µl
PS 21
PB 71v
PB 71 av
LL 60 v
LL60 av
CR 21
CP 40
Ps. tomato
Pf.
Xco.

Fig. 2. Hypersensitive response (HR) of intact plant (A) and callus culture (B) of ginger cultivars Yuak (GY), Phed (GH) and TaDang (GT)>. HR-like symptom was developed on GH and GT but chlorosis of typical wilt symptom occurred on GY after infiltration leaves of each cultivar with virulent strain of *Pseudomonas solanacearum* for 4-10 days. Brown or dark-brown color of callus tissue developed on both cultivars (GY and GH) when inoculated with incompatible bacteria; avirulent (AV) *P. solanacearum*, *P. fluorescens* (P.f) and *Xanthomonas campestris* pv. *oryzae* (X.c.o) whereas light brown was observed when inoculated with compatible virulent (v) strain of *P. solanacearum* and creamy white or yellow was occurred in treatment controls with autoclaved virulent (CV) and water (H_2O).

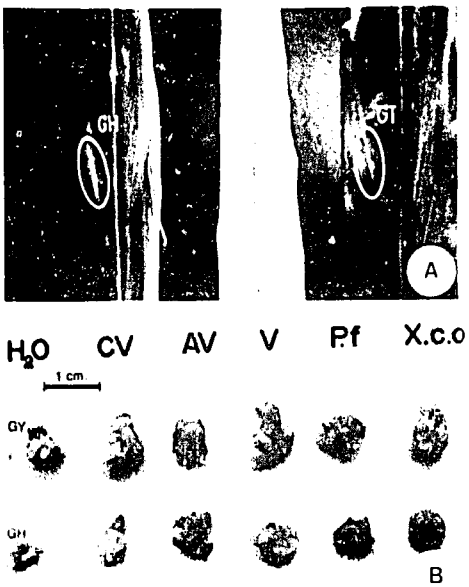

GH
GT
A
H_2O
CV
AV
V
P.f
X.c.o
1 cm
GY
GH
B

Fig. 3. Scaning electron micrograph of callus cells of ginger cultivar Phed (GH) inoculated with water (A), autoclaved cells (B), virulent (C) and avirulent (D) of *Pseudomonas solanacearum* with *P.fluorescens* (E) and *Xanthomonas campestris* pv. *oryzae* (F). Incompatible reaction (D) showed more bacterial cells attachment and fibrous material than in compatible (C) reaction.

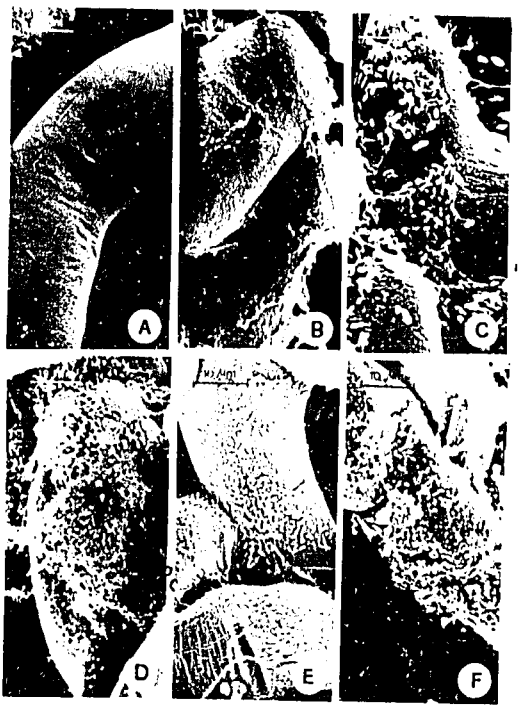

A
B
C
D
E
F

Fig. 4. Time course study of cell death (A) and electrolyte leakage (B) of susceptible cultivar Yuak (GY) and moderate resistant cultivar TaDang (GT) after inoculation with virulent (compatible) and avirulent (incompatible) strains of *Pseudomonas solanacearum*. A significant different of cell death and electrolyte leakage was observed between incompatible and compatible reaction in GY but only cell death was different in GT.

