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In
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F. Y. 1995

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INDIVIDUAL TRAINING REPORT Group Training Course In Plant Genetic Resources F. Y. 1995 November, 1995

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ホルヘ


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バヤルスク


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CONTENTS

1. CUBA

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TECNICAL REPORT

TRANSFORMATION OF SWEET POTATO USING *AGROBACTERIUM TUMEFACIENS*

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GROUP TRAINING COURSE OF PLANT GENETIC RESOURCES
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I would like to express my gratefulness to all the staff members of the Sweet Potato Laboratory Breeding of National Agricultural Research Center, because they have given my much of their time and all the facilities to make my stay in this laboratory very nice.

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I- INTRODUCTION

The sweet potato, *Ipomoea batatas*(L.),is the sixth most important crop in the world after wheat, rice, corn, white potato and barley(Vietmeyer, 1986), with an estimated production value of 10 billion dollars(FAO YEAR BOOK 1984). It is a valuable source of nutrition and sustenance to people, especially in the developing world(Walter *et al.* 1984). Using conventional breeding methods specific cultivars have been developed with improved characteristics. However, the use of conventional breeding for sweet potato improvement is specially difficult due to the hexaploid nature of its genome, low production of viable seeds, and problems of sterility and incompatibility(Carelli *et al.*1992). Genetic engineering techniques have considerable promise in an increasing agricultural productivity around the world because of their potential to genetically redesing crop plant. Gene transfer technology is already enable to introduce many novel value-added traits such as improved nutritional quality, resistance to pest and disease, and tolerance to stress factors, into a few crops(Goodman *et al.*, 1987). Gene transfer involves direct insertion of DNA constructs into a genome. This can be accomplished in a number of ways including bacteria or viral mediated transfer, absortion, electroporation or bombardment,(Collinns , 1992).

The most commonly used vector for introduction of foreign genes into plant genomes is the soil bacteria *Agrobacterium tumefasciens(Zambryski*,1989). Agrobacterium is capable of transfering defined segments of DNA to plant genomes. The foreign DNA sequence introduced into plant using this technique have shown to be stably inherited in a mendelian manner(Otten *et*

al.,1981;Horsh *et al.*, 1984).

The succesful use of *Agrobacterium tumefaciens* in others crops had been more divulgated, such as the transformation potato cv. Mantiqueira(Figueira *et al*, 1994), genetic transformation and plant regeneration of watermelon(Choi *et al.*, 1994), effect of plant genotype on the transformation of cultivated alfalfa(Du *et al.*, 1994), plant regeneration from leaf explant of *Cichorium intybus* (Genga *et al.*, 1994), tranformed by *A. tumefaciens*, and genetic transformation of *Populus nigra*(Confalonieri *et al.*, 1994).

In the case of sweet potato there are a few report about transformed plant using *Agrobacterium tumefaciens*(Al-Juboory and Skirbin, 1991; Lowe *et al.*, 1994; Carelli *et al.*, 1992; Prakash and Varadarajan, 1992), with limited success. A recent report, however describes the succesful method for producing transgenic sweet potato using *Agrobacterium tumefaciens*(Newet *et al.*, 1995).

This paper summarizes the preliminary results of our studies testing the effect of different antibiotics concentration on root emergence and growth, the effect of acetosilingon on infection of *A.tumefasciens*, and the use of *A.tumefasciens* to introduce foreign genes into the sweet potato with the aim to obtain transgenic plants as a part of Plant Genetic Resources Trainning Course stay during 5 months in the Laboratory of Sweet potato of NARC in 1995.

2-Materials and Methods

2.1-Plant Material

Chugoku 25 lines were maintained in plant proliferation medium(PP) containing MS(Murashige and Skoog, 1962) medium with sucrose(30 g/l, agar(8 g/l) at pH 5.8. Nodes cultures were kept during 16 hours a 26 C. Nodes and leaf disc obtained from proliferated plants were used as source material for following experiments.

2.2-Effect of growth regulator on root emergence.

