Individual Training Report

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Technical Report

PLANT GENETIC RESOURCES

The use of RFLP markers for the chromosomal location of rice blast resistance

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The use of RFLP markers for the chromosomal location of rice blast resistance

Introduction

Oryza sativa, rice, is the most widely-grown crop plant world-wide and constitutes an important part of the diet of more than half of the world's population (Coffman and Herrera, 1980). The rice blast, caused by Pyricularia oryzae, is one of the most important disease of rice. It cause large losses under favorable conditional and it is present in all rice-growing areas. Resistant cultivars generally has been considered the most economical and effective way to control this disease (Ziming Wang, et al 1989).

Study on the inheritance of reaction to different phathotypes indicate that blast resistance are generally dominant and one to three genes are involved in the inheritance of resistance (Padmanbhan, 1974)

The molecular markers are being studied for their potential to enhance selection efficiency in plant breeding. With the development of molecular marker linkage maps, the restriction fragment length polymorphism (RFLP) markers have been used to locate an manipulate loci affecting expression of simply and quantitatively inherited traits(Tanksley et al. ,1989). The RFLP

for disease resistance, as well as quantitative traits loci.

On this study, RFLP analysis was used to identify chromosomal region related with blast field resistance gene. The molecular markers were studied by screening genomic DNA from F3 generation which parents were the resistant cultivar Owarihatamochi and the susceptible cultivar Nipponbare.

Materials and methods

Plant materials

The field resistance to blast fungus was tested with F3 families from F2 individuals at Hokuriku National Agricultural Experimental Station, Joetsu, Niigata Prefecture. The degree of symptoms was scored for each family four times. The field resistance was standardized as compared of checks varieties and the families were classified into "R:resistant", R-M:intermediate" and "S:susceptible".

From this F3 population, 94 families were selected to be used in this study: 14 resistants, 45 intermediate and 35 sucepti-bles. The parents were the resistant cultivar Owarihatamochi and the susceptible cultivar Nipponbare.

Methods

DNA extraction

DNA extraction have been done from the leaves of plants of 45 days old. They were harvested from 60 plants of each F3 population.

The freeze-dried leaves were ground to a fine powder. The powder was mixed with 1x CTAB solution and incubated at 55°C for 30 minutes to destroy nuclear membrane and to inactive nuclease enzyme.

The chloroform:isoamylalcohol (24:1) was put on the mixture, to remove proteins . This was mixed by inverting for 30 min and spinned at 2500 rpm for 15 min. The upper phase was poured into fresh 50 ml tube. The lower phase was reextracted with 1x CTAB and mixed for 30 min and then spinned at 2500 rpm for 15 min. The upper phase was poured into the tube with the first extraction.

Into the total extraction, 1/10 volume of 10% CTAB was added and mixed with chloroform:isoamylalcohol and mixed by inverting for 30 min. Then spinned it at 2500 for 15 min. The upper phase, containing DNA, was transferred to fresh 50ml tube each with disposable pipette and added an equivalent volume of precipitation buffer. The mixture was mixed slowly by inversion and left it for more than 30 min.

The mixture was mixed by two or three inversions and spinned

at 2500 rpm for 15min. The solution was thrown away and the tube with precipitate was left up side down to drain well. High salt-TE was added to the tube then the suspension was incubated at 55°C until the precipitate was completely dissolved . The tube was centrifugated at 2500 rpm for 10 min. The undissolved DNA and others contaminants were precipitated.

The solution was poured into a fresh tube and the DNA was precipitated with the same volume of isopropanol. The DNA fiber was transferred to 1.5 ml with disposable pipette and washed twice with ethanol, then dissolved with 1/10 TE plus RNase. The tubes were left at room temperature until the pellet was completely dissolved.

The dilutions of the isolated DNA were quantified by electrophoresis with 0,8 agarose gel and compared with the lambda DNA of known concentration. The original extracts were diluted with sterilized distilled water and adjusted to 300ng/ul.

Digestion of DNA

DNA from each parental line and 94 F3 population was digested with five kinds of restriction enzymes: Hind III, Kpn I, Bam HI, Bgl II, Eco RV. For each reaction, 1.5 ng of DNA was digested with 6 units of restriction enzymes with the buffer recommend by supplier in the volume of 8 ul. The reaction mixture was incubate at 37°C during 12 hours. The complete digestion was checked on

0.8% agarose gel. The digest was mixed with 1/10 volume of tracking dye. The tracking dye was made of 0.25% of Bromo Phenol Blue.

DNA fragments digested with restriction enzyme were separated on 0,6% agarose gel. The digested was loaded by 1-1.5ng/lane and run at 75V for 30 min and at 25V for 16 hours. The electrophoresis was finished when BPB marker reached to the anode edge of the gel.

Southern Blotting

After the DNA fragments were fractionated by agarose gel electrophoresis, they were was transferred out of the gel to a membrane filter by the method called Southern blotting.

The sheets of filter paper (Whatman 3 mm) and a sheet of nylon membrane (PALL,Biodine B) were prepared. A transfer tray with alkaline transfer buffer (0.4M NaOH) was prepared and a supporting platform was made with a glass. The platform was covered with a sheet of filter paper. Then the gel was put on a sheet of filter paper and was transferred to the platform , then the nylon membrane was placed on the top of the gel. The surface of the membrane was stroke to squeeze out the bubbles under the membrane, specially around the wells. Two filter paper wetted with alkaline transfer buffer, a stock of absorbent paper towel and a light weight were put over the membrane. The capillary blotting was performed overnight.

The blotting stack was dismantled down to a membrane. The membrane and gel were removed together . And the membrane was marked with a soft-lead graphite so the face carrying the DNA was distinguished. The membrane was washed in 2X SSC to neutralize, and to remove any adhering agarose. This was dried complety on a sheet of new filter paper. Finally the membrane was put between two filter paper and baked into a oven at 120°C for 20 min. Afterwards, the membrane was placed into a plastic bag.

DNA hybridization

Prehybridization

The hybridization buffer was made with ECL hybridization buffer (R), Na Cl and the blocking reagent. It was stored under -20°C. Two sheets of membrane with the size of 7 x 20 cm was used for 1 hybridization. Eighteen milliliter hybridization buffer was warmed in a 42°C water bath and poured to a plastic bag containing the membrane filter. The bags were sealed without bubbles and incubated for 1 hour at 42°C.

Preparation of the probes

DNA fragments derived from rice genomic DNA or cDNA library was used as a DNA.

More than 100 probes polymorphic between Nipponbare cultivar and Kasalath cultivar were used for primary screening and only the probes polymorphics between Nipponbare and Owarihatamochi were used to F3 analysis.

The probes were amplified by the polymerase chain reaction (PCR). One microliter of plasmid solution with the insert was used as a template of reaction mix. The reaction mix contained 10 mM Tris-HCl ph 8.3, 50 mM KCL, 1.5 mM MgCl, 0.01% gelatin, 10uM dNTPs, 0.4uM forward primer (mainly M4), 0.4uM reverse primer (mainly RV) and 5U Tag polymerase. Some probes were amplified with modified reaction mix with the change the MgCl2 condition, ranging between 0.75 mM to 2.5 mM.

The amplification was performed in 30 cycles, each cycle consisted of 1min at 55°C and 3 min at 72°C. The 30 cycles were followed by 7 min at 72°C.

The amplified fragments were purified by ethanol precipitation. The 1.5 ml tube with 100 μl of PCR product and 350 μl of AA-ethanol was mixed and stored at -80°C for more than 30 min. Then it was desfrozen at room temperature, spinned the tube at 12000 rpm during 15 min and decanted the solution. The tube with the precipitate was filled with 1 ml of 70% ethanol, spinned and

decanted the solution and left the tube open to fly ET.OH away.

The purified and amplified probes were quantificated by agarose gel electrophoresis in comparation with lamba-DNA of known concentration. The probes were adjusted to 10 ng/μl with the addition of sterilized distilled water and stored at 4°C.

Preparation of labeled probe

The total amount of DNA required depends on the size of the membrane. On case of F3 analysis, 150 ng of probe DNA was used. It was put into 1.5 ml tube, then boiled for 5 min to make the double stranded DNA denature completely. Immediately the DNA was cooled on ice for 5 min and labeling reagent was added in an equivalent volume of DNA volume, briefly mixed and spinned. Then glutaraldhehyde solution was added in equal volume of labeling reagent, mixed and spinned. The mixture was incubate at 37°C for 10 min was added 2.5 μlof lambda-DNA digested with Hind III.

Hybridization

One side of the plastic bag with the membrane was cut to transfer the hybridization buffer to the 50 ml tube where the probe DNA solution was added and mixed. The solution was poured back to the plastic bag. Squeezed out the bubbles from the plastic bag and sealed. The plastic bag with the membrane was incu-

bated at 42°C, overnight.

Washing the membrane

The membranes were transferred to the container and washed with 0.5X primary wash buffer that contains 6 M urea, 0.4 % SDS, and 0.5X SSC. Then incubated with agitation for 20 min at 42°C. 0.5X. Then, wash buffer was discarded and replaced with fresh wash buffer and incubated for 20 min at 42°C. The buffer was discarded again. The membranes were placed in a fresh container, then an excess amount of secondary wash buffer (2X SSC) was added and shaked for 5 min at room temperature. Finally it was discarded another secondary buffer was added and incubated with agitation for 5 min at room temperature.

Signal detection

The detection reagents were prepared by mixing an equal volume of detection solution 1 (12 ml) with detection solution 2 (12 ml). That is enough amount to cover 8 sheets of membrane.

The membranes were put on a paper towel to remove excess of wash buffer, then transferred to the container with the mixed detection reagent for a minute at room temperature . The excess of detection buffer was drained on a paper towel. The membranes were mounted on the screen, DNA side up. They were wrapped with

Saran Wrap and then placed inside the cassette. A sheet of X - ray film was placed on the top of the membrane and the cassette closed . All this procedure has been done in the darkness.It was exposed for 2-6 hours. Finally, the film was developed.

Results - Discussion

The cosegregation of the clones with the traits of the interest was verificated on the F3 population segregating for blast resistance. The parents of the F3 were the resistant cultivar Owarihatamochi and the susceptible cultivar Nipponbare.

The digests of parental DNA were surveyed with the polymorphic probes between the cultivar Nipponbare (Japonica rice) and cultivar Kasalath (Indica rice). Out of 110 probes, 33 probes revealed difference between the hybridization pattern of parents. Then 22 probes and 5 restriction enzyme (Table 1) were used in the RFLP analysis among F3 population (Fig. 2).

DNA markers generated with these probes has been mapped on rice genetic linkage map (Fig 3).Most of the chromosomes, except chromosome 7 and 8 were surveyed with these probes.

The F3 population showed a single band or double band pattern. The band B was present in the resistant parent and represent the BB type, Owarihatamochi. The band B was present in the susceptible parent and represent the AA type, Nipponbare. The double - band represent the H (heterozygous) type, Owarihata-

mochi/Nipponbare.

The correlation between the genotype of each DNA markers and blast field resistance in F3 families was shown in Fig. 4. If there are some correlation between the genotype of RFLP markers and field resistance, the group with A type (Nipponbare) in F3 families, should have more susceptible family than resistant families. And conversely, B type should have more resistant family than susceptible families. As it was shown in Fig.4, the marker on chromosome 4 and 11 had the tendency prior mentioned. On marker 27, 78 % of A type the F3 family was susceptible and all of the B type had resistance. On the marker 189 of the chromosome 11, 73 % of the A type was susceptible and the B type had some extent of resistance. And the H type (heterozygous) intermediate frequency between A type and B type.

These observation could imply that the factor with some association with the blast field resistance, is located near the markers 27 and 189. The fact of heterozygous type was present in the resistant, susceptible and intermediate blast field resistant, could indicated this factor doesn't have dominant effect.

For the other markers analyzed,some association was not observed between the frequency of genotype of the marker and the blast field resistance.

The number of markers useful between parents is limited,and the chromosome 7 and 8 have not been surveyed yet.

In this study, it was found that two chromosomal region

could be related with blast resistance. Another markers located on these region are required to specify the region.

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Probe 66

Probe 131

Probe 187

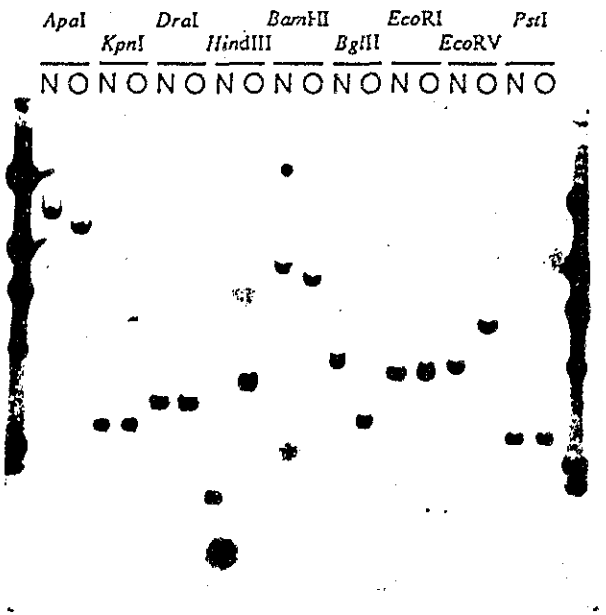


Fig. 1 Primary screening of probes

Table 1 Probe/Enzyme combinations and chromosomal location of DNA probe

		ENZYME				
CHROMOSOME	PROBE	Hind III	Kpn I	Bam HI	Bgl II	Eco RV
1	XNpb 113					
	7				X	
2	117					X
	XNpb 116	X				
	XNpb 132				X	
3	124		X			
	131					X
4	31					X
	27	X				
	XNpb 203					X
5	XNpb 25			X		
6	152	X				
	44	X				
9	56	X				
	57	X				
10	179					X
11	186					X
	187					X
	189	X				
	XNpb 189	X				
12	192			X		
	193					