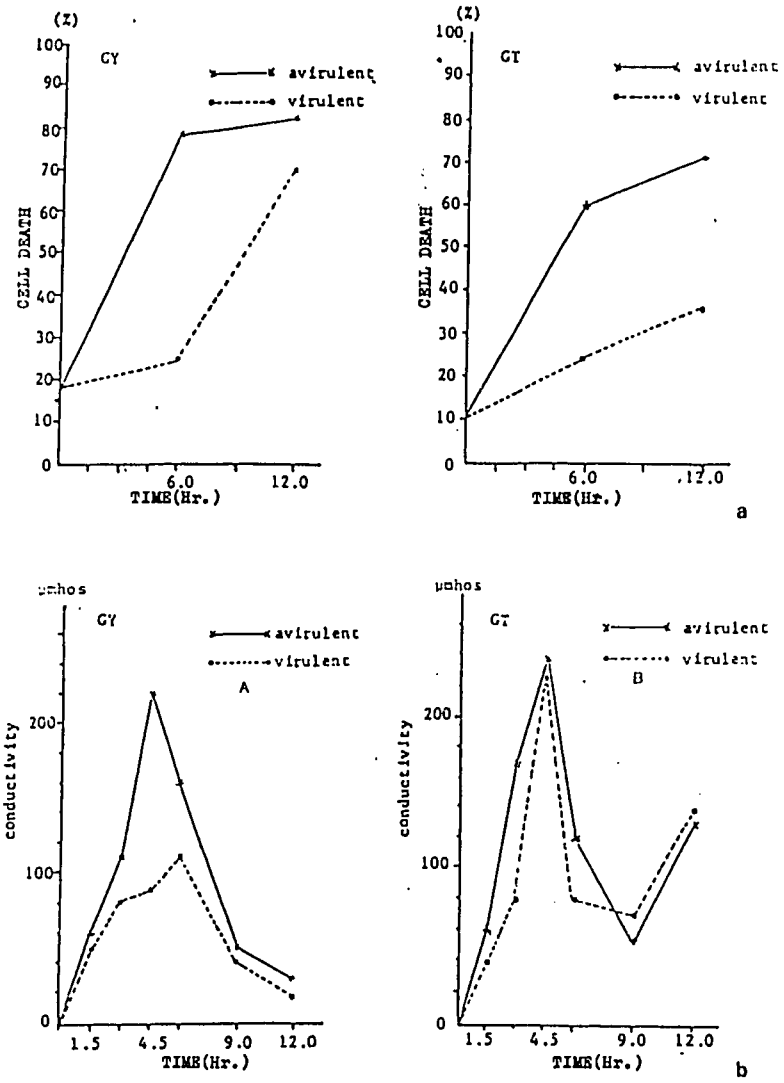

(%)
GY
avirulent
virulent
CELL DEATH
TIME(Hr.)
(%)
GT
avirulent
virulent
CELL DEATH
TIME(Hr.)
a
μmhos
GY
avirulent
virulent
A
conductivity
TIME(Hr.)
μmhos
GT
avirulent
virulent
B
conductivity
TIME(Hr.)
b

Fig. 5. Esterase isozyme pattern of ginger cultivars TaDang (A) and Yuak (B) after treated with avirulent (incompatible) and virulent (compatible) strains of *Pseudomonas solanacearum* at various time intervals. There was no different in an isozyme patterns at each time course of avirulent and virulent treatments in cv TaDang. But in cv Yuak avirulent strain induced esterase enzyme slightly higher than virulent strain at 12-72 hr after inoculation.