The effect on leaf disc rooting was determined by culturing leaf segments on MS medium with IAA(0.1, 0.5, 1.0 and 5,0 mg/l or NAA(0.1, 0.5, 1.0, and 5.0 mg/l) and sucrose(30g/l), Gellan Gum(2 g/l) at pH 5.8. Control treatment without growth regulator was included. Two replicates were used in each treatment. After 15 days the rooting was recorded by visual evaluation.

2.3-Antibiotic sensitivity assays.

Tissue sensitivity to kanamycin and hygromycin was independently tested in separate experiments. Kanamycin and Hygromycin inhibition of root formation was determined by culturing nodes explants on PP medium suplemented with kanamycin at concentration of 12.5, 25, 50, 100 and 200 mg/l and with hygromycin at concentration of 5, 10, 20 and 40 mg/l. In this experiment a control treatment lacking antibiotic was included. Three replicates were used in each treatment. After 21 days, the number of regenerated roots, root elongation, number of explants died and fungi contamination was recorded(Table 2).

2.4-Plasmid DNA and *Agrobacterium* proliferation.

The plasmids pBI333(Fig.1) and pBI121(Clonetech Laboratories, Palo Alto, Ca.(Fig. 2))was employed as source of exogenous DNA,

containing selectable markers for expression in plant cells (Jefferson *et al.*1987) under the regulatory control of the CaMV 35S promotor and the terminator from the neopaline synthase(nos) gene that encode for the enzyme neomycin phosphotransferase II, which confers resistance to aminoglycoside amntibiotic such as kanamycin, neomycin and G-418. The npt gene in pBI 121 is fused to promoter and terminator sequence from the neopalin synthase(nos) gene. *Agrobacterium* with pBI121 or pBI333 were proliferated by overnight incubation at 200 rpm in Luria broth medium(LB, Rodriguez and Tai, 1983 modificated)containing:1 % Triptone(Difco), 0.5 % bacto yeast extract(Difco), 0.5% Nacl. LB medium was suplementedwith kanamycin 50 mg /l, ryfampicin 10 mg/l and streptomycin sulfate 250 mg/l.

2.5-Leaf disc transformation and selection.

Preculture leaf disc were placed in the resuspended bacterial solution for about 3 minutes. Explants were blotted on sterile paper and cocultivated in four different medium containing MS+ O.5 mg/l IAA; MS + 0.5 mg/l IAA+ 100mg/l acetosilingon (AS), MS+ 0.5 mg/l NAA,and MS+0.5 mg/l NAA+100 mg/l AS, two days later the explants were transferred to fresh medium containng 200 mg/l Claforan(Cf)+0.5 mg /l IAA or NAA for one week, then 24 explants were randomly taken for GUS assays and the remain were put into a selective medium containing MS+0.5 mg/l NAA+Cf+10 mg/l higromycin(Hy) or 50 mg/l Kanamycin(Kn), change the medium every two weeks.

2.6-B-glucoronidase assays.

A histochemical assay for B-glucoronidase was carried out as

described by Jefferson et al.(1987). Leaf disc were flooded with the Gus assay solution and incubated overnight at 37 C, consisted of X-Glu solution(5,4-Bromo-chloro 3-indolyl B-D-glucoronic acid, and Dimethyl formamide; X-Glu Buffer(50 mM sodium phosphate;100mM ethylene diamine tetraacetic acid;0.1% Tryton X-1000, and 0.1 % Sarkosil(N-Laurylsar cosing sodium salt).and methanol(Mc Cabe *et al.* 1988). Gus activity was visually indicated by intense blue color.

Results and Discussion

Antibiotic sensitivity assays

Plant cells are known to be sensitive to a number of antibiotic including kanamycin and hygromycin(Pollock *et al.*, 1983 ; Catlin, 1990). To determine the sensivity of roots development to kanamycin levels, nodes explants were cultured in PP medium containing different concentrations of Kanamycin.

Kanamycin concentration of 50 mg/l completely inhibited root formation from nodes explants after one week culturing(Table 1), and the percentage of root regeneration was the lowest with 33.3 %(Fig. 3a). A similar study,carried out to determined hygromycin effect on root regeneration, showed that 40 mg/l inhibited root formation after one week(Fig. 3b), but in this case hygromycin even at the lowest concentration showed all explant died after two weeks culturing.