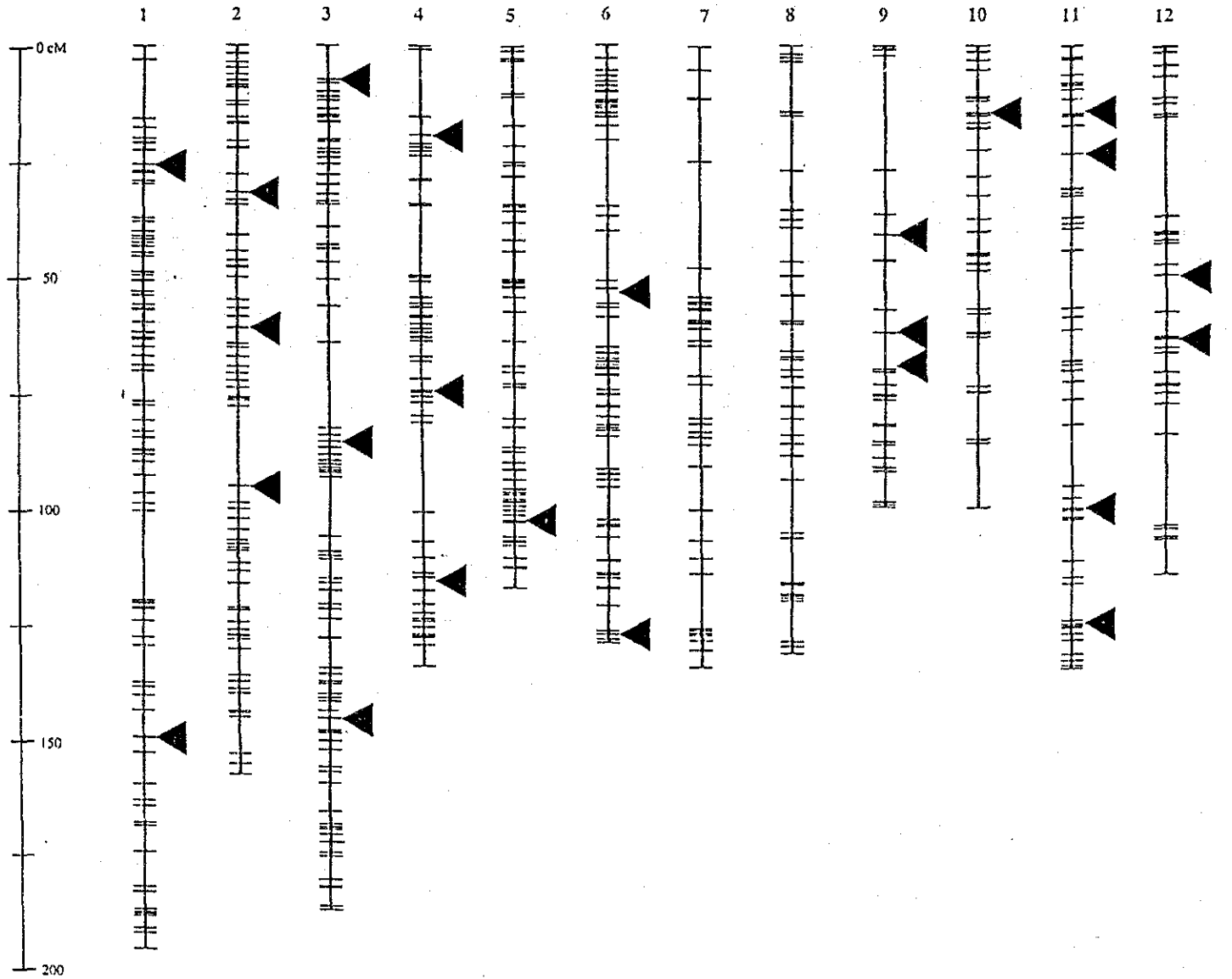
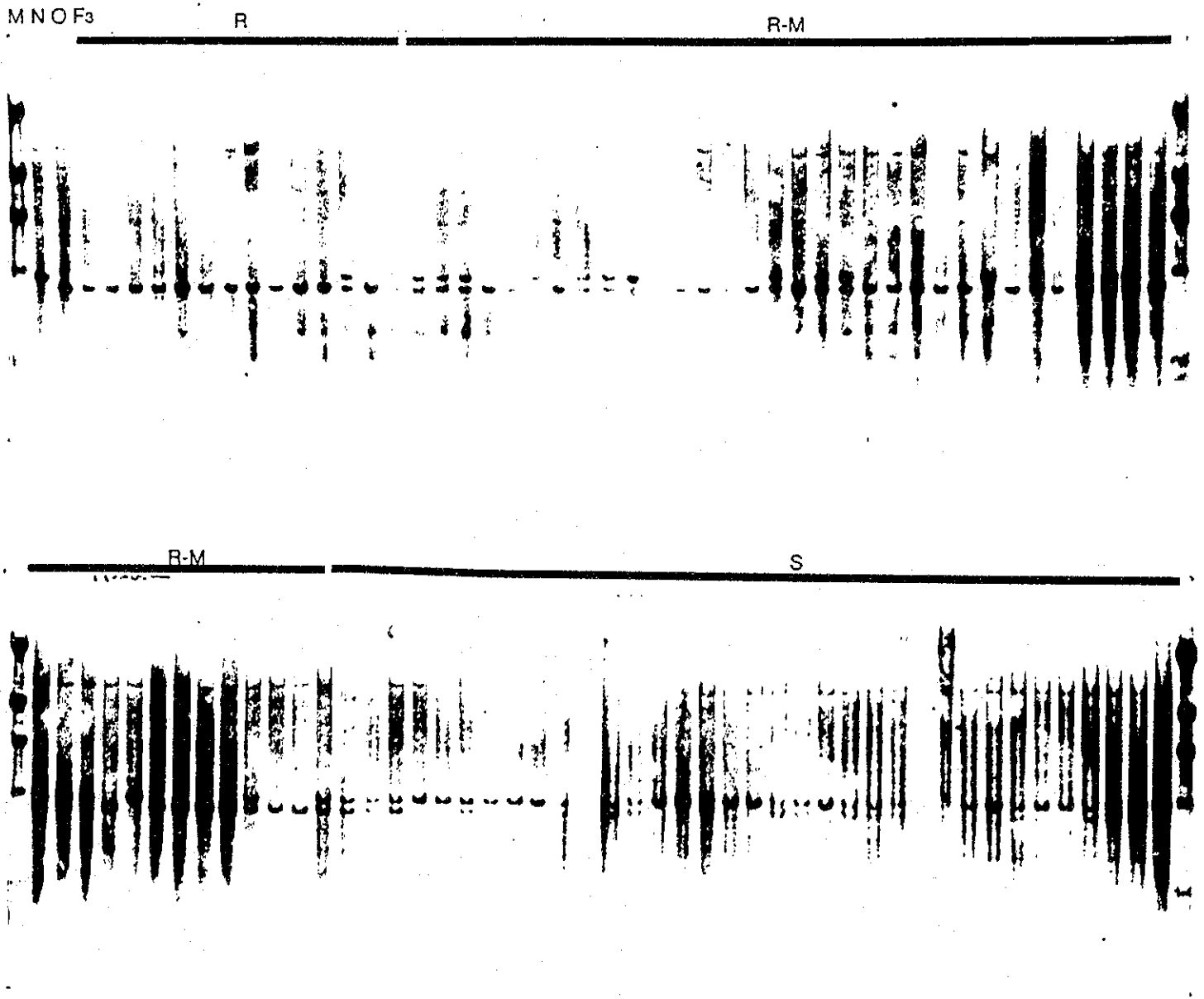


Fig. 2 Localization of the probes used in F3 analysis



Probe 27

Fig. 3a F3 analysis

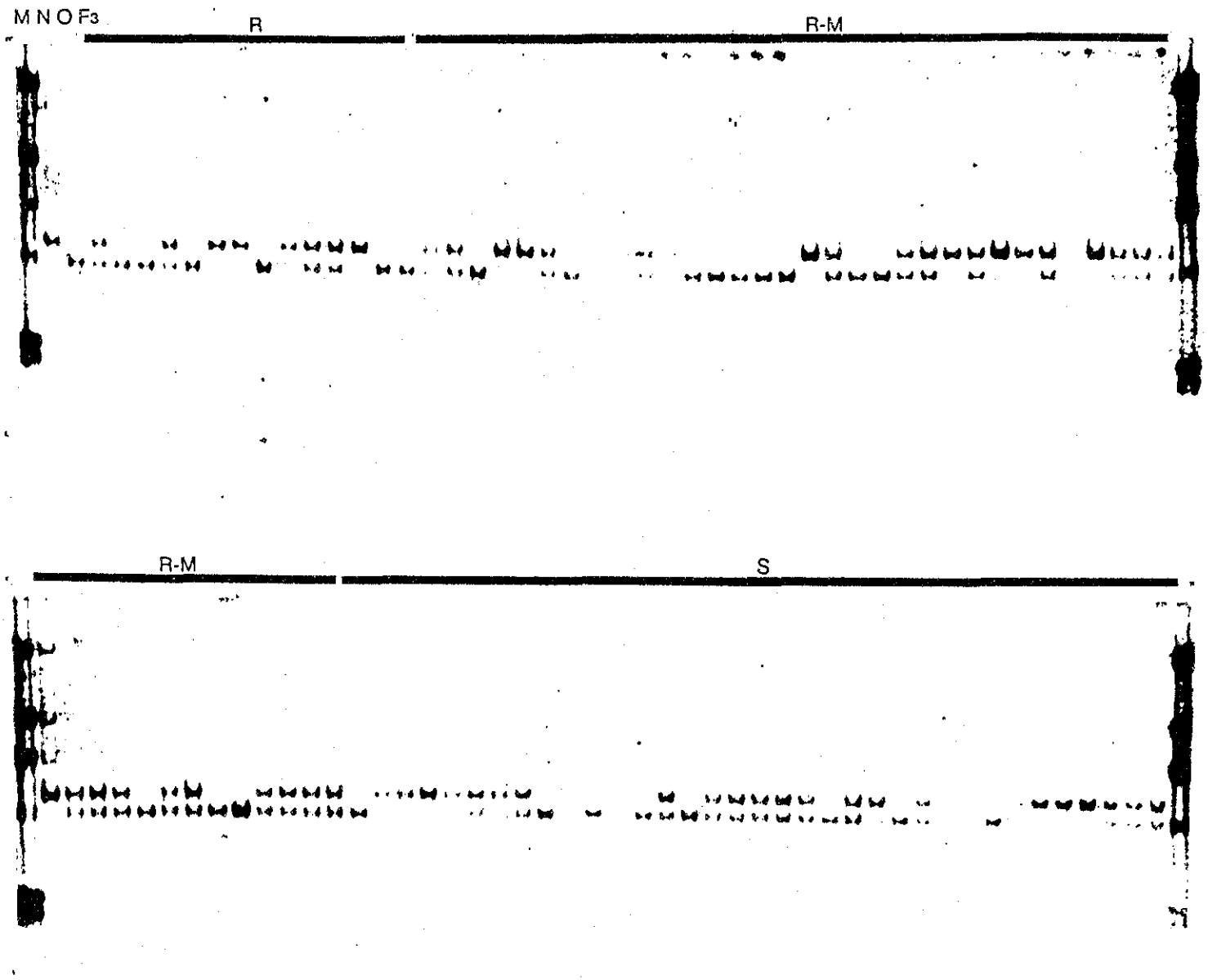


Fig. 3b F3 analysis

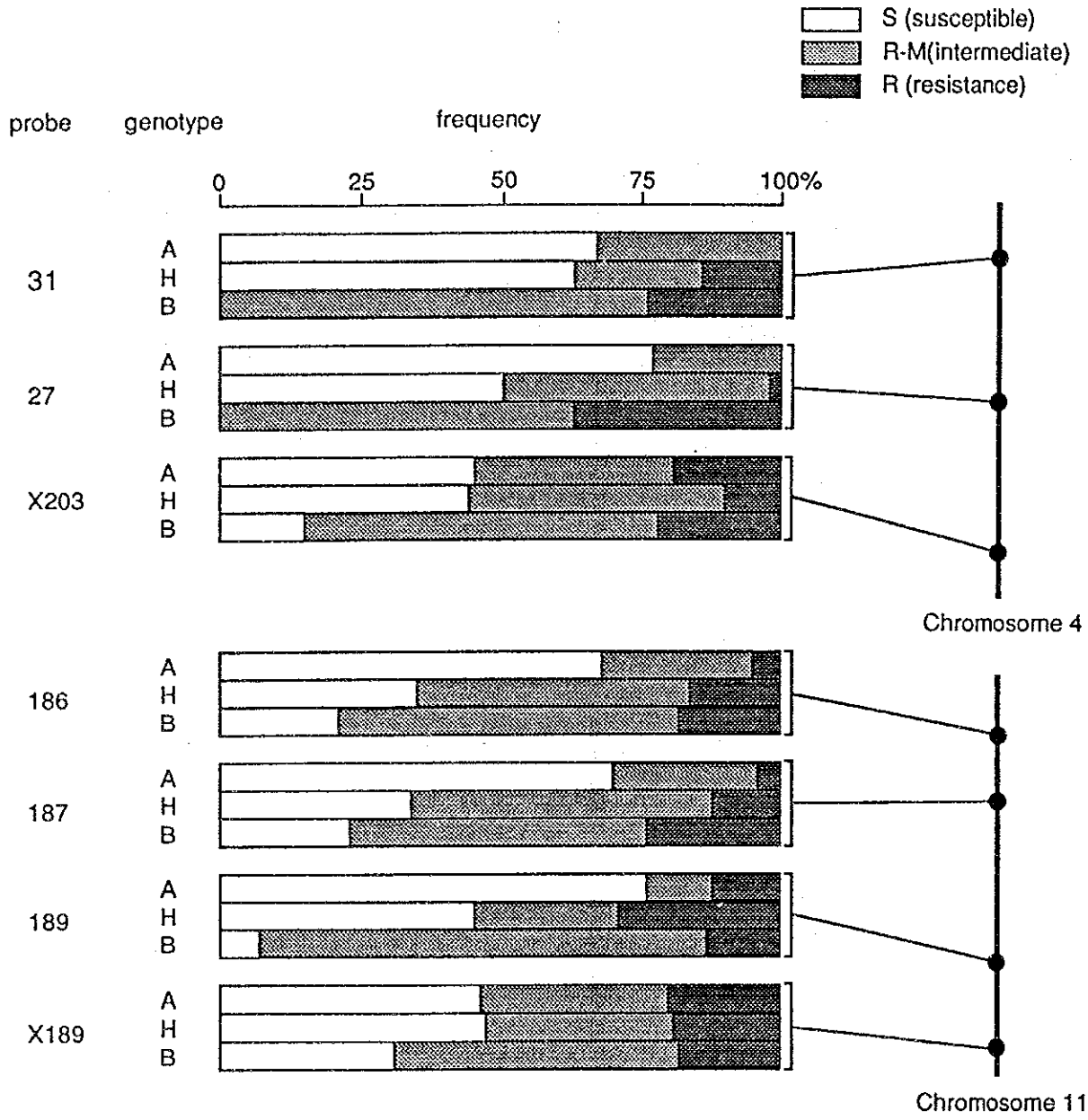


Fig. 4 Correlation between genotype of RFLP markers and blast field resistance in F3

Cryopreservation of Rice(*Oryza sativa*,L.)Callus

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ABSTRACT

In this work,conditions allowing for the cryopreservation of rice(*Oryza sativa*,L.)callus were defined.Survival rates higher than 60% could be obtained with callus in glass cryotubes at 10%(w/v)DMSO,10%(w/v)sucrose and 5%(w/v)glycerol for 60 minutes,crystalizing manually at -8°C and freezing at 0.3°C/min(cultivar 12)or 0.5°C/min(cultivar 1) to -30°C followed by immersion in liquid nitrogen. After rapid thawing in a 36°C to 40°C water bath and washing with 3% sucrose,the callus were allowed to grow in semisolid N6 medium and the growth of the cryopreserved callus was similar to that of the unfrozen control.In the case of using plastic cryotubes,results of the experiment was not consistent and there are still some problems to be resolved for successful cryopreservation of rice callus practically.

INTRODUCTION

There have been increased recent efforts to ensure that germplasm of plants is collected and preserved for future use.Seeds can be used to preserve rice(*Oryza sativa*, L.)

germplasm,but there is also a need to maintain clonal selections for which scientists used in laboratory.

For long-term storage of *in vitro* collections, elimination of routine subculturing is desirable,because 1),as long as cell divisions occur ,mutations may arise;2),at every subculturing event there is a risk of loss due to contamination or human error;3) even if subculturing can be reduced to once per year, a lot of labour will still be required to maintain a large number of collections.

Therefore,cryopreservation would be an ideal solution for long-term preservation of germplasm collections.Under conditions of storage in liquid nitrogen(-196°C),there is a complete cessation of all chemical reactions in the cell.Consequently,no cell division,cell degeneration or genetic change can take place with time.

MATERIALS AND METHODS

Cell cultures

Calli derived from nine cultivars of rice were used in our experiments.They were partly embryogenic.The calli were maintained in semisolid N6 medium whose PH was ajusted to 5.8 before autoclaving.They were incubated in the dark at 25°C and subcultured every 20 days.

Cryoprotection

The cryoprotectants used were composed of 10%(w/v)DMSO, 10%(w/v)sucrose and 5%(w/v)glycerol.After preparing,the cryoprotectants were autoclaved.Calli of about 0.1 gram

were transfered to 10 ml glass centrifuge tubes or 2 ml plastic cryotubes.To find optimal exposure time,specimens were treated with 1ml of cryoprotectant for 30 min,60 min and 90 min.After exposure treatment finished, cryoprotectants of 0.5 ml were removed and then containers with samples were put into a refrigerator set at -8°C and kept there for 20 to 30 min.Treated controls were cryoprotected but not frozen. Controls were neither cryoprotected nor frozen.

Crystalization

After 30 min, aseptical ice inoculation of cryoprotectant solution was made by using a liquid nitrogen cooled steel rod at -8°C for 10 seconds.For practical cryopreservation using plastic cryotubes,we also tested another two methods to induce spontaneous ice inoculation and they can make the work for plenty samples easier if effctive.The first method was that we put 1,2 or 3 pieces of leaves or stems into tubes containing cryoprotectants and samples before they were put into refrigerator.The second method was that we put containers with samples and cryoprotectants into ethanol bath of -20°C, -25°C,-30°C and -40°C and kept them there for 30 seconds.

Freezing

Then the samples were transferred to ethanol bath set at -8°C.The bath was cooled manually at a rate of 0.3°C,0.5°C or 1°C/min to -20°C,-30°C or -40°C by adding liquid nitro-

gen.The samples were held at final temperature for 10 min followed by plunging into liquid nitrogen.

Thawing and dilution

After 1 h in liquid nitrogen,the samples were thawed rapidly by placing the tubes in a waterbath of 36°C to 40° C for about 40 seconds(glass tubes) or 2 min(plastic tubes) with constant agitation until most of the ice was melted.

To remove cryoprotectants used,dilution were made by dropwise addition of 3% sucrose to 3 ml in 15 min or more followed by plating on a fresh N6 culture medium.

Viability test and regrowth

After 30 days of reculture,viability was determined by either visaul grading or rating the growth.The visual rating of viability of callus regrown was expressed as percent survival over the nontreated,nonfrozen control. The surviving rate was calculated with the following formular:

$$X(aWi)+(1-a)Wi=Wf$$

In the case of unfrozen control,a=1.So X was determined by final weight and innoculum of unfrozen control.

$$X=\frac{Wf(Control)}{Wi(Control)}$$

$$a=\frac{Wf/Wi-1}{X-1}$$

Wf:final weight;

Wi:innoculum;

a:surviving rate.

RESULTS

Effect of exposure time of cryoprotectants on the survival of rice callus cultivar 1 and 12 is shown in Fig.1 and Fig.2.