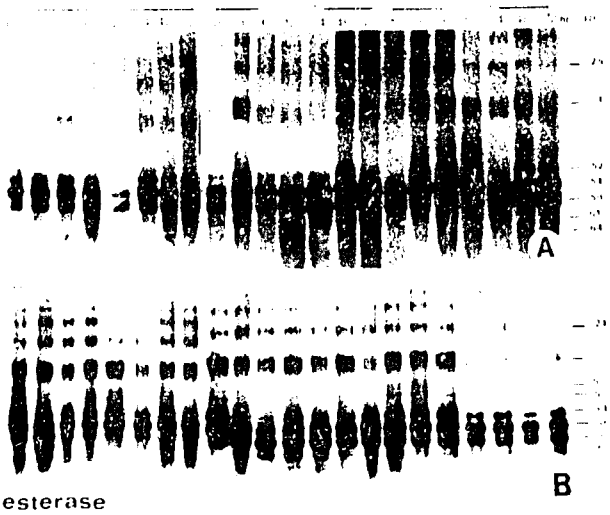
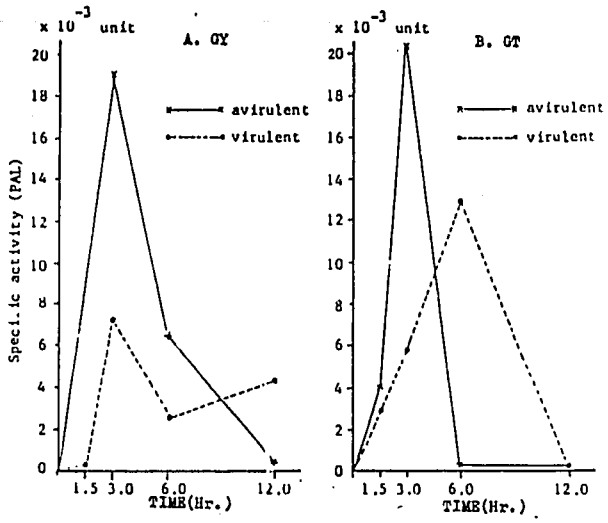

A
B
esterase

Fig. 6. Phenylalanine ammonia-lyase (PAL) activity of ginger cultivar Yuak (GY) and TaDang (GY) after infiltration ginger leaves with avirulent and virulent strains of *Pseudomonas solanacearum* showing a great induction of PAL in incompatible (avirulent) than in compatible (virulent) reaction for both ginger cultivars. But the activity in compatible reaction of cv GT was higher than cv GY at 6 hr after inoculation.

Fig. 7. Schematic diagram for *in vitro* production of ginger cultures for disease-free and disease resistant plantlet through induction mutation and somaclonal variation.



In vitro Production and Screening for Bacterial wilt Resistant in Ginger

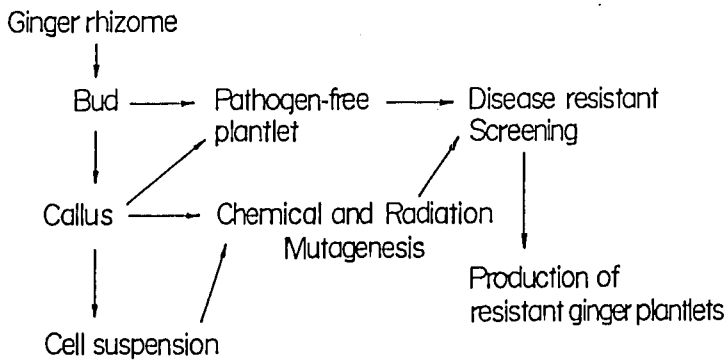


Fig. 8. Clonal multiplication from bud and callus cultures from various explants of ginger for production of disease-free and screening resistant plantlets. A, Production of disease-free plantlets from bud tissue growing on MS medium amended with 3 % sucrose, 0.5 $mg.l^{-1}$ NAA and 1.5 $mg.l^{-1}$ BA for 15(2), 25(3) and 45(4) days. B, Callus cultures from young leaves (1), leaf sheath (2), basal bud tissue (3) and root tip (4) on SH medium amended with 3 % sucrose, 0.5 $mg.l^{-1}$ 2,4-D, 0.1 $mg.l^{-1}$ kinetin and 2.0 $mg.l^{-1}$ CPA for 45 days which was successful only from basal bud (3) and root tip (4). The media were MS (Murashige and Skoog, 1962) and SH (Schenk and Hildebrandt, 1972) and growth regulators were 2,4-D (2,4-dichlorophenoxy acetic acid), NAA (1-naphthaleneacetic acid), BA (6-benzaladenine), kinetin (6-furfurylamino purine) and CPA (p-chlorophenoxyacetic acid)