A comparison table among control, hygromycin and kanamycin concentrations for root emergence, root elongation, number of root per explant and explant died showed that kanamycin 50 mg/l was the best treatment in our experiment(Table 2). This result is different from *Carreli et al.*(1992), that reported an inhibitory effect of Kanamycin on root regeneration using 100 mg/l.

Effect of growth regulator on root emergence

In an initial experiment to induce root formation was used IAA 5 mg/l based on Tabei(personal comunication), but root failed to growth into the medium(Fig. 4), then a second experiment was divised. In this experiment we analized the effect of two auxins at different concentrations on leaf disc in order to choose the best one.

The results showed that IAA at any concentration promote root emergence on leaf disc of sweetpotato(Table 3). In the case of NAA after evaluation, the concentration of 0.5 mg/l showed a good root formation(Fig. 5), and the major acount of roots with 13. In the control treatment, without auxins, we can not find any root emergence(Fig. 6).

Transformation studies and selection

Two different experiments were carried out with the aim to establish a method of transformation sweepotato plants. First one using pBI121 resistant to kanamycin, and selective medium Cf + Kn, and second one using pBI333 resistant to hygromycin and selective media Cf + Hy. In both case we included acetosilingon as a promoter of *Agrobacterium* infection.

The leaf discs inoculated with pBI121 followed by acetosilingon treatment exhibited signs of tranformation when tested by GUS histochemical assay(Fig. 7). Occasional roots were formed but failed to grow into the medium. Leaf disc became yellow before any shoots formed.

Leaf discs assayed for GUS activity showed 12 of 24 explant with GUS expression indicated by the intense blue color on the surface of the leaf when incubated in B - glucoronidase, but we atributed that those plants are not transgenic because of failure to produce good roots, then we consider that this intense blue color is due to the reaction of Agrobacterium on the surface of leaf disks with a B- gluc. Similar result was obtained by Carelli *et al.*(1992) using *A. tumefaciens* strain pEha101 in sweet potato line "Jewel".

The leaf disc exposed to a rooting medium without acetosilingon failed on root and shoot formation. The GUS assay showed only two

leaf disc with Gus expression by blue color(Fig. 8).

In this experiment we could appreciate the good effect of acetosilingon as a promoter of *Agrobacterium* infection.

Since no good roots were obtained using the first transformation protocol, the second experiment was devised. In this experiment we used NAA to induce roots from leaf discs, and pBI333. In this case the leaf disc exposed to pBI333 followed by acetosilingon treatment and without it, exhibited no signs of transformation, when tested by GUS histochemical assay(Fig. 9). Only one root was formed, but failed to grow in the selective medium.

The,results of both experiment suggest that another experiment using different lines or cultivars of sweet potato is required to carry out a new one using different line of sweepotato to study the behavior of rooting, because the growth of roots depends on genotype.

Conclusions

Kanamycin antibiotic at 50 mg/l concentration can be used for transformation experiment in sweet potato.

Acetosilingon could be used to promote *Agrobacterium* infection in the transformation of sweet potato.

The NAA as growth regulator showed better results than IAA in analized parameters.

A new experiment using different lines of sweet potato is required with the aim to study the behavior of rooting.

Table 1 Kanamycin inhibition of root emergence on sweet potato nodes explants after two weeks on culture.

Kanamycin(mg/l)	Root development one week	two week
12.5	+++	++
25	++	++
50	-	-
100	++	-
200	+	+

Legend:- no roots
+ light root emergence
++ moderate root emergence
+++ vigorous root emergence

Table 2 Comparative table between control and two antibiotic at different concentration for root emergence, root elongation, number of root per explant and explant died in sweet potato nodes explant.