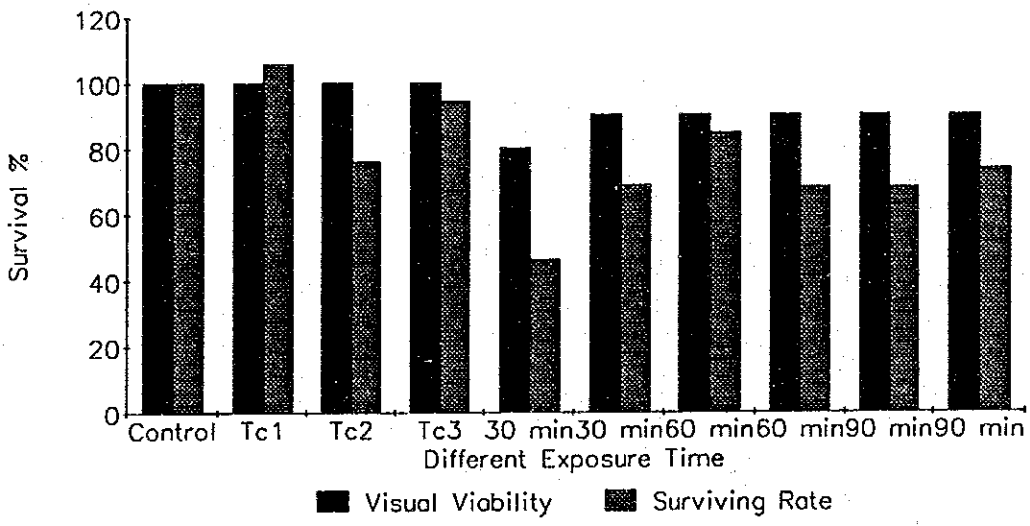

Fig.1 Effect of exposure time of cryoprotectants on the survival of rice cultivar 1.Ten-d-old callus were sampled into glass cryotube,cooled 0.3°C/min to -30°C and stored 12h in LN.

All treatments showed good cryoprotective effects.The lowest visual viability were 70% for cultivar 12 exposed for 30 min. The highest visual viability were 90% So the cryoprotectants used were good for protecting cells to survive the freezing and storage in LN.Longer exposure time(60,90min)gave similar survival.60 min was used in the following experiments.

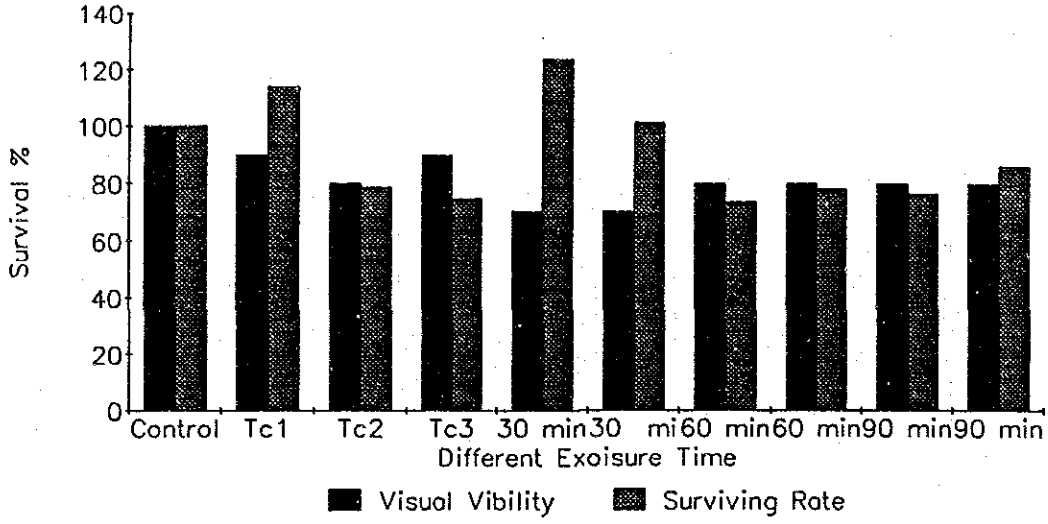

Fig2. Effect of exposure time of cryoprotectants on the survival of rice cultivar 12.Ten-d-old callus were sampled into glass cryotube,cooled 0.3°C/min to -30°C and stored 12h in LN.

The optimal cooling rate appears to be 0.3°C/min for cultivar 12(Fig.3) and 0.5°C/min for cultivar 1(Fig.4).

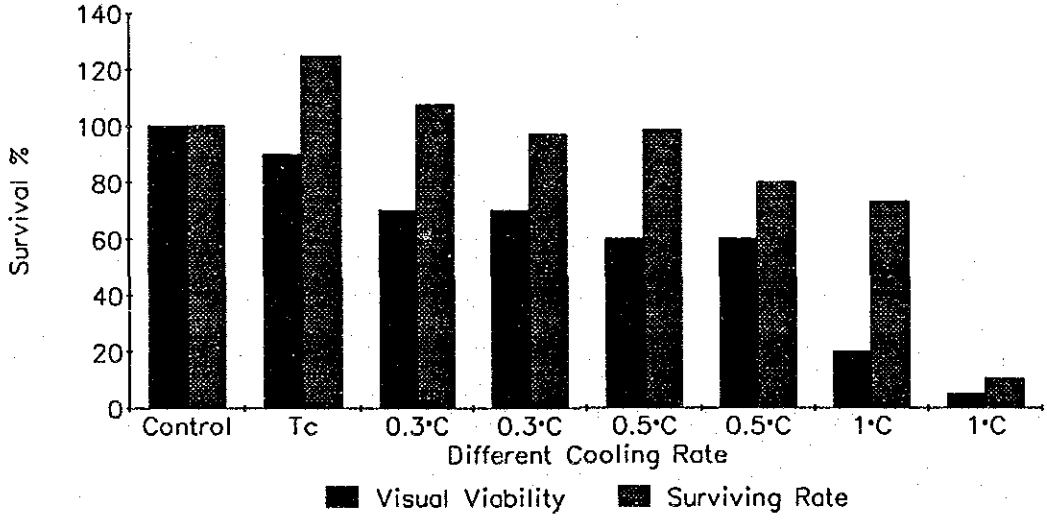

Fig.3 Effect of cooling rate on the survival of rice cultivar 12 frozen and stored in LN for 12h using glass cryotube.Ten-d-old callus were cryoprotected as the method described above,cooled at various cooling rates to -30°C and then immersed in LN.

In the case of cultivar 1,cells cooled at 0.3°C/min exhibited an significant lower visual viability than those cooled at 0.5°C/min. On the other hand,faster cooling rates greater than 1°C/min resulted lower survival .In the case of cultivar 12, cells cooled at 0.5°C/min exhibited a lower visual viability than those cooled at 0.3°C/min.The fastest cooling rate(1°C/min) resulted in poor survival.

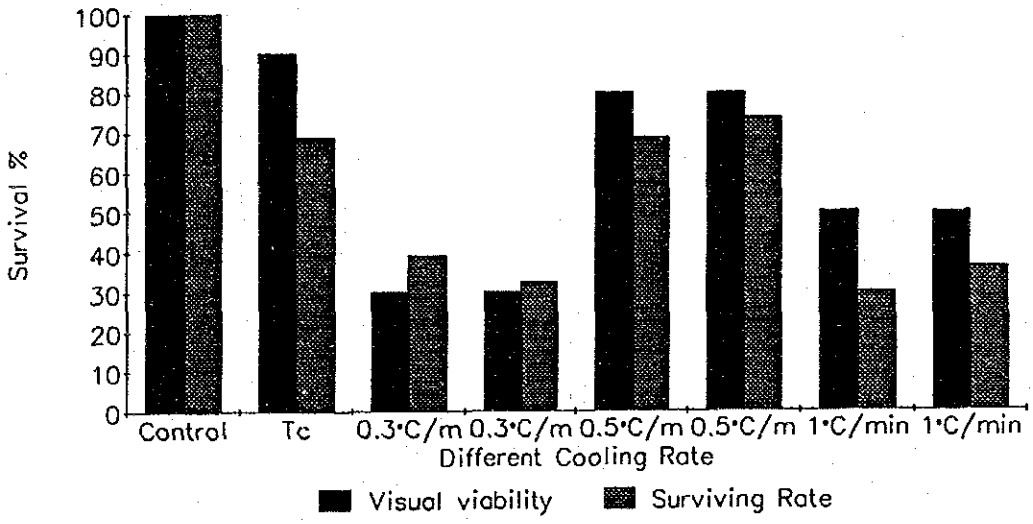

Fig.4 Effect of cooling rate on the survival of rice cultivar 1 frozen and stored in LN for 12h using glass cryotube.Ten-d-old callus were cryoprotected as the method described above,cooled at various cooling rates to -30°C and then immersed in LN.

The effect of the terminal freezing temperature prior to immersion in LN on cell survival is shown in Fig.5 and Fig.6.For cultivar 12,the visual viability with the terminal freezing temperature of -20°C to -40°C did not show much difference. But the callus of cultivar 1 exhibited poor survival at -20°C and lower terminal freezing temperatures resulted in much higher viability.

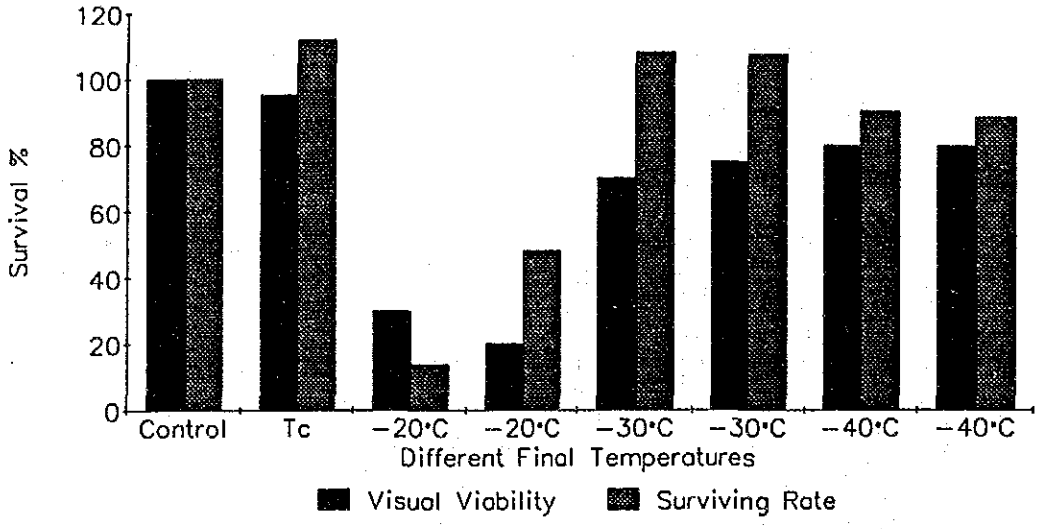

Fig.5 Effect of terminal temperature prior to immersion in LN on the survival of rice cultivar 1.Ten-d-old callus were sampled into glass cryotusbe,cooled 0.3°C/min to various temperatures and stored 1h in LN.

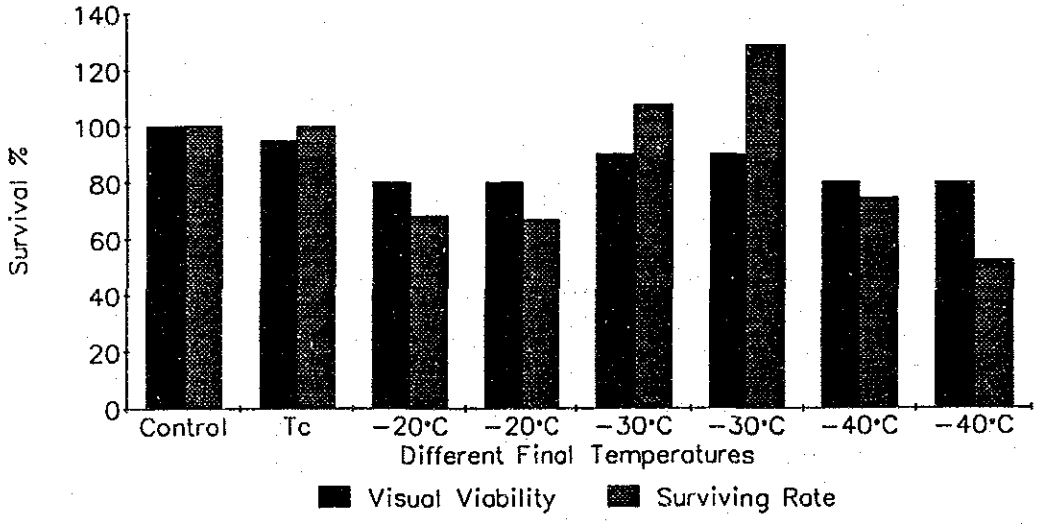

Fig.6 Effect of terminal temperature prior to immersion in LN on the survival of rice cultivar 12.Ten-d-old callus were sampled into glass cryotube,cooled 0.3°C/min to various temperatures and stored 1h in LN.

Fig.7 shows the survival of different cultivars stored in LN for 12h.Callus of cultivar 4 exhibited the lowest

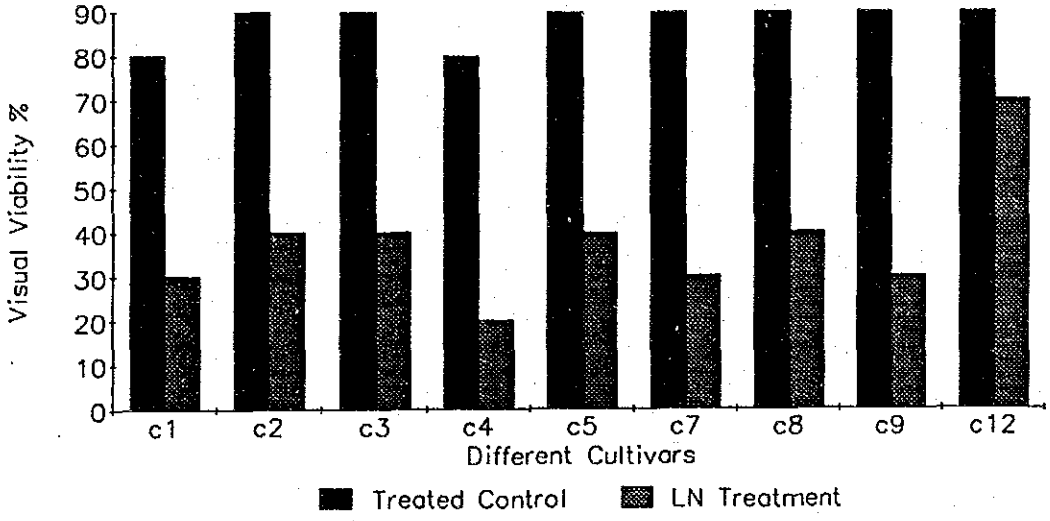

Fig.7 Performance of different cultivars of rice callus frozen slowly at 0.3°C/min to -30°C before plunging into liquid nitrogen.

visual viability(20%),cultivar 12 showed the highest viability(70%).Viabilities of 4 cultivars (cultivar 2,3,5 and 8) were 40% .So different cultivars had different survivals under experimental conditions given. So there may be different optimum for different cultivars.

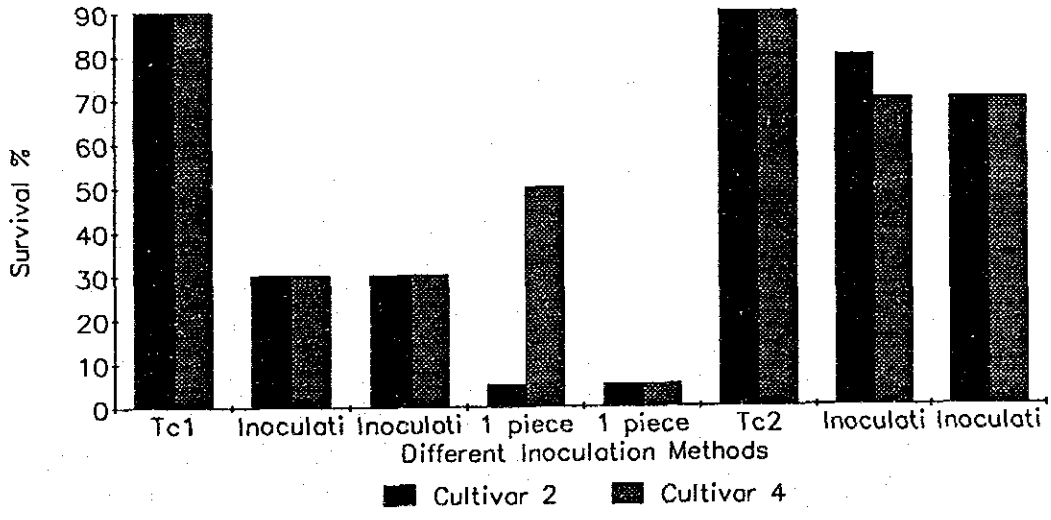

Fig.8 Survival of rice cultivar 2 and cultivar 4 using different containers and different ice

inoculation methods .Samples of Tc.1 and the following treatments were in plastic cryotube.Samples of Tc.2 and the following treatments were in glass cryotube.Specimens were cooled 0.3°C/min to -30°C and stored 12 h in LN.