19a

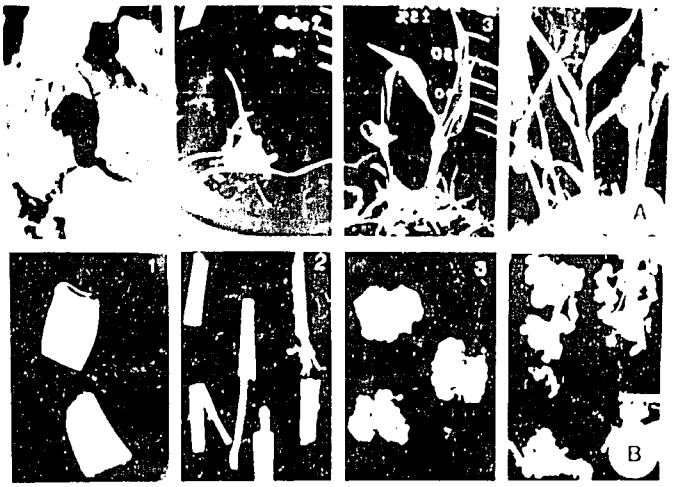


Fig. 9. Regeneration of callus and cell cultures into plantlets. A, Callus culture on solid medium SH (Schenk and Hildebrandt, 1972) amended with 3 % sucrose and 5 $g.l^{-1}$ ginger extract (left)regenerated into complete plantlets (right). B, Cell culture, on liquid SH amended with 3 % sucrose, 0.5 $mg.l^{-1}$ 2,4D, 0.1 $mg.l^{-1}$ kinetin and 2.0 $mg.l^{-1}$ CPA (1) for 4(1) and 8(2) weeks for callus induction and regeneration on SH amended with 3 % sucrose, 0.5 $mg.l^{-1}$ NAA, 1 $mg.l^{-1}$ zeatin for 2 weeks before subcultured on hormone free SH for 6-8 weeks showing roots and dome-like leaf primodia (3). Abbreviations: 2,4-D=2,4-dichlorophenoxyacetic acid, CPA=p-chlorophenoxyacetic acid, NAA=1-naphthaleneacetic acid, zeatin=6=(4 hydroxy-3 methylbut-2 enylamino)-purine.

20w

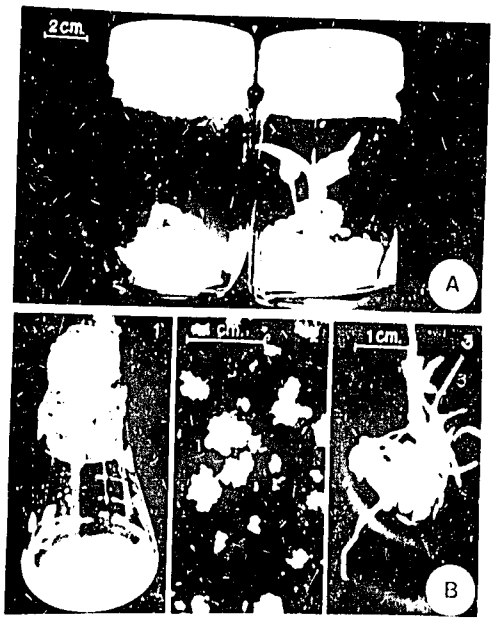


Fig. 10. Cultivation of ginger TC plantlets, TCR-1 rhizomes and production of TCR_2 rhizomes under greenhouse condition. A, Pots of TC plantlets (foreground) at about one month after transplanting and TCR-1 pots (background) growing nearly 7 months. B, Rhizomes of TC plantlets (TCR-1) from growing in plastic bag for 10 months. C, TCR-2 rhizomes obtained from TCR-1 rhizomes growing for 10-12 months in 8-10" clay pots.