Variants	Total explant	Conc. mg/l	Root emerg.	Root elong.	# root per exp.	Explant died
Control	15	-	15	14.2	4.26	-
Kanamycin	15	12.5	10	0.46	1.13	-
	15	25.0	7	0.35	0.66	5
	15	50.0	5*	0.23*	0.46*	-
	15	100	9	0.22	0.66	-
	15	200	12	0.43	1.13	-
Hygromycin	15	5	10	-	0.66	15
	15	10	4	-	0.26	15
	15	20	5	-	0.33	15
	15	40	1	-	0.06	15

Table 3 Effect of different concentrations of IAA and NAA on leaf disc rooting of sweet potato

Variants	Basal medium	Conc. of auxin(%) IAA	NAA	Root develop.	Explants with roots	Total explants
Control	MS	0.0	0.0	-	20	20
IAA	MS	0.1	0.0	-	20	20
		0.5	0.0	-	20	20
		1.0	0.0	-	20	20
		5.0	0.0	-	20	20
NAA	MS	0.0	0.1	-	0	20
		0.0	0.5	+++	13	20
		0.0	1.0	++	7	20
		0.0	5.0	+	5	20

Legend:- no root emergence
+ a few root emergence
++ moderate root emergence
+++ vigorous root emergence

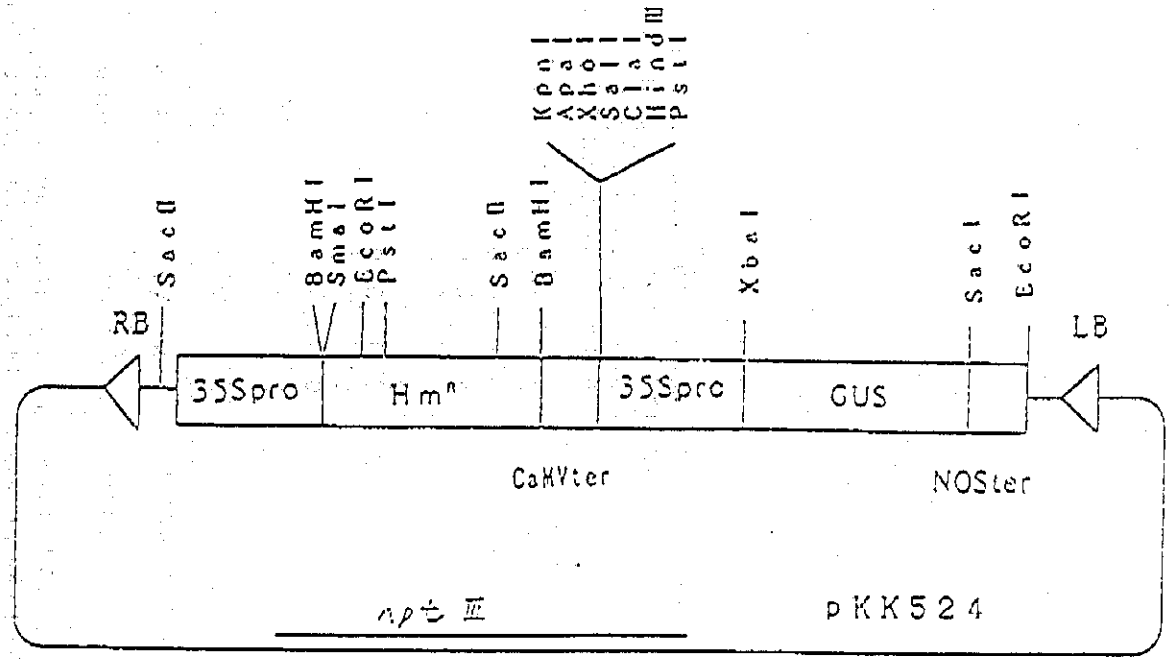
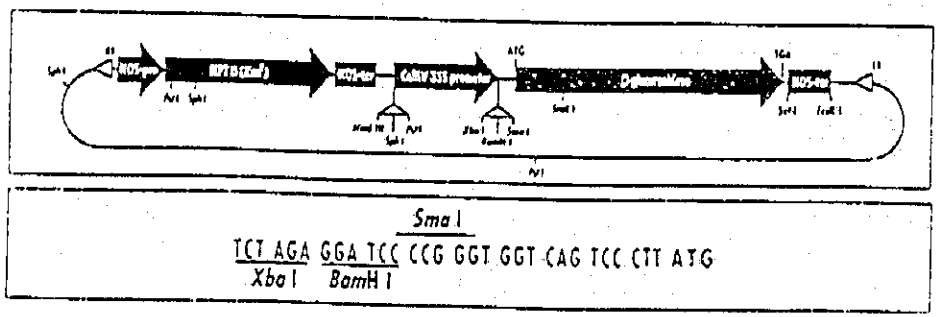