Fig.8 shows different survival of rice callus using different cryotubes.Survivals of treated controls are the same in spite of different containers.However,LN treated callus in plastic cryotube exhibited much lower survivals than those treated in glass cryotube for both cultivars under optical conditions.Cultivar 2 exhibited lower survival than cultivar 4 in plastic cryotube using 1 piece of plant leaves.

Effects of different ice inoculation method for plastic cryotube on the survival of rice callus are showed in Fig 9 and Fig 10.

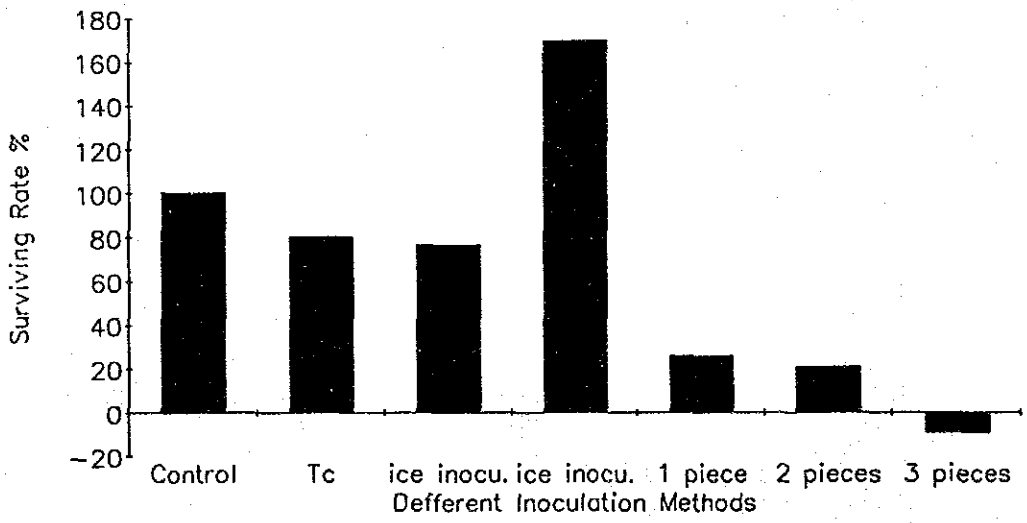

Fig.9 Effects of ice inoculation method on the survival of rice cultivar 1 frozen and stored in LN for 12h using plastic cryotube.Eleven-d-old callus were sampled,cooled 0.3°C/min to -30°C and then immersed in LN.

In Fig 9,cultivar 1 showed good survivals by using manual ice inoculation method but exhibited poor viability by using plant leaves to crystalize cryoprotectant solution.Nontheless,in Fig.10,cultivar 4 showed good results by using plant leaves.Other ice inoculation method had little effects on the survival of callus.

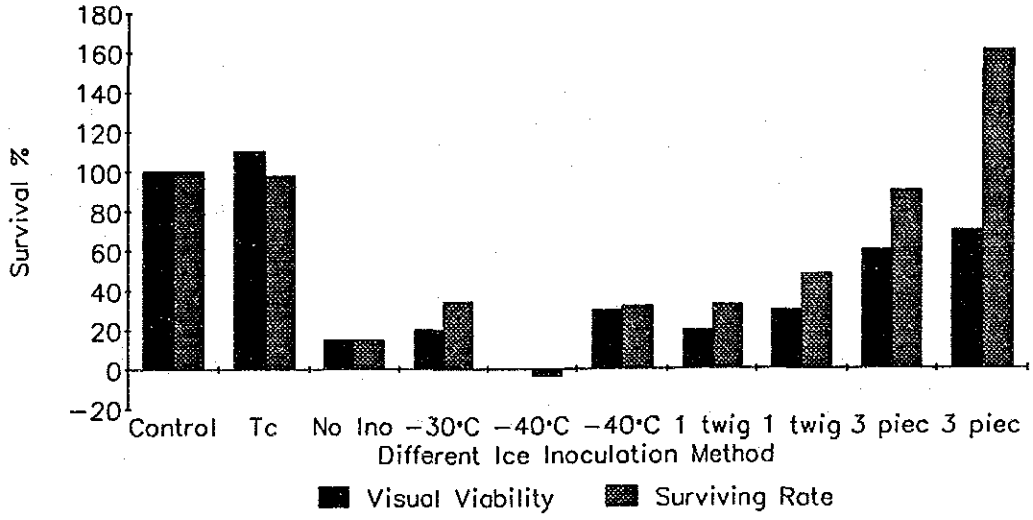

Fig.10 Effects of ice inoculation methods on the survival of rice cultivar 4.Specimens in plastic cryotube were cooled 0.3°C/min to -30°C and stored 12 h in LN.

DISCUSSION

The results obtained were quite primary and limited .We still have a lot of work to do to cryopreserve rice callus practically.After regrowth,average viability of callus treated in plastic cryotube is only 30% which is much lower than that of callus treated in glass cryotube.The reason may be the difference of containers. Plastic and glass have different conductivity of temperature .So the

technique received by using glass cryotubes may be to some degree have problems when applied to plastic cryo-tube.So we need to do more work using plastic cryotubes to find optical conditions to cryopreserve rice callus.

We have studied some main factors(exposure time,cooling rate and terminal temperatures) which affect the survival of rice callus stored in LN.That is not sufficient.For example the age of callus sampled is also a critical factor which contributes greatly to survival of rice callus stored in LN and regrew in N6 medium.Because in some phase in growth cycle,cells have big capacity to survive freezing and thaw-ing. In other phases,cells are sensitive.Results described above have showed some differences between cultivars.We need more work to find optical conditions for successful cryopre-servation of each cultivar.

From figures above,we can find that sometimes the results are not uniform.One of the features of rice callus is that clumps are mixed with cells of different ages because of growing in a semi-solid medium.This may be one of the reasons why the results somtimes were not consistent.To receive better results,we'd better try to purify callus by preculturing in specific medium or use cell suspencion cultures of rice.

ACKNOWLEDGEMENTS

I am greatly indebted to Dr.M.Ishikawa,senior research scientist on physiology of plant cold hardiness & cryopreservation in NIAR, for his guidance and help during this study.I feel also obliged to JICA and NIAR for providing me the facility to work in Japan.

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Technical Report

seyed mohsen hessamzadeh hejazi (IRAN) 1993

JAHAD DANESHGAHI UNIVERSITY OF TEHERAN

course:

Plant Genetic Resources

IMPROVEMENT OF " MAIZE METHOD " FOR WHEAT HAPLOID PRODUCTION.
- Effects of casaminoic acid, yeast extract and malt extract on the growth of haploid plants from haploid embryos

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INTRODUCTION

The haploid breeding method is of great value to plant breeders, since it enables to obtain the homozygous lines in short period. In wheat (Triticum aestivum L., 2n=6x=42), there are two different methods to produce haploid plants systematically. One is the anther culture method and the other is the immature haploid embryo culture method following the hybridization with maize (Zea mays L., 2n=2x=20) or Hordeum bulbosum L. (2n=4x=28). Though the haploid embryo culture method has a higher frequency of haploid production than anther culture method, the application of "Hordeum bulbosum method" is restricted by the wheat genotype. The wheat genotype carrying the cross-incompatibility gene Kr1 and/or Kr2 can not produce the haploid embryo by the hybridization with Hordeum bulbosum (SNAPE et al 1979). On the other hand, the hybridization with maize can produce haploid plants even from the wheat genotype carrying the Kr gene(s). Most of the wheat cultivars except those of Asian origin have Kr gene(s) (FALK and KASHA 1981). Therefore, the

"Maize method" is thought as the most suitable haploid production method for practical breeding.

For the application of maize method to the practical breeding, it is important to increase the efficiency of production of haploid plants. In this paper, the effects of the supplements such as casaminoic acid, yeast extract and malt extract to the medium for the embryo culture were investigated.

MATERIALS AND METHODS

Crossing procedure

Two wheat genotypes , Norin 61 and Chinese Spring , were grown in the field and used as female parents. The F1 hybrid maize B14 x CI64 was grown at 18 - 25 °C in a green house and used as the pollen donor. The wheat spikes at the stage of 1 or 2 days before flowering were emasculated by the heat shock emasculation method. The heat shock emasculation was carried by putting the spikes into the hot water for 5 minutes that was kept at 43 °C . The emasculated spikes were detached and cultured in water for one day. One day after emasculation, wheat florets were pollinated with the freshly collected maize pollen and covered with the paper bags. After the pollination, the detached spikes were cultured under the conditions of 24°C and 16-hour photoperiod in the liquid medium that contained 100 mg 2,4-Dichlorophenoxyacetic acid (2,4-D), 10 ml ethanol, 8 ml sulfurous acid and 40 g sucrose per liter.

Embryo culture

Fourteen days after the pollination, seeds were removed from the florets and immersed in 70 % ethanol for one minute and subsequently in 1 % antiformin for 5 minutes. Following a rinse with sterile water , the embryos were asceptically taken out and cultured in petri dish.

Eight kinds of solid mediums were used in this experiment. All mediums contained the half strength MS (MURASHIGE and SKOOG 1962) salts and vitamins, 2 % sucrose, 0.02 % gelrite, but differed with each other in the supplements. Casaminoic acid (100, 500 or 1000 mg/l), yeast extract (100 or 1000 mg/l) or malt extract (100 or 1000 mg/l) was used as the supplement.

For first 7 days, the embryos were cultured at 25 °C in dark and after that under the conditions of 20 °C and 12-hour photoperiod . At 40 days after the embryo rescue, the numbers of embryos with normal shoot and with vitrificated shoot ,and embryos forming callus with root(s) and without root, and non-developed embryos were recorded.

Experiment plan and data analysis

For the investigation of the effects of medium and cultivar on the normal shoot formation, all treatment blocks had two replications except the block of (Chinese Spring - yeast extract 1000 mg/l).The embryos cultured were derived from at least 2 spikes, usually more than 10 spikes for each replication. Analysis of variance was performed after the arcsin

transformation for all data, but the data for the blocks of the medium with yeast extract 1000 mg /l were not used for the variance analysis because one replication was lacked in Chinese Spring.

RESULTS AND DISCUSSION

The frequencies of the non-emasculated floret, the seed set from the emasculated florets and the embryo obtained from seeds are shown in Table 1. The non-emasculated florets were easily distinguished by the milky endosperms, because the emasculated and pollinated florets with the maize pollens did not have milky endosperm. The frequencies of the non-emasculated florets were as small as 1.91 % in Norin 61 and 14.06 % in Chinese Spring. Therefore the heat shock treatment was thought as the labor-saving emasculation method, but further investigation is necessary to know the influence of the heat shock emasculation on the development of haploid embryos. There was a large varietal difference in the frequency of seed set from florets, but small varietal difference in the frequency of embryo obtained from seeds.

The frequencies of shoot and callus formation are shown in Table 2 and the frequency of normal shoot formation is shown in Fig. 1. On all mediums, the frequencies of normal shoot formation in Chinese Spring were higher than in Norin 61 . The variance analysis also indicated that Chinese Spring had a higher frequency of normal shoot formation than Norin 61 at 1% level of significance (Table 3).

Although the effect of medium on the frequency of normal

Table 1. Frequencies of the non-emasculated floret, the seed set from florets pollinated and the embryo obtained from seeds

cultivar	No. of florets pollinated	Frequency of non-emaculated floret (%)	Frequency of seed set (%)	Frequency of embryo obtained from seeds (%)
Norin 61	5723	1.91	74.73	23.78
Chinese Spring	2176	14.06	56.84	25.87

Note : For the calculation of the frequency of seed set, the number of non-emasculated florets was eliminated from the number of florets pollinated.

Table 2. Effects of medium and cultivar on the development of haploid embryo of wheat

1) medium	cultivar	Frequency (%)						
		shoot formation			callus formation			Non developed embryo
		(total)	normal	vitrificated	(total)	with root	without root	
1/2 MS (control)	N61	14.7	7.8	7.0	78.5	34.2	44.4	6.8
	C. S.	46.7	15.6	31.2	40.6	25.5	15.2	12.7
1/2 MS+CA 100mg	N61	25.6	5.5	20.1	67.1	40.5	26.6	7.4
	C. S.	22.8	9.1	13.7	62.1	36.4	25.8	15.2
1/2 MS+CA 500mg	N61	20.7	4.9	15.8	72.1	38.5	33.6	7.4
	C. S.	58.8	34.1	24.6	30.2	27.8	2.4	11.1
1/2 MS+CA 1000mg	N61	7.2	2.4	4.8	86.7	44.1	42.9	6.0
	C. S.	46.7	25.7	21.1	15.8	5.3	10.6	37.5
1/2 MS+YE 100mg	N61	10.4	1.0	9.4	87.6	40.7	46.7	2.1
	C. S.	16.7	8.4	8.4	76.0	28.3	47.8	7.4
1/2 MS+YE 1000mg	N61	10.1	2.6	7.6	87.5	34.3	53.2	2.4
	C. S.	36.8	15.8	21.1	52.6	0.0	52.6	10.5
1/2 MS+ME 100mg	N61	18.0	7.9	10.1	72.5	48.0	28.7	5.6
	C. S.	45.2	35.7	9.5	38.1	17.9	20.3	16.7
1/2 MS+ME 1000mg	N61	11.5	3.1	8.5	86.2	40.0	46.2	2.3
	C. S.	30.6	5.6	25.0	58.4	38.4	25.0	11.2

1) See Fig. 1.

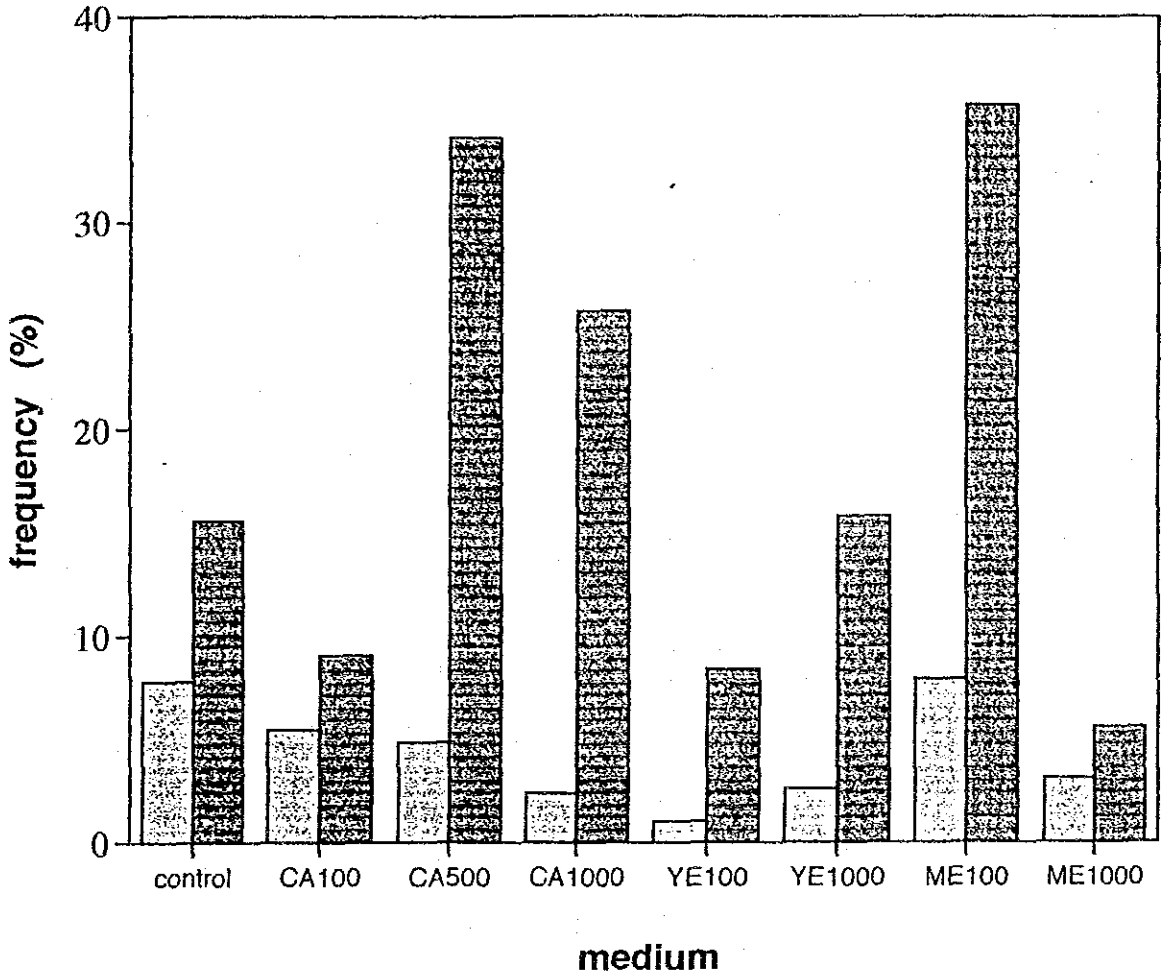

Fig. 1 Frequency of normal shoot formation

Norin 61 Chinese Spring

control : 1/2 MS
CA100 : 1/2 MS+casaminoic acid 100mg/L
CA500 : 1/2 MS+casaminoic acid 500mg/L
CA1000: 1/2 MS+casaminoic acid 1000mg/L
YE100 : 1/2 MS+yeast extract 100mg/L
YE1000 : 1/2 MS+yeast extract 1000mg/L
ME100 : 1/2 MS+malt extract 100mg/L
ME1000 : 1/2 MS+malt extract 1000mg/L

Table 3. Results of variance analysis on the frequencies of each developmental type of haploid embryo

factor	degree of freedom	shoot formation			callus formation			Non developed embryo
		(total)	normal	vitrificated	(total)	with root	without root	
medium	6	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
cultivar	1	*	**	n.s.	**	*	**	n.s.
medium×cultivar	6	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.

note: Data obtained from the medium with yeast extract 1000 mg /l were not used in this variance analysis, because one replication in Chinese Spring was missing.

shoot formation was not statistically significant ($0.05<P<0.10$), there were fairly large differences of the effects among mediums. In both cultivars, the frequency of normal shoot formation was the highest on the medium supplemented with 100 mg/l of malt extract (ME100).