21a

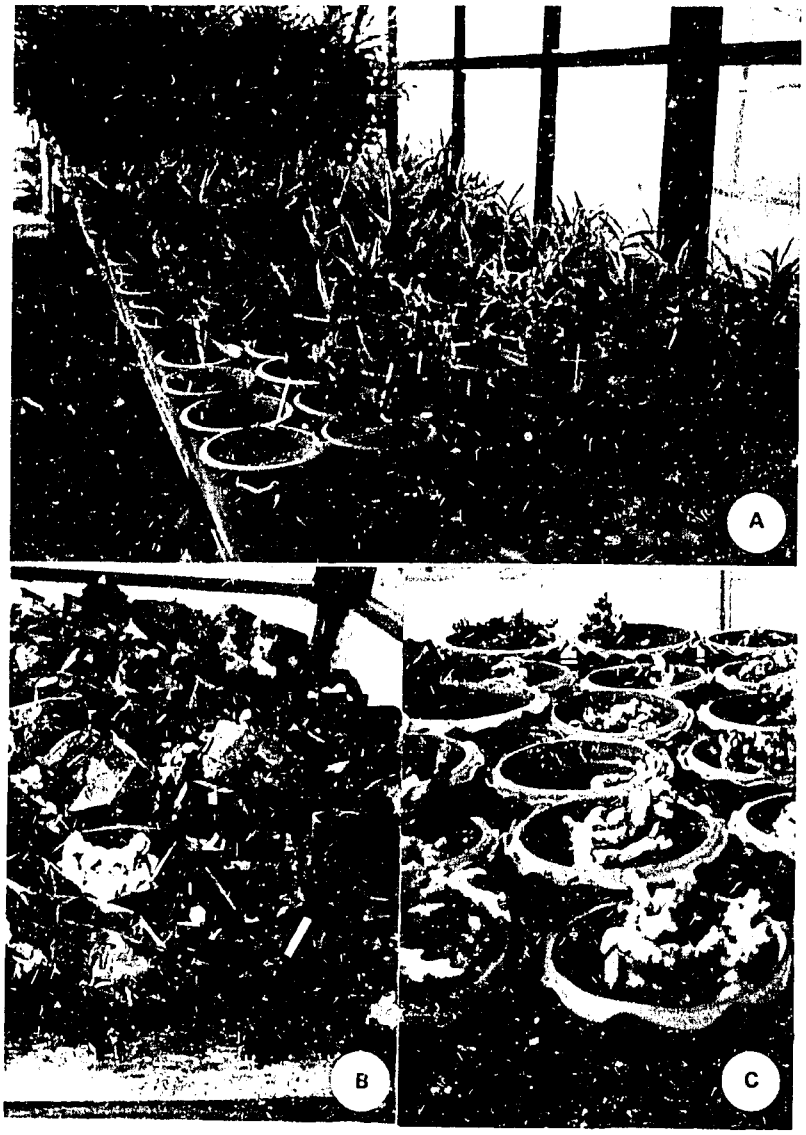
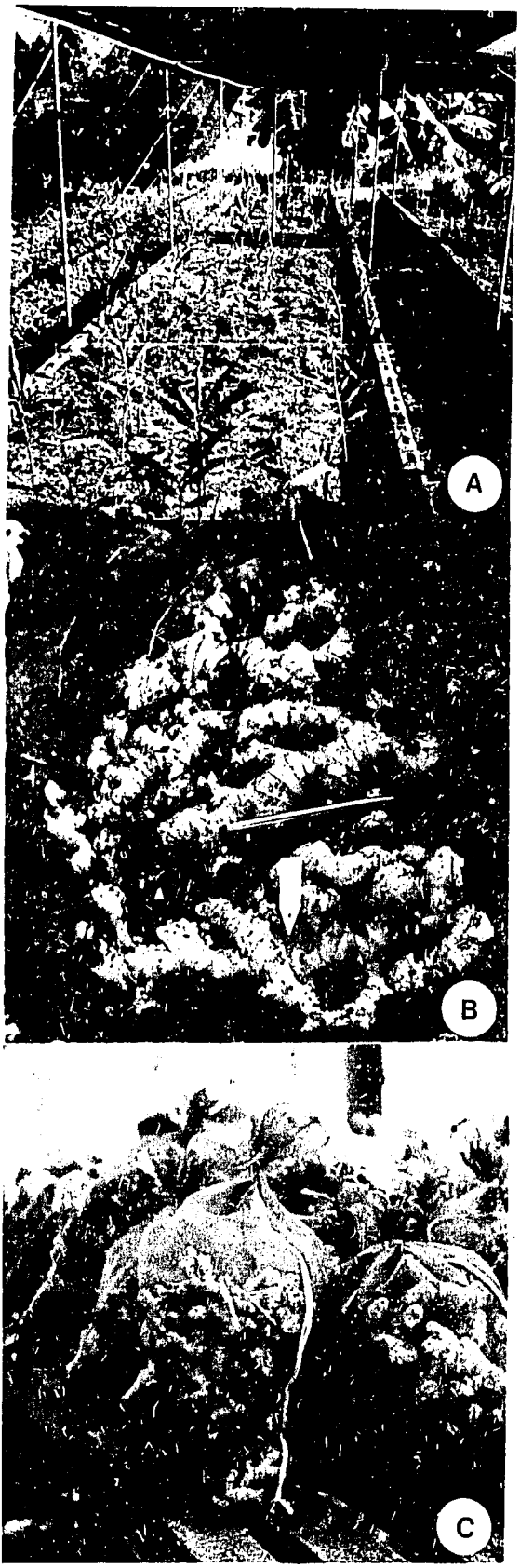