Figure 1 Schematic representation of binary vector used in these experiments. The T- DNA vector pBI333 carries genes for hygromycin resistant(hm) and B- glucoronidase activity, for expression in plant cells.

pBI121 Yesistant to Kanamycin



Product	Size	Cat. #
pBI121	5 µg	6018-1

Figure 2 Schematic representation of binary vector used in these experiment. The T- DNA vector pBI121 carries genes for kanamycin resistant and B- glucoronidase activity(GUS), for expression in plant cells.

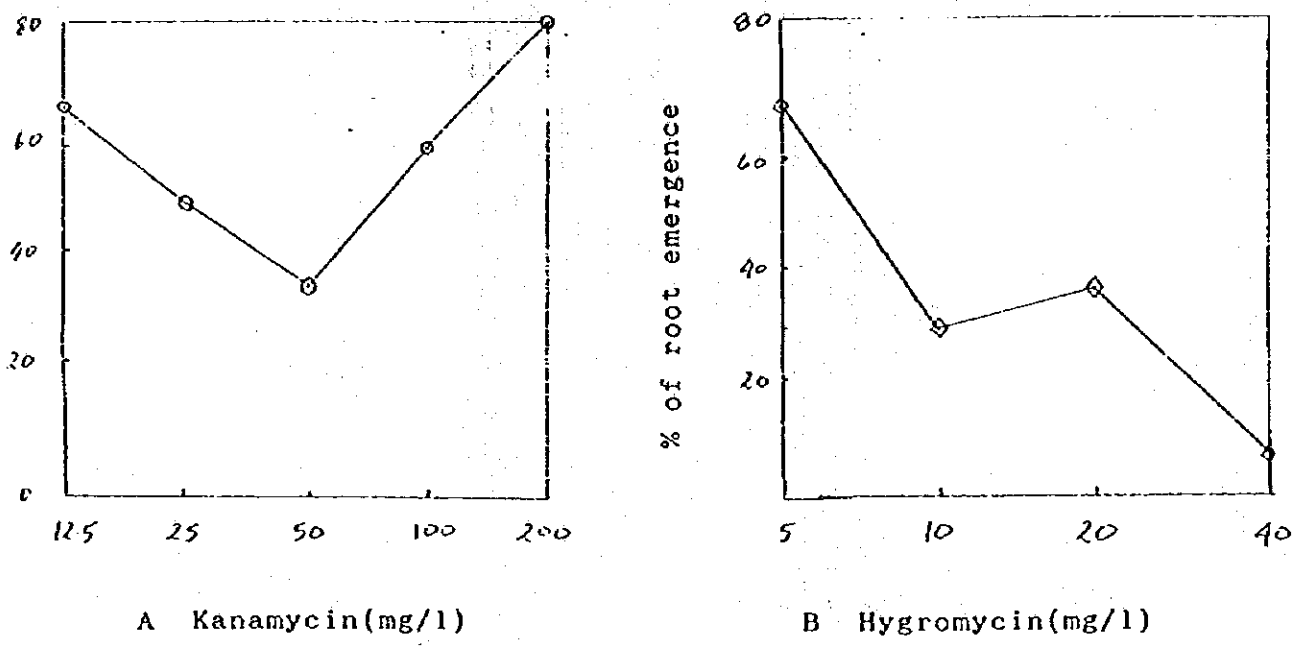


Figure 3 Kanamycin(A) and hygromycin(H) dose responses showing inhibition of root emergence from sweet potato nodes explants. Root percentage was obtained after two week culture.