In Norin 61, the frequencies of normal shoot formation on all mediums except ME100 were smaller than control. The frequencies of normal shoot formation in Norin 61 on the mediums supplemented with casaminoic acid were decreased along with the increase of its concentration . In a result, all the supplements used in this experiment were thought to inhibit the normal shoot formation in Norin 61 except for the low concentration of malt extract. The frequencies of normal shoot, vitrificated shoot and callus formation in Norin 61 are shown in Fig. 2. The depression of the frequency of normal shoot formation on the medium supplemented with casaminoic acid was attributed to the increase of vitrificated shoot formation on the mediums of casaminoic acid 100 mg/l (CA100) and 500 mg/l (CA500) and attributed to the increase of callus formation on 1000 mg/l (CA1000). The depression of the frequency of normal shoot formation on other mediums was also attributed to the increase of callus formation.

On the other hand in Chinese Spring, the frequencies of normal shoot formation on the mediums of CA500 and CA1000 in addition to ME100 were also higher than control (Fig. 1). Therefore, in Chinese Spring, middle and high concentrations of casaminoic acid were thought to stimulate the normal shoot formation. The stimulation mechanism of normal shoot formation in

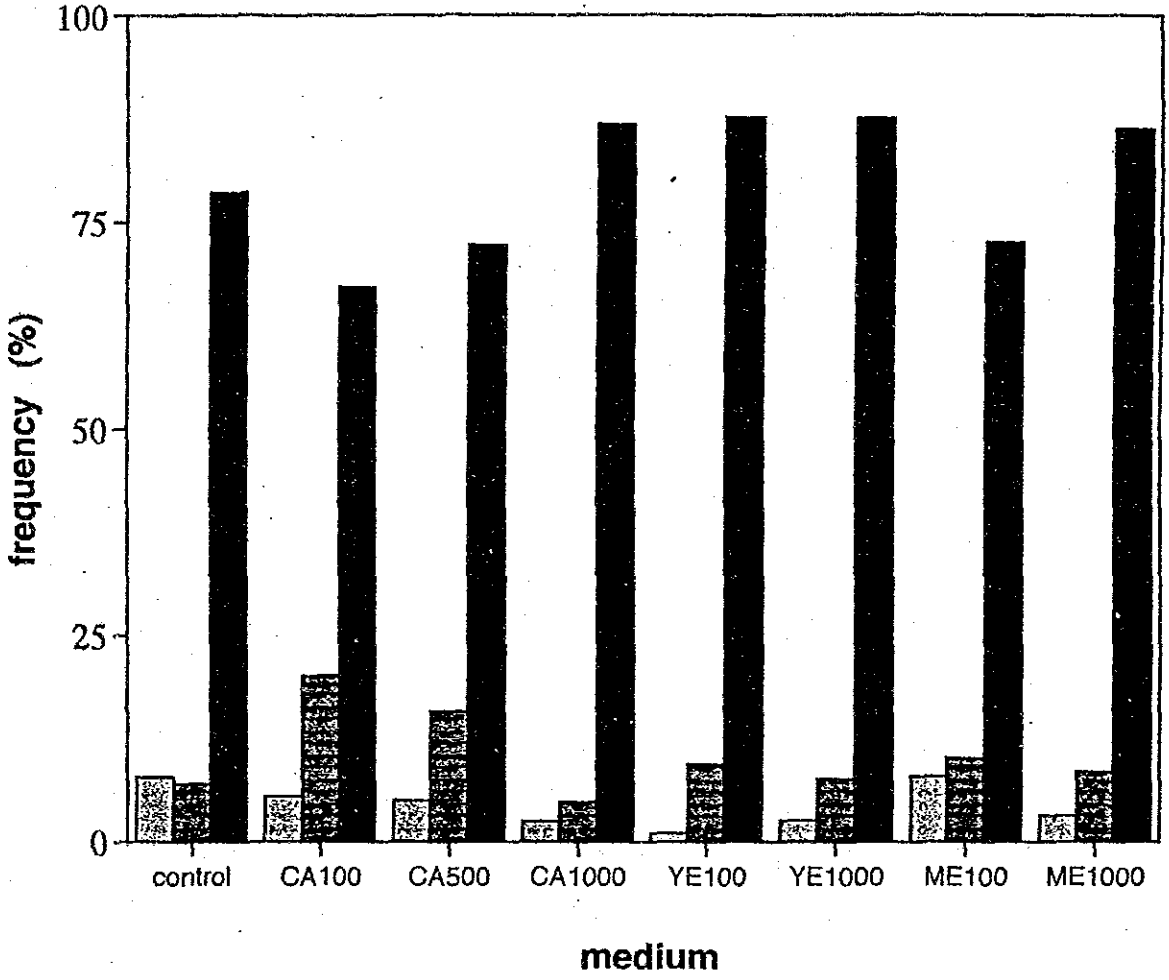

Fig. 2 Frequency of normal shoot,vitrificated shoot and callus formation in Norin 61

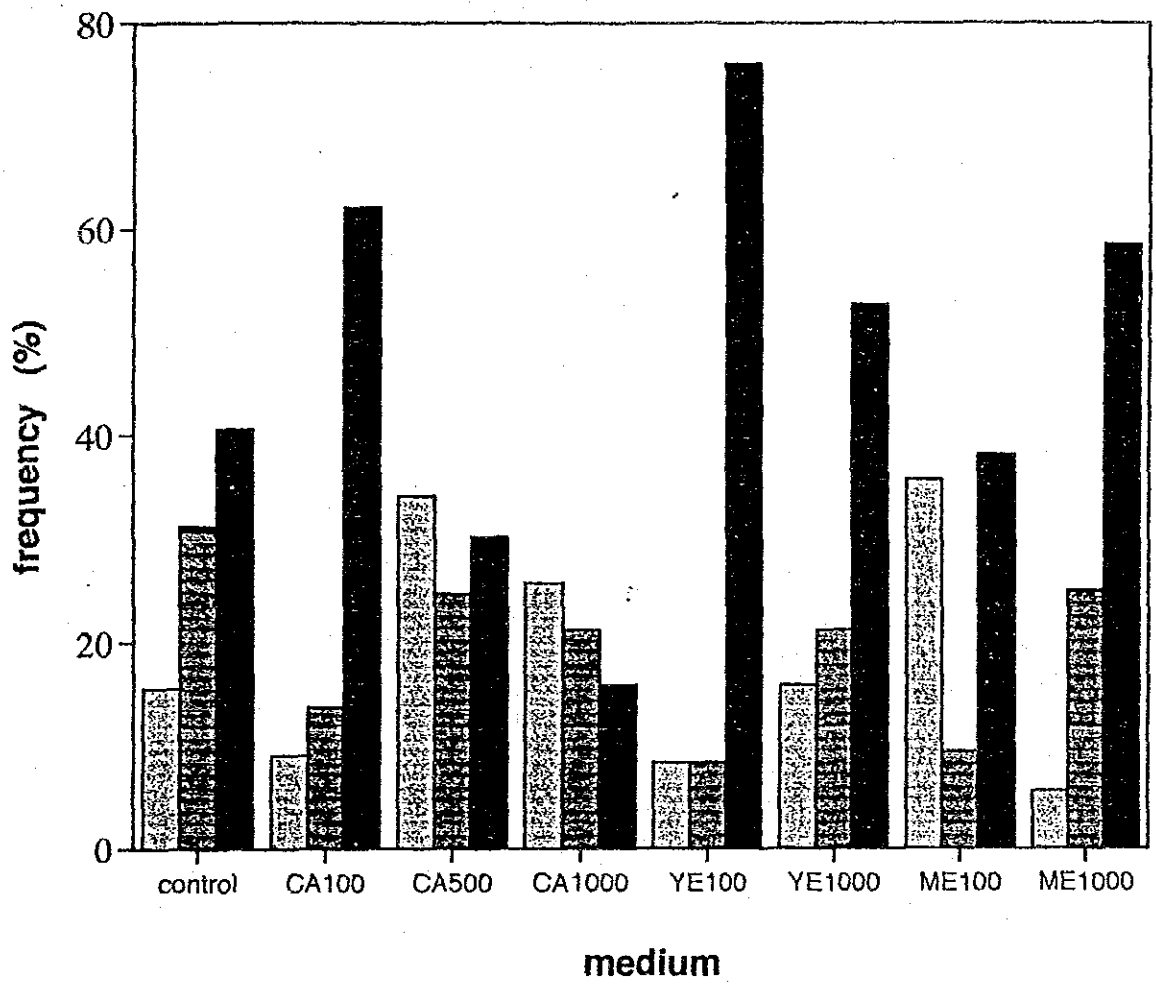

Fig. 3 Frequency of normal shoot,vitrificated shoot and callus formation in Chinese Spring

Chinese Spring was thought to be different among mediums with different supplements . On the medium of ME100, the frequency of vitrificated shoot formation was lower than control but the frequency of callus formation was almost the same (Fig. 3). The medium of ME100 increased the normal shoot formation by the decrease of vitrificated shoot formation, not by the decrease of callus formation. On the other hand, the mediums of CA500 and CA1000 decreased both of the vitrificated shoot formation and callus formation in Chinese Spring.

In a result, the effect of casaminoic acid was thought to be opposite by the wheat genotypes, Norin 61 and Chinese Spring. Therefore the application of casaminoic acid in practical haploid breeding is thought to be difficult. On the other hand, the malt extract is thought to be suitable to use in practical breeding because its effect was also different between the cultivars used, but it was not changed to the inhibitor of the normal shoot formation.

The main components of three kinds of supplements are the amino acids that derive from the hydolysis of milk protein, germination of barley or yeast. The differences of the effect of supplements on the normal shoot formation might depend on the differences of the amino acids composition and some minor element contained in supplements. The superior effect of malt extract was thought natural because it might contain the components necessary for the germination of barley and they might also be effective for the germination of wheat haploid embryos.

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RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS OF PSEUDOMONADS ISOLATED FROM RICE SEED AND SEEDLINGS

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ABSTRACT:

The seedlings of nine rice accessions showing the symptoms of seedling rot were collected from nursery beds of National Institute of Agrobiological Resources, Kannondai, Tsukuba. From six accessions, white pigmented colonies were isolated while three accessions had both white and yellow pigmented colonies. Out of 66 isolates, 42 were found to be pathogenic to rice. The genomic DNA from the strains of white colonies was extracted and processed for RFLP analysis using EcoR1 and BamH1 as restriction enzymes. For hybridization of DNA, rRNA 16 +23 S from E. coli was used as probe. The banding patterns of DNA from pathogenic strains of bacteria isolated from the rice seedlings were quite distinguishable from those of the genomic DNA of Pseudomonas glume, P. fuscovaginae, and P. avenae. Based upon this study, it is suggested that P. glume, P.fuscovaginae or P. avenae were not the casual agent for this disease. The cause of this disease may be bacteria belonging to group I or II isolated from rice seedlings or it may be a complex disease.

INTRODUCTION:

The bacterial diseases cause a considerable loss in rice yield worldwide. The extent of yield loss depends on pathogen, locality, season, weather, and cultivar (Mew 1987). In Punjab, Haryana, and western Uttar Pardesh States of India, major epidemics occured in 1979 and 1980 due to bacterial blight and total rice crop failure was reported (Durgapal 1985). Recently rice bacterial diseases have attracted more attention and many new diseases have been discovered during the last 30 years. The introduction of high yielding varieties, farm mechanization, and change in cultural practices have also caused new diseases in rice. The literature reports indicate at least 15 bacterial diseases of rice which are caused by Xanthomonas, Pseudomonas, and Erwinia species (Ou 1985 and Mew 1987). The diseases of rice caused by bacteria are presented in Table 1. The Xanthomonas species causes 4 diseases including bacterial leaf blight and leaf streak. Pseudomonas causes 7 diseases mostly of seed and seedlings while Erwinia species are responsible for foot rot and palea browning. It is intersting to mention that 10 diseases out of 15 were recorded first from Japan.

For epidemiological studies of bacteria, disease causing agent must be identified to the species level. Identification of bacteria based upon morphological features is difficult beacuse of their very small size. The virulence, restriction fragment length polymorphism (RFLP), protein electrophoretic patterns, and fatty acid profiling techniques have often been used to study pathogenic diversity and in addressing taxonomic problems in species of bacteria(De Vos and De Ley 1983, Grimont and Grimont 1986). Now

molecular approaches are used increasingly in taxonomy and epidemiology of different species of bacteria. Restriction fragment length polymorphism analysis of genomic DNA, based on hybridization with probes, have been used to differentiate the bacteria(Gottwald et al.1991 , Hartung and Civerolo 1989).

During the year 1992 and 1993, a disease having different symptoms than previously known bacterial diseases was observed on rice seedlings in nursery beds at National Institute of Agro-biological Resources (NIAR), Tsukuba, Japan. The symptoms of the disease were appearing in the form of necrotic spots on the leaves which were enlarging in size and ultimately causing the death. The present study aimed to isolate the casual organism, study their pathogenicity and taxonomic identification.

MATERIALS AND METHODS :

The seedlings of nine rice accessions namely 8700055(line 38, Thailand origin), 8700172 (line 85, Nepal origin), 8700294 (line 106, Malaysia origin) 8700279 (line 109, Malaysia origin), 8700302(line 113, Malaysia origin), 8700312 (line 119, Malaysia origin), 8700313 (line 120, Malaysia origin), 8700324 (line 128, Malaysia origin) and 8710002 (line 141, Burma origin) affected by a disease were collected from rice nursery beds at National Institute of Agrobiological Resources, Tsukuba, Japan in May 1993. The research work was carried out at Microorganism Germplasm Evaluation Laboratory of Plant Genetic Resources Centre.

ISOLATION OF BACTERIA AND PATHOGENICITY TEST: The diseased plants

collected from the field were crushed and seven dilutions upto 7^{-1} were made. One ml of extract from dilution no.4-7 was taken in sterile petri dish and 3ml of PSA medium was added in each petri dish. The petri dishes were kept in incubator at 25° c overnight. Next day pure representative colonies were transferred in test tubes on slants of Wakimoto Agar medium. The test tubes were put in incubator at 25° c for 24-48 hours for bacterial growth. The pathogenicity of bacteria was tested by soaking the rice seeds in bacterial suspension for 24 hours and germinating them on sterile soil in petridishes.

EXTRACTION OF DNA: Selected pathogenic strains of bacteria isolated from rice seedlings were grown separately on 8 ml of PSA liquid medium for 14-16 hours by shaking at 25° c. Bacterial cells were harvested from suspension by centrifuging at 5000 rmp for 10 minutes. The pellet of bacteria was washed with STE buffer and centrifuged again for 10 minutes at 5000 rmp. Pellet of bacteria was resuspended in TE buffer(10mM Tris Hcl,1mM EDTA,pH) and treated with 260µl of 10 % SDS buffer and 26 µl of proteinase K (20 mg/l) for lysis and then incubated at 37° c for 1 hour. Eight hundred eighty µl of 5M Nacl and 690 µl of CTAB was added for extracting polysaccharides and then incubated at 65° c for 10 minutes. Deproteinization was performed by sequential chloroform- isoamyl alcohol(24:1) and phenol-chloroform+ isoamyl alcohol(25:24:1) extraction. The upper layar was then extracted twice with ether. Precipitation of DNA was carried out by propanol (1:0.6 v/v)and washed with cold 70 % ethanol. The precipitates of DNA were dried and suspended in 100 ul of TE buffer. One ul of RNase(10 mg/l)was

added in each sample and stored at 4° c.