Fig. 11. Production of disease-free TCR-3 rhizomes of ginger cultivars Yuak (GY) and Australia (GA) in concrete block containing methyl bromide fumigated soil (A) and TCR-3 rhizomes from one plant (B) and disease-free stock TCR-3 rhizomes (C) collected after planting 10 months.


A
B
C

Impact, Relevance and Technology Transfer

In regard to the ultimate goal of this project, development of bacterial wilt resistant ginger through tissue culture technology and establishment of a rapid and reliable *in vitro* screening for bacterial wilt resistant is suceess in some extents. Technology for disease-free ginger rhizome production,multiplication and disease resistant screening *in vitro* is now available. Establishment of genetic improvement of ginger for disease resistant especially bacterial wilt is possible through somaclonal, radiation and chemical treatments. Eventhough resistance is not quite promising under greenhouse test with a high pressure of bacterial inoculum, however, it may perform well in farmers field which has yet to be clarified. In addition, improvement of disease resistance can possibly be obtained by retreating with either chemical or radiation. Therefore, the findings of this project may provide a significant impact for the developing countries to apply these techniques in their home countries to solve a similar disease constraint.

In Thailand, bacterial wilt of ginger is still be considered as the most serious disease due to causing tremendous yield losses each year. Based on information of this project indicated that an importance primary source of inoculum is infected seed rhizomes instead of infected soils because farmers traditionally rotated to new growing areas or left fallow land for 2-3 years. If the implement of disease-free rhizome of some selected resistant clones obtained from this project is possible, it may certainly decrease destruction of ginger from bacterial wilt disease. Therefore, a 3-year collaborative program to produce disease-free plantlets and rhizomes for solving the wilt problem is initiated between Department of Plant Pathology, Kasetsart University (KU) and Institute of Plant Tissue Culture, Department of Agricultural Extension (DOAE), Ministry of Agriculture and Cooperatives. The project is still under discussing in details of work plan which anticipate the implementation next year. KU will transfer technology and provide some selected resistant clones to the DOAE for multiplication. Total of 300,000 TC plantlets will produce by each institute in the first year and 500,000 TC plantlets per year in the next2 years. If survival of TC plantlet is 75 %, about 2 millions of the plantlets will obtain for producing TCR-1 rhizomes to contribute to farmers. A training program for farmers to improve cultivation and management of diseases will be established before they receive the stock rhizomes for planing. The proper practices not only provide a high yield and elite disease-free ginger but also reduce dissemination of the disease. The collaborative project will also increase experience of scientists and technicians for both institutes especially on the management of disease-free ginger TC plantlets in a large scale production that will greatly support for future commercial production by private enterprises.