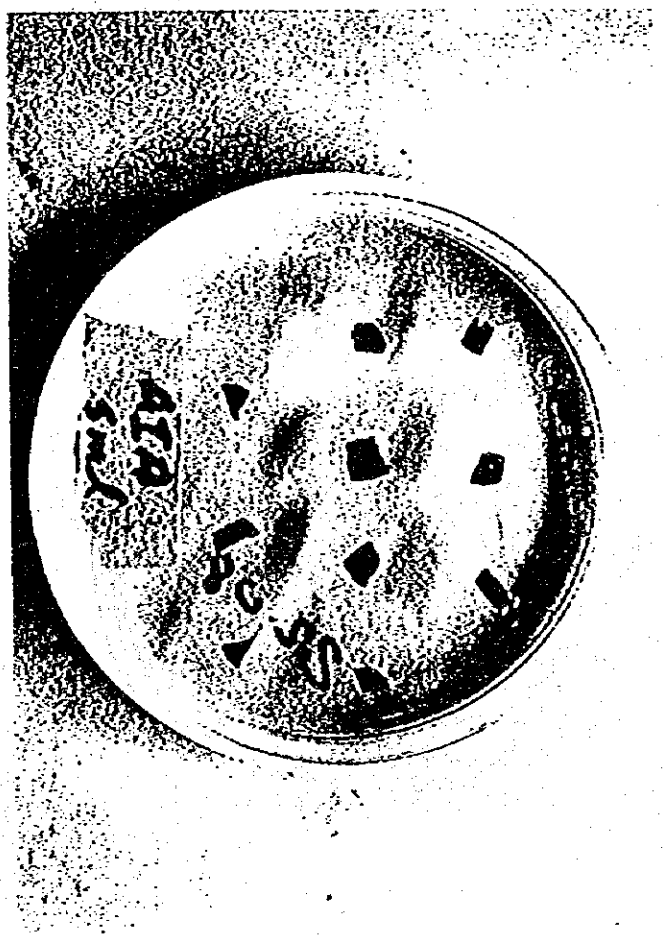


Figure 4 Total inhibition of root formation at 5 mg/l IAA concentration on leaf disc of sweet potato.

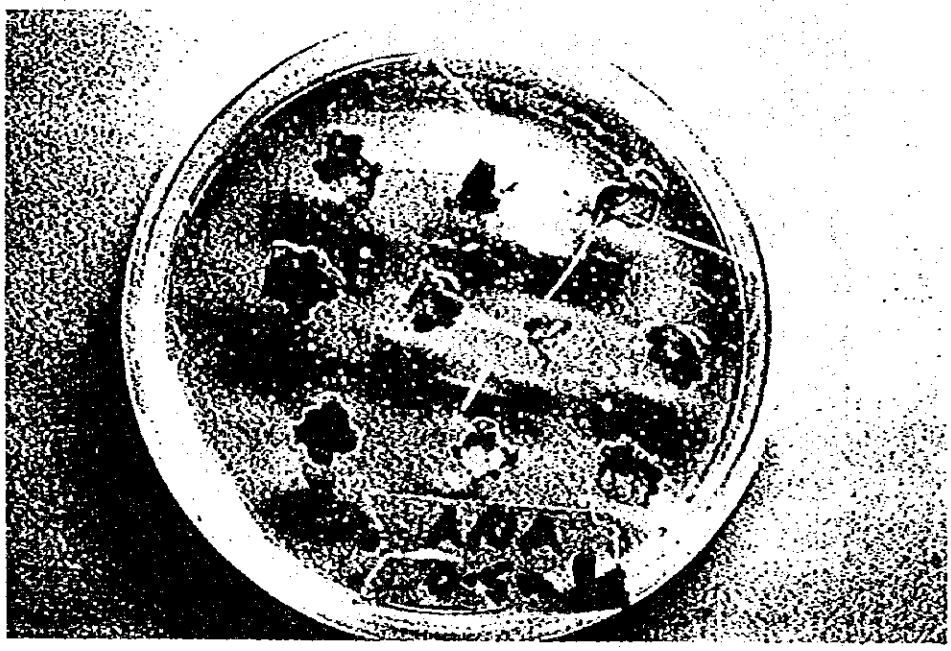


Figure 5 Development of root on leaf disc of sweet potato using 0.5 mg/l of NAA.