DIGESTION, GEL ELECTROPHORESIS & TRANSFER: Five micro gram of each DNA sample was cleaved for 5 hours at 37° c by restriction endonucleases EcoR1 or BamH1 and 1.5µl of reaction buffer. Horizontal gel electrophoresis of DNA samples was carried out as described by Maniatis et al (1982) by using 0.8 % agarose gel in Tris_ borate buffer. As DNA marker, DNA λH3 and 1Kb was included. Transfer of DNA to a BA 83 nitrocellulose membrane was carried out as described by Southern (1975).

PREHYBRIDIZATION, HYBRIDIZATION & PROBE: The transfer membranes were prehybridized at 42° c for 4 hours with shaking in a solution of 5 X SSC(50% formamide, 50mM Na-phosphate buffer pH 6.5, 5mM EDTA &0.2 % SDS), 5 Denhardt s solution (1 Denhardt s solution is composed of 0.2 mg/ ml polyvinyl pyrollidone 350, 0.2 mg/ml of Ficoll 400, 0.2mg/ml of bovine serum albumin(BSA) & 0.2 mg/ml denatured DNA(ex- Calf Thymus, herings sperma). The membranes were hybridized for 18 hours at 42° c with 4 volume of prehybridized solution + 1 volume 0.5g/ml dextran sulphate. Photobiotin labelled 16+ 23 S r RNA from E. coli was used to detect fragments in the genomic DNA.

RESULTS AND DISCUSSIONS

PATHOGENICITY TEST:

The bacteria having white or yellow pigmented colonies were isolated from all the nine accessions of rice seedlings collected from the field. The kind of bacterial colonies isolated from each accession are listed in Table 2. From 6 accessions, only white

pigmented colonies were isolated while from seedlings of 3 accessions, two types of colonies having white or yellow colour were recorded. When the seeds of the accessions were processed for bacterial isolation, only two accessions were found having seed borne bacteria (data not shown). From all 9 accessions of seed and seedlings, 66 pure representative colonies were isolated. The pathogenicity test revealed 42 strains to be pathogenic (Table 3), white pigmentd colonies being more in percentage as regards their pathogenicity. The pathogenic strains also varied in their degree of pathogenicity ranging from high to low.

RFLP ANALYSIS:

The banding patterns of genomic DNA from pathogenic strains of bacteria isolated from rice seedlings are distinguishable from the genomic DNA of Pseudomonas glume, P. fuscovaginae, and P. avenae as shown in Fig.1.The patterns after digestion of DNA with EcoR1 and Bam H1 as well as after using the probe rRNA from E. coli are showing different patterns than the Pseudomonas species under this investigation. The banding patterns of genomic DNA among P. glume, P. fuscovaginae and P. avenae are also clearly distinct from each other suggesting the usefulness of this technique in taxonomic identification. The probe rRNA hybridized multiple discrete bands in the RFLP profiles which indicates that there is a high frequency of repeated sequences. The banding patterns of strains isolated from rice seedlings after cleaving the genomic DNA with restriction enzyme Bam H1 could be divided in two groups excepts the strains 113-VI and 120-1-R which had unique banding patterns. The four

strains of bacteria isolated from rice accession no. 8700055 (Thailand origin, line 38) have the same banding pattern(group 1) while the strains isolated from accessions no. 8700294 , 8700312 (Malaysia origin, line 106 and 119) can be put in another group (group II) based upon their similar banding patterns. The banding patterns after cleaving the DNA with restriction enzyme EcoR1 could also be put in two groups similar to BamH1. The strain 120-I-R isolated from accession no. 8700313 and strain no. 38-IV isolated from accession 8700055 had unique banding patterns differing from other isolates and three species of Pseudomonas. Among the two groups classified , the differences in banding patterns were noted at one or two sites. There is possibility that the strains isolated from rice seedlings are different races of one species or the differences may be intraspecific. The results confirm the previous investigations of Hartung and Civerlo 1989 and Gottwald and Alvarez 1991 that bacterial strains could be differentiated from each other based upon banding patterns after RFLP.

It is suggested from the present investigations that the disease observed in rice nursery beds was not caused by Pseudomonas glume, P. fuscovaginae or P. avenae. The casual agent for this particular disease may be bacterial species belonging to group I or II isolated from rice seedlings or it may be a complex disease where different bacteria have been involved.

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TABLE: 1. BACTERIAL DISEASES OF RICE

S.No.	Diasese	Bacterium	Reported from	Year	By Whom
1	Bacterial leaf blight	Xanthomonas compestris pv oryzae.	Japan	1884	Farmers
2	Bacterial leaf streak	X. compestris pv.oryzicola	Philippi -nes.	1918	Reinking
3	Bacterial brown stripe	Pseudomonas avenae	Japan	1961	Goto & Ohota
4	Sheath brown rot	P.fuscovaginae	Hungary	1955	Klement
5	Bacterial grain rot	P. glume	Japan	1956	Goto
6	Bacterial foot rot	Erwinia chrysanthemi	Japan	1979	Goto
7	Bacterial palea browning	E. herbicola	Japan	1931	Iwadara
8	Brown stripe	Erwinia species amylosa group	___	___	___
9	Glume blotch	P. syringae	Australia	1974	Gother
10	Bacterial black rot	X. itoana & E. herbicola	Japan	1931	Iwadara
11	Cinnamon speck of rice	X. cinnamona	Japan	---	Miyake Tsunoda
12	Black eye spot	X. atroviri digenum.	Japan	---	Togami & Mizukami
13	Seedling blight	P.plantarii	Japan	---	Nishiya -ma et.al
14	Bacterial holo blight	P.syringae pv.oryzae	Japan	1985	Kuwata
15	Bacterial sheath rot	P. syringae	---	---	Ou

Table 2. BACTERIAL COLONIES ISOLATED FROM RICE SEEDLINGS.

S.no.	Accession no.	Bacterial kinds	Colour of colonies	Dominant colonies
1	8700055 line 38	1	white	white
2	8700172 line 85	1	white	white
3	8700294 line 106	1	white	white
4	8700297 line 109	2	white & yellow	yellow
5	8700302 line 113	2	white & yellow	yellow
6	8700312 line 119	1	white	white
7	8700313 line 120	1	white	white
8	8700324 line 128	1	white	white
9	8710002 line 141	2	white & yellow	white

TABLE:3. PATHOGENICITY OF DIFFERENT STRAINS OF BACTERIA ISOLATED FROM RICE SEEDLINGS.

S no.	Strain no.	Pathogenicity	Degree of pathogenicity	Colour of colonies
1	38-I	+	medium	white
2	38-II	+	medium	white
3	38-III	+	medium	white
4	38-IV	+	medium	white
5	38-V	+	medium	white
6	119-I	-		white
7	119-II	+	medium	white
8	119-III	-		white
9	119-IV	+	low	white
10	119-V	+	medium	white
11	119-VI	+	high	white
12	85-I	+	low	white
13	85-II	+	low	white
14	85-III	+	low	white
15	85-IV	+	low	white
16	85-V	+	low	white
17	85-VI	-		white
18	85-VII	-		white
19	113-I	-		yellow
20	113-II	-		yellow
21	113-III	-		yellow
22	113-IV	-		yellow
23	113-V	-		yellow
24	113-VI	+	medium	white
25	113-VII	+	medium	white
26	128-I	-		yellow
27	128-II	-		yellow
28	128-III	-		yellow
29	128-IV	+	medium	yellow
30	128-V	+	low	yellow
31	141-I	+	medium	yellow
32	141-II	+	medium	yellow
33	141-III	+	low	white
34	141-IV	+	low	white
35	141-V	-		white
36	106-I	+	medium	yellow
37	106-II	-		yellow
38	106-III	+	high	white
39	106-IV	+	high	white
40	109-I	+	medium	yellow

41	109-II	+	medium	yellow
42	109-III	+	medium	yellow
43	109-IV	-		yellow
44	120-I	+	high	yellow
45	120-II	+	medium	golden
46	120-III	+	low	white
47	120-IV	+	low	yellow
48	120-V	-		white
49	106-I-R	-		white
50	106-II-R	+	low	white
51	106-III-R	-		yellow
52	106-IV-R	+	high	white
53	109-I-R	-		yellow
54	109-II-R	+	low	white
55	109-III-R	+	medium	white
56	109-IV-R	+	medium	yellow
57	109-V-R	+	low	white
58	120-I-R	+	low	white
59	120-II-R	-		white
60	120-III-R	+	high	white
61	120-IV-R	+	low	white
62	141-I-R	-		yellow
63	141-II-R	-		yellow
64	141-III-R	-		yellow
65	141-IV-R	-		yellow
66	141-V-R	+	low	yellow

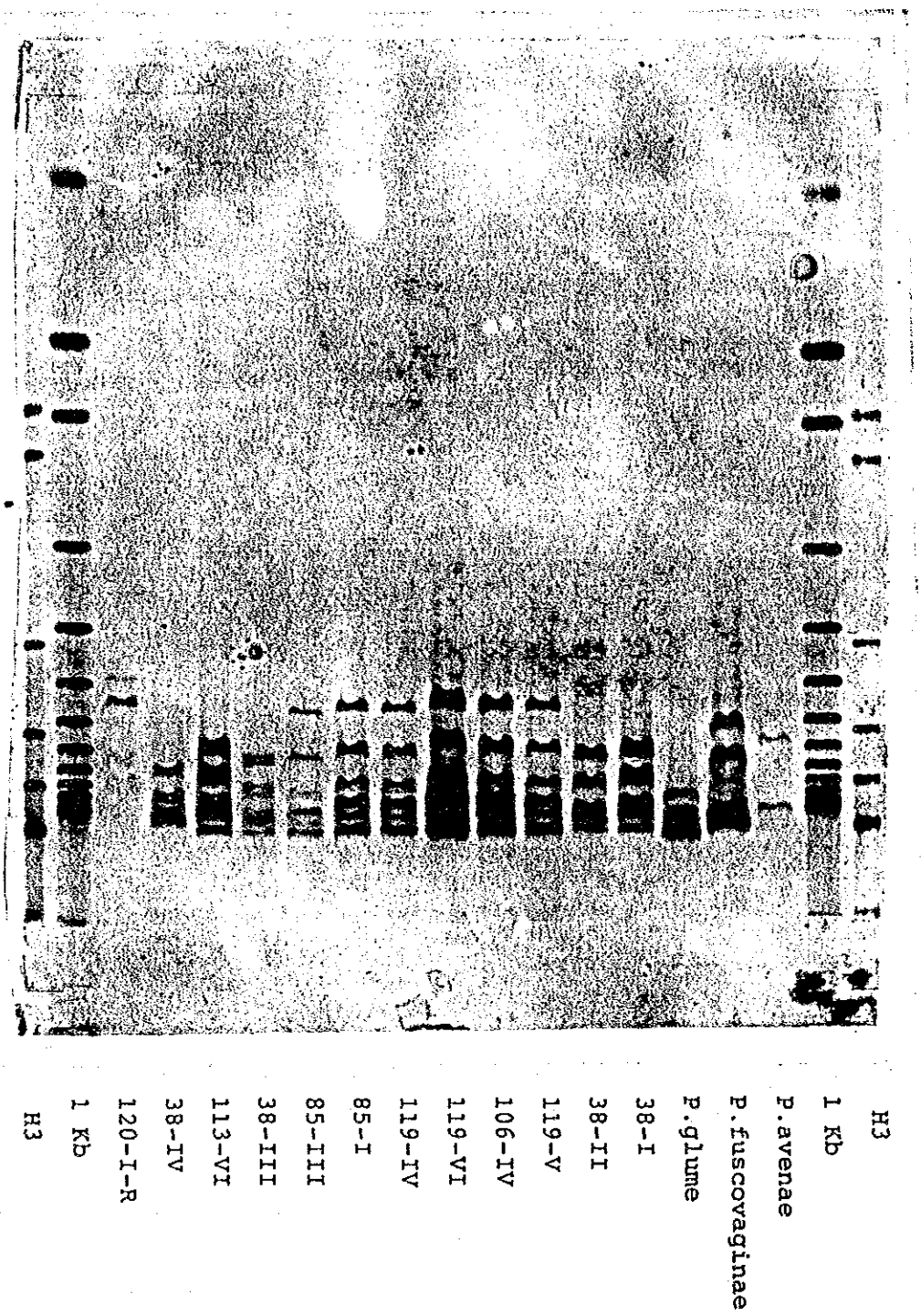

Fig1. Southern hybridization of genomic DNA after digestion with restriction enzyme Bam H1 and hybridized with rRNA from E.coli.

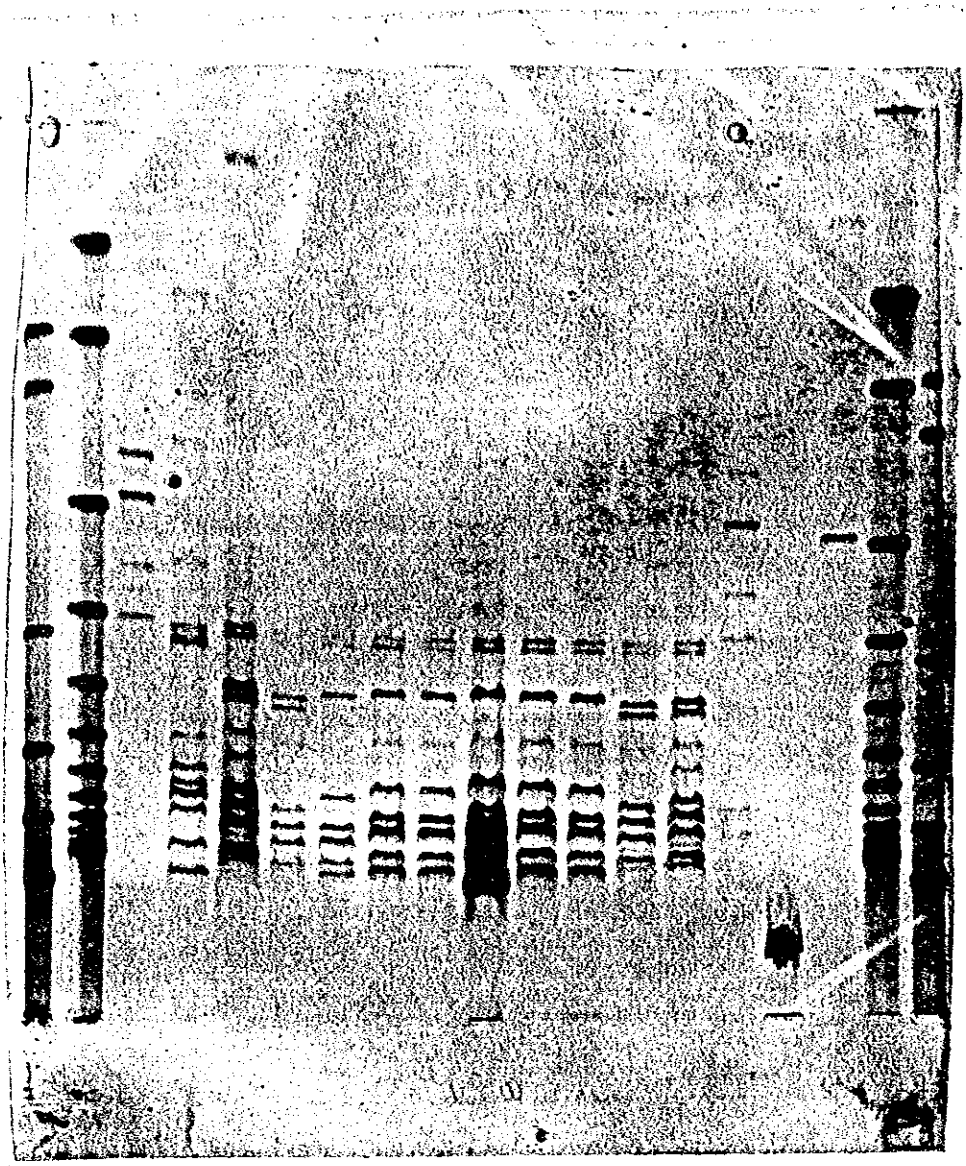

Fig 2. Southern hybridization of genomic DNA after digestion with restriction enzyme Eco R1 and hybridized with rRNA from E.coli.

PLANT GENETIC RESOURCES

TRAINING COURSE

- 1993 -

DATA MANAGEMENT SYSTEM

Manuel S.Paniagua - Paraguay

1-Introduction

This report contains a brief explanation about the use of INFORMIX-SQL in the management of data on genetic resources. Generally the management systen for genetic resources comprises,conservation data management,passport data management,and characterization and evaluation data management.
The reason why the management systen should be divided into three parts is to obtain hight efficiency of the data management and the accession numbers are adopted as the key to connect the categorized data one another.
In management of genetic resources,the most important work is management of the conservation data after that the basic is management of passport data and the characterization and evaluation data should become in the most useful information for users.