Besides collaborative program with the DOAE, all of facilities and equipments obtained from the project strongly support an opportunity to collaborate the Asian Vegetable Network (AVNET) research on bacterial wilt of tomato among 5 countries; Indonesia, Malaysia, the Philippines and Asian Vegetable Research and Development Center in Taiwan which was granted by Asian Development Bank. In

addition, a bilateral project on molecular biology of plant disease resistant of pepper including bacterial wilt disease between Malaysia and Thailand is presently established and will implement in this coming year. In accordance with these research activities make a great contribute to us by having reputation and recognition as ASEAN scientists working on bacterial wilt disease of crop plants caused by *P. solanacearum* which was the most serious bacterial disease in this region. Furthermore, these equipments are being used not only for research but also for teaching undergraduate and graduate courses. Thus, the project contributed a strong strengthening to our research and teachning in the areas of molecular and biotechnology in plant pathology. Several graduate students successfully received graduate fellowships from the National Genetic Engineering and Biotechnology Center,Ministry of Science, Technology and Energy. In conclusion, this research project is definitely benefit to scientists and students in advancement of science and technology and also to farmers and the country as well

Project Activities/Outputs

The outcomes of project activities conducted during the course of research granting are included as follows:

a. Grant-related workshops/conferences/seminars

1. Dr.N. Thaveechai. Participating in His Majesty's Five Cycle Commemorative Conference of "USAID Science Research Award Grantees". Nakhon Pathom, Thailand (July 24-26, 1987).
2. Dr.W. Kositratana. Participating in Scientific Conferences on "Biotechnology for Health and Agriculture". Washington D.C., U.S.A.(June 6-9, 1988) and Presenting the poster on "Bacterial Wilt of Ginger and Prospects of Control".
3. Dr.W. Kositratana. Participating in Workshop on AID/SCI Funded Research in "Agricultural Biotechnology", Kasetsart University Bangkok, Thailand (Aug 16-18, 1990)

b. Workshops organized by investigator

1. Dr.N. Thaveechai, coworkers and AVRDC. AVNET-I Germplasm Improvment Subnetwork Workshop on "Laboratory Course on Bacterial Wilt". Kasetsart University, Kamphaengsaen Campus, Nakhon Pathom,Thailand (October 18-27, 1989).
2. Dr.N. Thaveechai and AVRDC. AVNET-II Germplasm Improvement Subnetwork Workshop on "Bacterial Wilt Disease Management". Asian Vegetable Research and Development Center, Shanhua, Tainan, Taiwan (April 25-May 3, 1993).

c. Submitted papers at scientific meeting/conferences

1. Dr.N. Thaveechai. Oral presentation in "Pacific Division Annual Meeting of American Phytopathological Society". Hilo, Hawaii, U.S.A. (Jun 19-21, 1990). The title was shown in abstract paper below.

Thaveechai, N., Akarapisan, A., Kositratana, K., and Schaad, N.W. 1990. Responses of callus and cell cultures of ginger during early interactions with *Pseudomonas solanacearum*. Phytopathology 80:892. (Abstract)

d. Attending at scientific meeting/conferences.

1. Dr.N. Thaveechai. Attending in "The 5th International Congress on Plant Pathology". Kyoto, Japan (Aug 20-27, 1988).
2. Dr.N. Thaveechai. Attending in "Annual Meeting of American Phytopathological Society". Grand Rapids, Michigan, U.S.A. (Aug 5-8, 1990).

e. Training/Visiting received and provided

Training/Visiting recevided

1. Ms A. Akarapisan. Training on "Protoplast and Cell Culture". Tissue Culture Unit, Kasetsart University Research and Development Institute, Kamphaengsaen, Nakhon Pathom, Thailand (Oct 10-25, 1988).
2. Dr.N. Thaveechai. Training on "Detection and Isolation of Metabolites during Hypersensitive Response". Harris Moran Seed, Co., California, U.S.A. (Jun 23-Aug 3,1990).
3. Dr.N. Thaveechai. Visiting for "Tissue Culture Research on Callus Development and Regeneration". Kyoto University, and National Institute of Agrobiological Resources, Tsukuba, Japan (Aug 29-Sep 4, 1988).

Training/Visiting provided

1. Dr.N. Thaveechai. Farmers' training on "How to Prevent Bacterial Wilt of Ginger". Prachaub Khirikhan, Thailand (Mar 18, 1989).
2. Dr.N. Thaveechai. Farmers' consulting on "Mangement of Bacterial Wilt of ginger" Sisaket, Thailand (Sep 4, 1989).

f. Theses/Special Problems involving to the project and equipments

Theses.