2-Use of INFORMIX-SQL in Data Management Systen

The systen provides two ways to create database and tables, on the one hand is using the main Menu and on the other hand is using RDSQL query language.
Firstly, I explain the use of main Manu:
Selecting Database option on the main Manu I created a database named GENKO,after that the step was to create tables,in this sense we made up seven tables and all they are refered to passport table, as following:

A-Passport

Column Name	Type	Length	Index	Nulls	Refer to
acc_number	char	6	index	no	
reg_date	date			no	
organ_code	char	5		no	B
lab_code	char	5		no	E
plant_code	char	5	index	no	C
culti_name	char	60		no	
others_name	char	60		yes	
status	char	1		no	
source	char	1		no	
origin_code	char	3	index	no	D
intr_place_code	char	3		no	D
intr_organ_code	char	5		no	B
intr_year	char	4		no	
former_number	char	8		no	
cons_class	char	1		no	
cons_form	char	1		no	
remark	char	60		yes	

B-Organization Code

Column Name	Type	Length	Index	Nulls
organ_code	char	5		no
organ_name	char	40		yes
short_organ_name	char	70		yes

C-Plant Code

plant_code	char	5		no
plant_name	char	40		yes
plant_sci_name	char	70		yes

D-Origin Code

origin_code	char	3		no
origin_name	char	60		yes
spanish_name	char	60		yes

E-Laboratory Code

lab_code	char	5		no
lab_name	char	15		yes
short_lab_name	char	10		yes

F-Address

acc_number	char	6		no
loc_number	char	6		no
quantity	decimal	6,2		no
germ_ratio	decimal	5,2		no
germ_test_date	date			no
last_takeout_date	date			yes
operator_name	char	20		yes
remark	char	60		yes

_G-Distribution

a-user

distr_number	char	8		no
user_name	char	20		no
user_address	char	60		yes

b-variety

distr_number	char	8	no
acc_number	char	6	no

Each tables ~~and tables~~ consist in rows and columns that may be store many different types of data,such as accession number,organization code,plant code,cultivar name,the list is endless.
To create indexes for the columns in a table help us to find information more quickly.
The keyword,Not Null means that this column must have been input.
The easiest way to store data in a table is using a screen form which contains blank spaces called fields and look like this:

```
acc_number     [       ]
reg_date       [          ]          organ_code    [       ]
plant_code     [      ]              lab_code      [      ]
culti_name     [                                                ]
others_name    [                                                ]
status         [ ]                    source       [ ]
origin_code    [       ]             intr_place_code  [    ]
intr_organ_code    [      ]          intr_year     [     ]
former_number  [         ]
cons_class     [ ]                  cons_form     [ ]
remark         [                                                ]
```

---

* screen form of passport data table.

What we can enter in a field depends on the data type of the column to which the field corresponds.There are four major types of field,NUMERIC (smallint,integer,smallfloat,float, decimal,serial or columns);CHARACTER;DATE and MONEY.
PERFORM does not allow to enter data of the wrong type in the field.
Creating a screen form is a two step process.First,to create a form specification,and then compile it.Once the specification files is compiled the form is used in PERFORM. These steps must be repeated for each created form.
After stored some data in a database,the same screen form can be used to inquire about the data.The Query option let us to find information filling one or more fiels on the screen form with the data that appears in the row or rows we want to find.
Relational and range operator,wildcard characters,highest-to-lowest may be used.

Secondly,I explain the use of RDSQL query language.
Selecting Query Language option on main Manu we can access RDSQL and enter one or more statements,which consist in a

simply instruction that tells RDSQL what we want to do and display only the information requested.
RDSQL statements contains instructions that define the characteristics of the table.

After named the table,enter a name and data type for each column ,the following is an example of create table statements:

```
Create table address
(
  acc_number      char (6) not null,
  loc_number      char (2) not null,
  quantity        decimal (6,2),
  germ_ratio      decimal (5,2),
  germ_test_date  date,
  last_takeout_date  date,
  oprator_name     char (20),
  remark           char (60),
);
```

---

* table address with statements.

Once entered and run create table statement successfully,we have to save in a command file and use again at any time.
Select statement serve to query a database and display the results on the screen form,select has seven clauses being the most important Select and From,both are required while the others are optional (Where,Order By,Group By,Having, Into Temp).
For example we can search all row and column from a table using this format:

```
Select * from passport          (see appendix 1)
```

and also search for data from specific columns,for example:

```
Select   acc_number,quantity,operator_name
from  Address                   (see appendix 2)
```

To search for some,but not all rows in a table include a Where clause in a Select statement,is use to specify the search criteria,RDSQL uses to determinate which row to retrive,for example:

```
Select   acc_number,quantity,germ_ratio
From   Address
Where   germ_ratio <=89            (see appendix 3)
```

Also can be retrieved data from more than one table in a query as long as all the tables we are working with belong

to the same database.By joining columns of the table has in common,we can query all they at the same time and find information about.For example:

```
Select   Passport.culti_name,Passport.reg_date,
         Passport.intr_year,Plant.plant_sci_name,
         Address.last_takeout_date,Address.operator_
         name
From     Passport,Plant,Address
Where    Passport.acc_number = Address.acc_number
         and Passport.plant_code = Plant.plant_code
```

After executing this statement,we can get culti_name, reg_date,intr_year,plant_sci_name,last_takeout_date, operator_name.Method like this is named normalization of tables.(see appendix 4)
To create Indexes for the columns in a table is necessary to enter a create index statement,such as:

```
Create unique index plnt_idx
       on Plant(plant_code)
```

This statement must be entered for each index,the word unique is with the purpose to prevent users from entering duplicate information in an index column.

REPORT is another important point available on main Menu, which is useful in management systen.
Some time is necessary to get database information,arranged and formated according to our instructions,and display, printed on paper,or store in a file for future use.
To create and display a report,we have to create a report specification,compile it,and run.
The following is an example of report specification,using NEW option on report Menu:

```
Database genko end
Output report to printer end
Select  intr_year,plant_code,culti_name
From  Passport
Order by intr_year
end
Format
Page header
print column 15,"cultivares"
skip 2 lines
print column 50,"july 25,1993"
skip 2 lines
print "intr_year",
column 15,"plant_code",
column 30,"culti_name"
skip 1 line
```

```
On every row
print intr_year
column 15,plant_code,

column 30,culti_name
After group of intr_year
skip 1 line
end
```

(see appendix 5)

All report specification must contain at least three section and they are:Database,Select,Format.
Database contains the information we want to use in the report.
Select is used to specify which rows and columns contain the information that we want to include in the report.
Format controls the apperance of a report.
Another way to create report is using a default report specification through Generate option on report Manu but it does not take adventage of many of powerful features available with ACE.
To send report to printer,we have to use the Report to Printer statement in the Output section as shown above in the report specification.

3-References

INFORMIX-SQL,Relational Database Management Systen (user guide)
INFORMIX-SQL,Reference Manual.(version 2.10)

UMEHARA,M.Data Management Systen for Plant Genetic Resources in the Gene Bank of N.I.A.R

acc_number	000016
reg_date	1986/06/13
organ_code	01121
lab_code	2128
plant_code	52291
culti_name	itapua 1
others_name	
status	1
source	3
origin_code	008
intr_place_code	007
intr_organ_code	01121
intr_year	1986
former_number	000016
cons_class	1
cons_form	2
remark	

acc_number	000017
reg_date	1978/12/24
organ_code	01121
lab_code	2128
plant_code	52291
culti_name	itapua 5
others_name	
status	1
source	3
origin_code	008
intr_place_code	007
intr_organ_code	01121
intr_year	1978
former_number	000017
cons_class	1
cons_form	2
remark	

acc_number	000018
reg_date	1985/05/17
organ_code	01121
lab_code	2128
plant_code	52291
culti_name	itapua 25
others_name	
status	1
source	3
origin_code	008
intr_place_code	007
intr_organ_code	01121
intr_year	1985
former_number	000018
cons_class	1
cons_form	2
remark	

acc_number	000019
reg_date	1989/09/28
organ_code	01121

(Appendix 1)

acc_number	quantity	operator_name
000001	1256.00	jose matias
000002	2365.00	jose mesias
000003	2365.00	jose molas
000004	2365.00	jose rojas
000005	2365.00	jose rojas
000006	1236.00	jose rojas
000007	3654.00	jose rojas
000008	2356.00	lupo
000009	2365.00	lupo
000009	3265.00	lupo
000010	2304.00	lupo
000011	3261.00	lupo
000012	3265.00	lupo
000013	1235.00	lupo
000014	2456.00	lupo
000015	3264.00	lupo
000016	1356.00	lupo
000017	3265.00	lupo
000018	2365.00	lupo
000019	2563.00	lupo
000020	2356.00	lupo

(Appendix 2)

acc_number	quantity	germ_ratio
000001	1256.00	89
000004	2365.00	85
000005	2365.00	89
000007	3654.00	88
000008	2356.00	86
000009	2365.00	88
000010	2304.00	85
000011	3261.00	86
000014	2456.00	89
000018	2365.00	89
000019	2563.00	85
000020	2356.00	88

(Appendix 3)

culti_name	visoja
reg_date	1980/12/29
intr_year	1979
plant_sci_name	Glicine Max L.
last_takeout_date	1991/12/14
operator_name	jose matias

culti_name	parana
reg_date	1989/08/13
intr_year	1989
plant_sci_name	Glicine Max L.
last_takeout_date	1992/12/02
operator_name	jose mesias

culti_name	prata
reg_date	1986/03/27
intr_year	1986
plant_sci_name	Glicine Max L.
last_takeout_date	1991/05/23
operator_name	jose molas

culti_name	paranagoyana
reg_date	1988/05/25
intr_year	1987
plant_sci_name	Glicine Max L.
last_takeout_date	1990/06/27
operator_name	jose rojas

culti_name	santa rosa
reg_date	1968/08/13
intr_year	1968
plant_sci_name	Glicine Max L.
last_takeout_date	1992/09/26
operator_name	jose rojas

culti_name	ufv-1
reg_date	1976/06/08
intr_year	1976
plant_sci_name	Glicine Max L.
last_takeout_date	1992/07/09
operator_name	jose rojas

culti_name	bossier
reg_date	1968/09/23
intr_year	1968
plant_sci_name	Glicine Max L.
last_takeout_date	1991/07/05
operator_name	jose rojas

culti_name	cria-1
reg_date	1981/03/12
intr_year	1981
plant_sci_name	Glicine Max L.
last_takeout_date	1990/12/03
operator_name	lupo

culti_name	alas-60

(Appendix 4)

CULTIVARES

july 25,1993

intr_year	plant_code	culti_name
1968	53300	santa rosa
1968	53300	bossier
1968	53300	galaxia
1976	53300	ufv-1
1978	52291	itapua 5
1979	53300	visoja
1981	53300	cria-1
1985	52291	itapua 25
1986	53300	prata
1986	53300	alas-60
1986	53300	yguazu
1986	52291	itapua 1
1987	53300	paranagoyana
1989	53300	parana
1989	53300	pirapo
1989	53300	iac-8
1989	53300	campinas
1989	52291	ian 8
1991	53300	juan fe
1992	52291	itapua 40

(Appendix 5)

Phylogenetic Relationships of the Subgenus *CERATOTROPIS* Based on Random Amplified Polymorphic DNAs (RAPD)

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Abstract

Random Amplified DNA Polymorphism was detected for 24 different accessions of 12 different species in the subgenus *Ceratotropis* of the genus *Vigna*. Of the 52 RAPDs generated with 8 decamer primers only one was monomorphic and 51 were polymorphic. Each accession could be distinguished from each other by at least a single RAPD fragment. The cluster analysis based on UPGMA identified 2 major clusters at 70% dissimilarity level representing the azuki and mungbean groups. The 4 accessions of the 3 species in mungbean group were more divergent than the other 20 accessions of 9 species of the azuki group. *V. reflexo-pilosa* and *V. glabrescens* exhibited the closest affinity among the species. The results were in agreement with analyses based on RFLPs and isozymes.

Introduction

The subgenus *Ceratotropis*, which is called Asian *Vigna*, is a morphologically homogeneous and specialized group of Asian origin. The taxonomic treatment of this group has been confused. By the proposal of Verdcourt (1970), this group was transferred from the genus *Phaseolus* to the genus *Vigna*. Maréchal et al. (1978) followed Verdcourt and presented a monograph on the *Phaseolus-Vigna* complex. This monograph has now become the most widely accepted taxonomic treatment on the *Phaseolus-Vigna* complex.

According to the monograph presented by Maréchal et al. (1978), the subgenus *Ceratotropis* consists of 16 species which includes 5 cultivated crop species, i.e., mungbean (*Vigna radiata*), blackgram (*V. mungo*), moth bean (*V.* aconitifolia), rice bean (*V. umbellata*) and azuki bean (*V. angularis*). Maekawa (1955) suggested that *Ceratotropis* should be divided into two separate genera (*Azukia* and *Rudua*) on the basis of differences in seedling characters. Species within the genus *Rudua* show epigeal germination having a primary leaf with very short petiole, while species belonging to the genus *Azukia* show hypogeal germination having a primary leaf with a long petiole. Although this concept has not been supported by further studies, it is convenient to use this concept in this study. The genus *Rudua* includes mungbean, blackgram and moth bean, while the genus *Azukia* contains rice bean and azuki bean as cultivated species. Therefore, the genus *Rudua* and the genus *Azukia* are hereafter referred to as "mungbean group" and "azuki group", respectively in the present paper. The species of the "mungbean group" are distributed mainly in the Indian Subcontinent, while the species of the "azuki group" are found principally in East and Southeast Asia.

Since the available number of specimens for the wild species in "azuki group" is so limited, Maréchal et al. (1978) mentioned that the taxonomic treatment of the wild species in the "azuki group" should be rearranged by the further examination of the newly collected specimens. Recently, several wild species belonging to the "azuki group" were collected in Thailand, Japan and Malaysia by the exploration (Tomooka et al., 1991,1992; Egawa et al., 1990,1991,1992).

The objective of the present study is to apply the RAPDs (Randomly Amplified Polymorphic DNAs) method on the species in the subgenus *Ceratotropis*, and to clarify the phylogenetic relationships among the species.

Materials And Methods:

Materials

All the plants analyzed were grown in a green house at the National Institute of Agrobiological Resources, Tsukuba in the summer of 1993. Two accessions each from 20 species both from azuki and mungbean groups were included at the DNA isolation stage and one accession at RAPD detection. The young tender leaves harvested from mainly 1-2 week old seedlings or from somewhat mature plants were used. The species, accessions and their place of origin is given in table 1.

Extraction of DNA

Two methods were adapted in DNA isolation.

At the inception, isolation of DNA in larger quantities was attempted based on Murray & Thompson (1980). Tender leaves about 8g of fresh weight were collected in an ice container, cut into small pieces and frozen in liquid nitrogen and stored in a deep freezer at -80°c. They were freeze dried for 48 hours and ground to a fine powder using Cyclotec 1093 sample mill grinder.

The powdered leaf sample was transferred into a 50 ml vial and 15 ml of hot 1x CTAB buffer (1% cetyltrimethyl ammoniumbromide; 0.1% Tris-HCl,pH 8.0; 1.4M sodium chloride) was added into it and, then placed in a water bath at 57°c for 30 minutes. Proteins were separated into a heavier lower phase by centrifugation at 2500 rpm for 15 minutes after mixing 10 ml of 1 isoamylalcohol : 24 chloroform v/v solution. DNA was precipitated with an equivalent volume of precipitation buffer (1% CTAB; 50mM Tris-HCl, pH 8.0; 10mM EDTA) mixing with the upper phase and recovered by centifugation at 2500 rpm for 15 minutes. The pellet was completely dissolved in 10 ml of high salt-TE buffer (1M NaCl; 10mM Tris-HCl,pH 8.0; 1mM EDTA) at 57°c in a water bath for 3-4 hours. DNA was re-precipitated with an equal volume of isopropanol after centrifugation again at 2500 rpm for 10 minutes to remove undissolved contaminants and transferred carefully with a blunt-tipped disposable pipette into a 1.5 ml effendorp vial. The pellet was washed with 2-3 changes of 70% ethanol and finally stored at 4°c in a refrigerator in 0.1 TE + 100x RNAse.

However, since some accessions, at the dilution stage tend to produce smears and unclear bands, a small-scale modified method was attempted at DNA isolation (Doyle & Doyle, 1987). 0.3% mercaptoethanol was added to 1ml of 2x CTAB buffer in order to remove phenolic contaminants.

After chloroform 24:isoamylalcohol 1 extraction, DNA was precipitated using 1 ammonium acetate: 6 ethanol v/v solution. Re-purification of DNA isolates was accomplished using phenol-chloroform method (Sambrook et al., 1989).

DNA concentration was estimated by the mini-gel method in comparison with λ-DNA of known concentrations and adjusted to 5.0 or 0.5ng/μl with sterilized distilled water.

PCR Runs:

Polymorphic Chain Reaction was performed in a polycarbonate microplate (Techne Hi-Temp 96) of 12 x 8 wells but using only the centermost 48 well per run. 1ng of template DNA was first placed at the bottom of the well and subsequently 8 μl of premix consisting of 5.4 μl of sterilized distilled water, 1.0 μl of 10x buffer(10mM Tris-HCl, pH 8.3; 50mM KCl, 2mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2mM each of dATP, dCTP, dGTP, dTTP, 0.2 μl of 25mM magnesium chloride, 2μM of primer and 0.2 units of *Taq* DNA polymerase (Toyo,Japan) added and finally overlaid with a drop of mineral oil. The PCR apparatus (Techne PHC-Thermal Cycler) was programmed for 2 major cycles: the first one for 45 cycles each consisting of 93°c for 1 minute, 35°c for 2 minutes and 72°c for 3 minutes and the second cycle with 72°c for 7 minutes. The amplified DNA was electrophoresed on a submerged 1.6 or 2.0 % agarose gel at 50 v for 1.5 hours, stained with ethidium bromide and photographed under UV light.

Data Analysis:

Twelve primers (P1,P2,P3,P4,P16,P21,P22,P2,P28,P29,P31 and P34 maintained at the Plant Germplasm Introduction Laboratory, NIAR) each ten bases in length were selected from a pool of primers that gave reasonble numbers of amplification under the PCR reaction conditions described above. Each cultivar was scored for the presence or absence of reliable amplification product and the data entered into symmetrical data matrix. Coefficient of dissimilarity as the % of mismatched RAPDs were calculated on the basis of the formula:

$$D = 1 - [2n_{xy}/(n_x + n_y)]$$

where D equals the percentage of mismatched fragements between the pair of accession x and y and n_{xy} represents the matched fragments for both accessions. Cluster analysis using UPGMA (Unweighted Pair Group Method with Averages) was performed and a dendrogram was constructed as described by Sneath & Sokal (1973) using multivariate analysis.

Table 1 Accessions used in the experiment

ID. No.	Species Name	Accession Name	Origin
C1-1	*V. angularis* var. *angularis*	Kyoto Dainagon	Japan
C1-2	"	102	"
C2-1	*V. angularis* var. *nipponensis*	Sendai	"
C2-2	"	VA0001	"
C3-1	*V. umbellata* var. *umbellata*	89-531	Nepal
C3-2	"	NT9152	Thailand
Chan	"	Chanta Buri	"
C4-1	*V. umbellata* var. *gracilis*	6-1-1	"
C4-2	"	DKL	"
C5-1	*V. riukiuensis*	Irio-5	Japan
C5-2	"	TNH 25277	Taiwan
C6-1	*V. nakashimae*	Kankoku	Korea
C6-2	"	Ukushima	Japan
C7-1	*V. minima* var. *minima*	Fujieda	Taiwan
C7-2	*V. minima* subsp. *gracilis*	M27	Malaysia
C8-1	"	Species C	Thailand
C8-2	"	Somyot 23	"
C9-1	unidentified species	Species D	"
C9-2	"	Somyot 20	"
C10	*V. nepalensis*	Nepalen	Nepal
C11-1	*V. trinervia*	M18	Malaysia
C11-2	"	NT9144	Thailand
M3	"	M3	Malaysia
M12	"	M12	"
M36	"	M36	"
C12-1	*V. reflexo-pilosa*	M23	"
C12-2	"	Yona 5	Japan
C13	*V. glabrescens*	V1160	Philippines
C14-1	*V. radiata* var. *radiata*	Tanegashima	Japan
C14-2	"	250007	Iran
C15-1	*V. radiata* var. *sublobata*	TC1965	India
C15-2	"	TC1966	Madagasscar
C16-1	*V. mungo* var. *mungo*	Subsomotod	Thailand
C16-4	"	BC48	"
C17-1	*V. mungo* var. *silvestris*	TC2211	India
C17-2	"	Somyot 5	Thailand
C17-3	"	TC2210	India
C18	*V. grandiflora*	Species A	Thailand
C19-1	*V. aconitifolia*	Nilgiri	India
C19-2	"	2752.5	Pakistan
C19-3	"	2754.3	"
C19-4	"	2762.2	"
C20-1	*V. trilobata*	NI251	India
C20-2	"	NI1030	"
C21	*V. radiata* var. *setulosa*	NI 11135	"

Results

In isolation of DNA, the modified method served to be more cost and time effective than the mass method. Even though the yield of DNA was a little lower, it did not require freeze drying of material nor larger volumes of samples and reagents. The samples were directly crushed with a small volume of liquid nitrogen into a powder and DNA could be extracted the same day saving 2-3 days of time usually spent on isolation with the original method.

Of the species of sub-genus *Ceratoropis* studied, isolation was easier with accessions of azuki bean group but accessions from the mungbean subgroup were not amicable with isolation. They either yielded no DNA at all or only minute amounts of fragmentized DNA. The modified isolation method improved the situation and it turned out DNA even from the above difficult species. However, many accessions of the mungbean group produced poor results at amplification and as a result all the accessions could not be included in RAPD analysis. Thus differences between azuki and mungbean groups were indicated even at the stages of DNA isolation and amplification. Since some of the accessions yielded pigmented DNA necessity of further purification is suggested.

The importance of the concentration of the DNA template used for the amplification was evidenced. Some failed to produce amplified products with 5ng/ μl templates. When dilutions of 1.0, 0.5 and 0.25 ng/ μl of the same sample were tested, they produced consistent RAPDs and consequently 0.5 ng templates were used in this study. The effect of concentration on AP-PCR (i.e. RAPD) has been reported by Welsh et al. (1991).

Altogether 12 primers were attempted and data on 8 repeatable and persistent primed events were taken into consideration; 2 of the primers, P4 & P29, were unable to result in any amplification. The screening of 8 decamer primers against 24 accessions of *Vigna* resulted in the amplification of discernible DNA fragments ranging from 2500 to 100 bp. The bands beyond this range which were neither prominent nor consistent were not considered in the statistical analysis.This resulted in an average of 6.5 fragments per primer. The size of amplified DNA was measured in comparison with Lambda-DNA digested with *Hind-III* and *Pst-I* restricion enzymes.

An example of a typical analysis is illustrated in Fig. 1. Oligonuleotide P1 primed the amplification of 6 fragments of which 5 were polymorphic and 1 is shared by all the accessions.

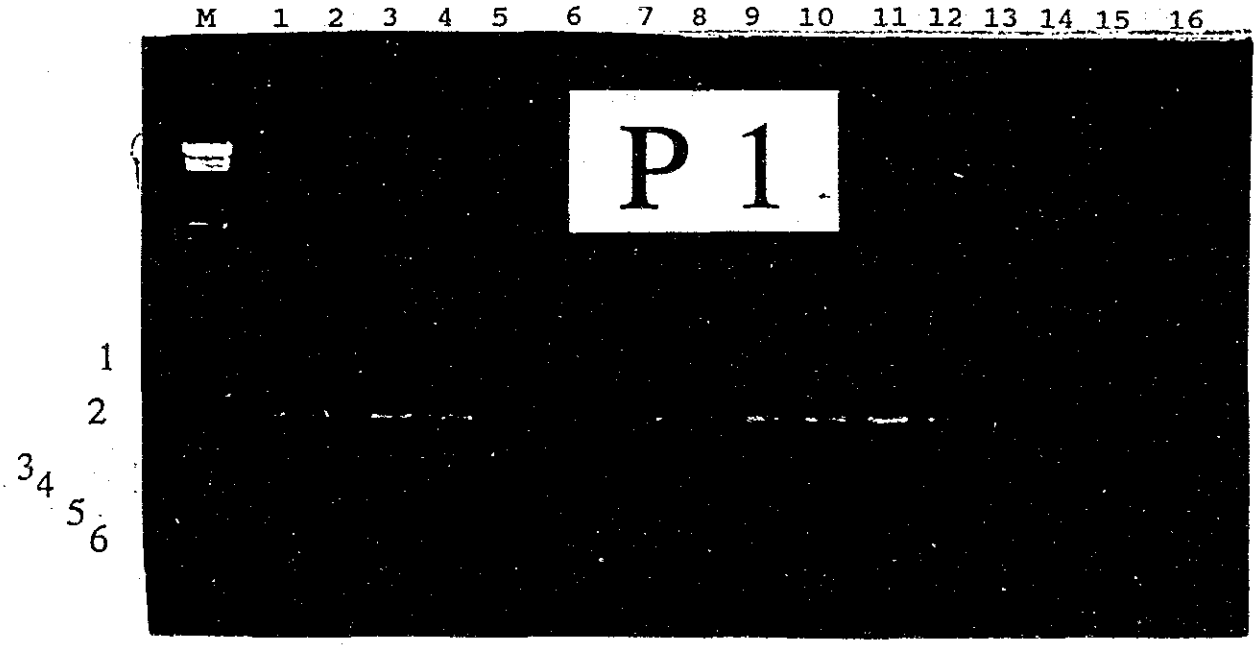

Fig. 1: Random Amplified Polymorphic DNAs (RAPD) generated by the decanucleotide primer P1 with base suquence 5'-GTCTGACGGT. Lanes: M= -DNA digested with *Hind-III*, 1=C1-1, 2=C1-2, 3=C2-1, 4=C2-2, 5=C3-1, 6=C3-2, 7=C4-1, 8=C4-2, 9=C5-1 10=C5-2, 11=C6-1, 12=C6-2, 13=C7-1, 14=C7-2, 15=C8-1, 16=C8-2.
Fragment 2 was monomorphic while 1,3,4,5 and 6 were polymorphic.

Discussion

RAPDs have become useful in determining relationships among various taxa and have been successfully deployed in studying polymorphism in wheat (Vierling et al, 1992), elucidating phylogenetics of cereals (Weining et al., 1991), identifying accessions of rice (Fukuoka et al., 1992), determining parentage of maize (Welsh et al., 1991) and tracing resistant gene markers in *Phaseolus* (Haley et al., 1993).

In the present study, a total of 52 RAPDs were recorded from the PCR experiments. Only 1 fragment of the P1 primer was monomorphic among all the accessions and, therefore almost all the observed amplification fragments were phylogenetically important and could be deployed in generating a cladogram.

The cluster analysis produced 2 major clusters at 70% dissimilarity level representing the azuki and mungbean groups (Fig. 2). The larger cluster with less divergence at 62% level consists of most of the studied species which belong to the hypogeal germination type. The separation of the 2 groups has been reported by RFLP analysis (Fatokun et al., 1993) and differences in carbohydrate composition (Yasui et al., 1985).

Of the accessions, the closest pair was *V. reflexo-pilosa* and *V. glabrescens* which show more than 96% of similarity. This supports the hypothesis that *V. reflexo-pilosa* is the putative ancestor of *V. glabrescens*. In the group, both of them are unique being the only tetraploids with 2n=4x=44, and successful crosses have been obtained between the two species inmplying the homogeneity of the two genomes. The next most closely related pair was the 2 varieties *angularis* and *nipponensis* of *V. angularis* as is expected. The pair forms a mini cluster with the two varieties of *V. umbellata* at a higher diverging point. The relative position of *V. angularis* and *V. umbellata* in azuki cluster and of *V. mungo* and *V. radiata* in the mungbean cluster is in good agreement with RFLP results(Fatokun et al., 1993). The isolation of the 4 accessions of *V.trinervia* into a common cluster at 31% level indicates their closeness to each other although 3 are from Malaysia and the fourth is from Thailand. This mini cluster identifies *V. reflexo-pilosa* and *V. glabrescens* pair as their closest relatives. However, placement of *V. trinervia* in the middle of the major cluster contradicts the belief that *V.trinervia* is intermediary between mungbean and azuki groups. The unidentified species, C9-1, exhibit congruity to wild *V. umbellata*, but the distant separation of wild *V. umbellata* from its cultivated counterparts and of C8-2 from other *V. trinervia* relatives is inexplicable.

The 4 members of the mungbean group show more divergence among themselves than their counterparts in the azuki bean group. Similar findings have been reported by Egawa and Tomooka(1993). *V. grandiflora* was the most divergent indivdual of all. Since it failed to produce AP-PCR with several primers, its position in relation to the others is not reliable.

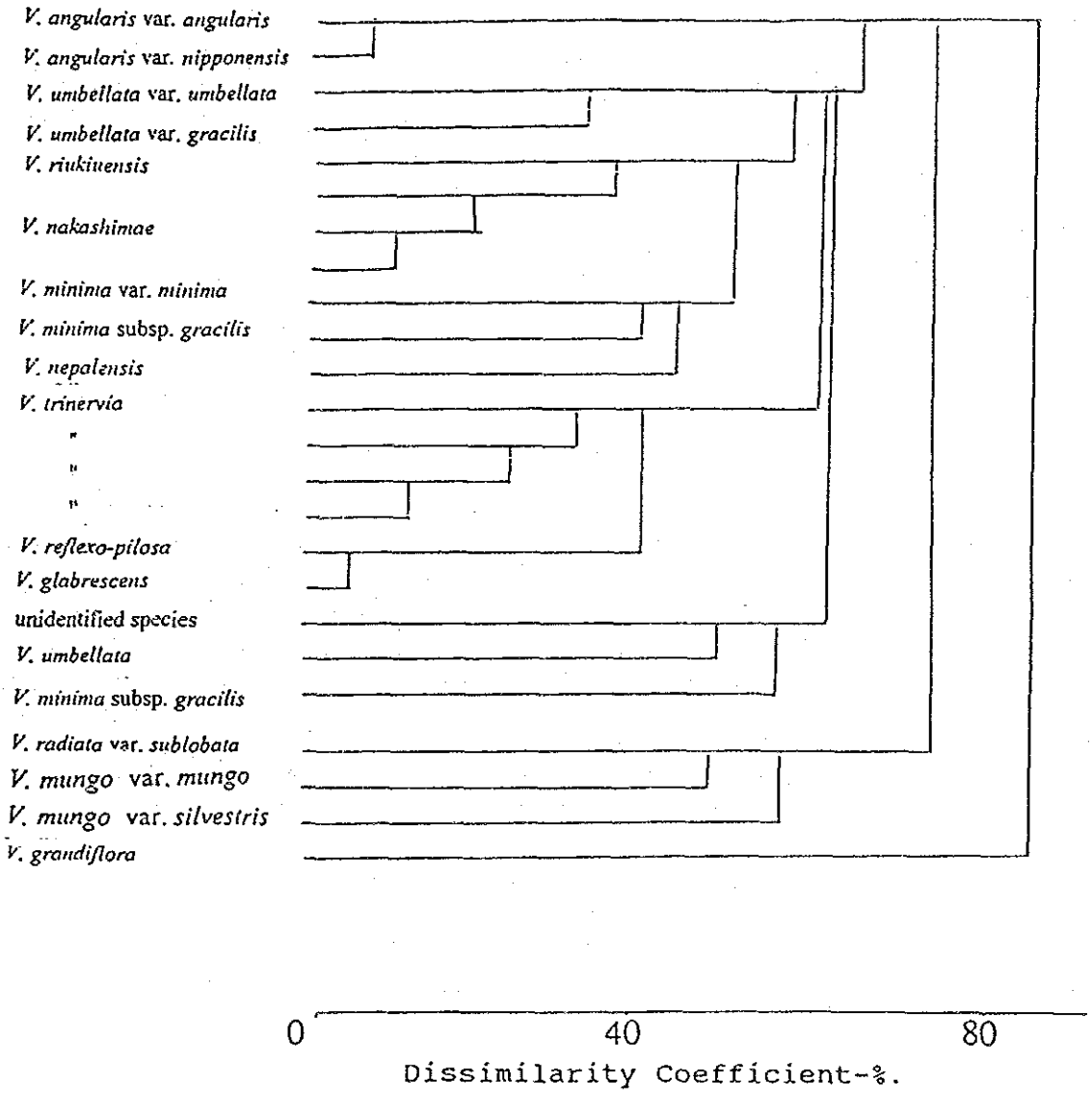

Fig 2. Dendrogram depicting the phylogenetic relationships of the 24 accessions of the *Ceratotropis* sub-genus.

In the present study only 4 accessions of the mungbean group could be included due to the inability of many others to produce RAPDs. The failure may have been caused by 2 factors. Either they require conditions and time periods for PCR different from the conditions which were suitable with azuki members. Or it may be due to some contaminants still remaining in the template even after attempts of re-purification of DNA which inhibt DNA from amplifying. Therefore differnt means of DNA isolation and more conducive conditions in PCR is suggested for the mungbean group.

RAPDs is much faster and more convenient method in the detection of polymorphism than the traditional RFLP analysis. RAPD data can be obtained within 2 hours after PCR as opposed to the Southern Blotting and DNA hybidization required in RFLP. Further it does not require specific base sequences identified by restriction endonucleases and thus any arbitrary sequences can be utilized. The linkage between RAPDs with RFLP and isozyme markers has been established in legumes (Torres et al., 1993) and consequently RAPDs in association with the latter would provide faster methods in gene mapping. Therefore the results presented here show promise not only academically but practically as well since they will be of assistance in breeding programmes of *Vigna* species.

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