1. Akarapisan, A. 1990. Protoplast and cell culture techniques for studying hypersensitive reaction of ginger cultivars to bacterial wilt pathogen (*Pseudomonas solanacearum* E.F. Smith). M.S. Thesis, Graduate School, Kasetsart University. (As Major Advisor)
2. Kasigumpaiboon, U. 1991. Potentail of root colonizing bacteria from tomato root and soil as biocontrol agents of tomato wilt bacteria (*Pseudomonas solanacearum* E.F. Smith). M.S. Thesis, Graduate School, Kasetsart University. (As Co-Advisor)
3. Rakkai, C. 1992. Development of bacterial wilt resistant tomato through plant cell culture technique. M.S. Thesis, Graduate School, Kasetsart University. (As Major Advisor)
4. Leksomboon, C. 1994. Investigation on bacterial wilt resistance of tomato and molecular cloning of hypersensitive reaction and pathogenicity genes of *Pseudomonas solanacearum*. Ph.D. Thesis, Graduate School, Kasetsart University. (As Major Advisor)

Special Problems (Undergraduate level)

1. Ramunt, S. 1990. Interactions of tomato roots and bacterial wilt pathogen (*Pseudomonas solanacearum*) in hydroponic culture. Special Problem. Department of Plant Pathology, Kasetsart Univesity. (As Advisor)
2. Rhokew, T. 1990. Biochemical characters and pathogenicity on host plants of *Pseudomonas solanacearum* strains from Thailand. Special Problem. Department of Plant Pathology, Kasetsart University, (As Advisor)
3. Maleepan, S. 1992. Growth of *Pseudomonas solanacearum* associated with ginger callus and tissue cultures. Special Problem. Department of Plant Pathology, Kasetsart University. (As Advisor)

g. Other outcomes

1. Display and demonstration on "Production and Screening for Bacterial Wilt Resistance in Ginger" at National Agricultural Fair, Kasetsart University, Bangkok, Thailand. (Jan 30-Feb 4, 1994) (Fig 12)
2. Produce a video tape on "Bacterial Wilt Disease of Ginger and Disease Management" for 30 min.
3. Provide an interview tape for radio program on "Diseases of Ginger and Their Controls".

Project Productivity

The research was not fully accomplished as the proposed goals. Development of callus culture was success but regeneration into complete plantlet was not consistent and reliable may be due to imbalance of growth hormone to induce shoot formation of callus culture. Abundance of roots was easily obtained from callus culture in all of tested culture media. Development of *in vitro* screening disease resistant using serological detection of metabolites producing during hypersensitive reaction was not completed as proposed because of metabolic products related to hypersensitive reaction could not effectively detect and isolate due to lack of highly resistant ginger cultivar. Furthermore, hypersensitive resporse of PAL enzyme in resistant cultivar was not always higher than in susceptible when observed in tomato. Therefore, it needs to be studied intensively for understanding mechanism of disease resistance of *P. solanacearum* especi ally at molecular level in order to find specific metabolite for production of antiserum to be used for resistant screening as proposed. The other constraint that made the project partial fulfillment due to ginger rhizome production was time consumed causing delayed of field evaluation and distribution of disease-free rhizome to farmers.

Fig 12 Display of research project on "Production of Disease-free rhizome and Screening for Bacterial Wilt Resistance in Ginger".

27ก

Future Work

Several future works have been proposed as mentioned earlier especially on molecular study of host-pathogen interaction during HR development or resistant response by using either tomato and pepper as models. Method of detection and isolation of specific metabolic products of host defense mechanism is necessary required for further use to screen disease resistance. Regeneration of callus into plantlet is another topic needed to be continued for a better acheivement of inducing and screening disease resistance *in vitro*. Evaluation of selected bacterial wilt resistant clones under farmer's field condition is also necessarily to be done to confirm the results. Improvement of selected resistant clones for high degree of resistant by repeated treatments with either chemical and radiation is also interested to carry on. If all of those aspects are fully acheived, establishment of disease resistant and a rapid and reliable screening disease resistance should be accomplish. Therefore, control of bacterial wilt disease is possibly managable.

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