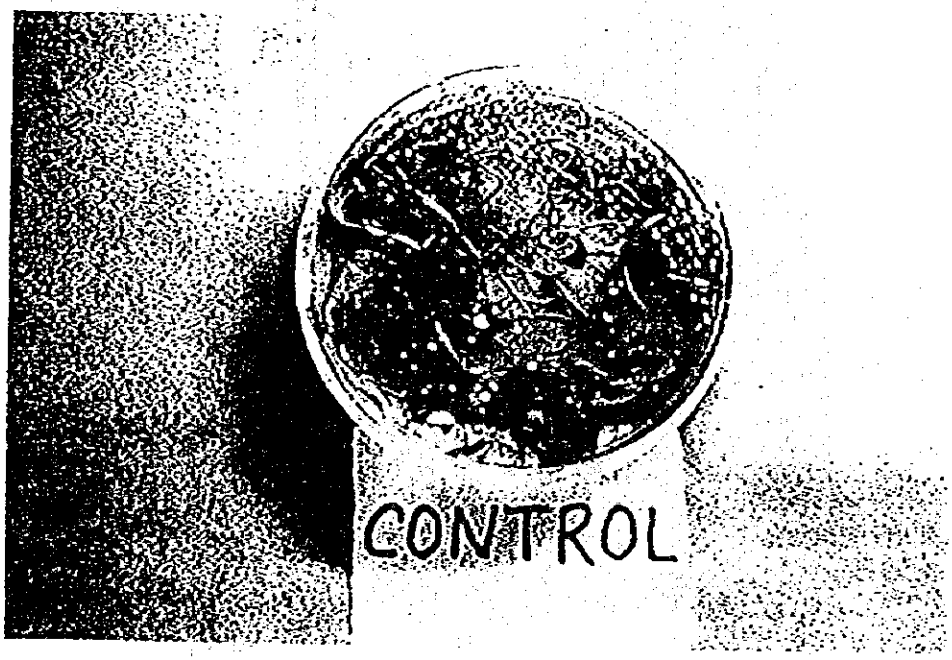


Figure 6 Good root development in the control treatment without antibiotic.

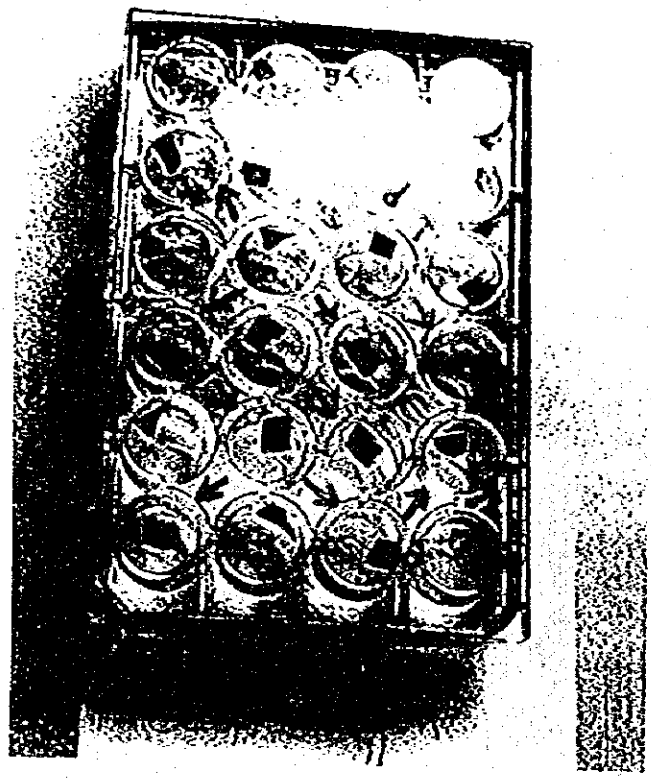


Figure 7 Histochemical GUS staining leaf disc under kanamycin selection with acetosilingon.Twelve of 24 explants showed Gus expression but failure to produce roots.

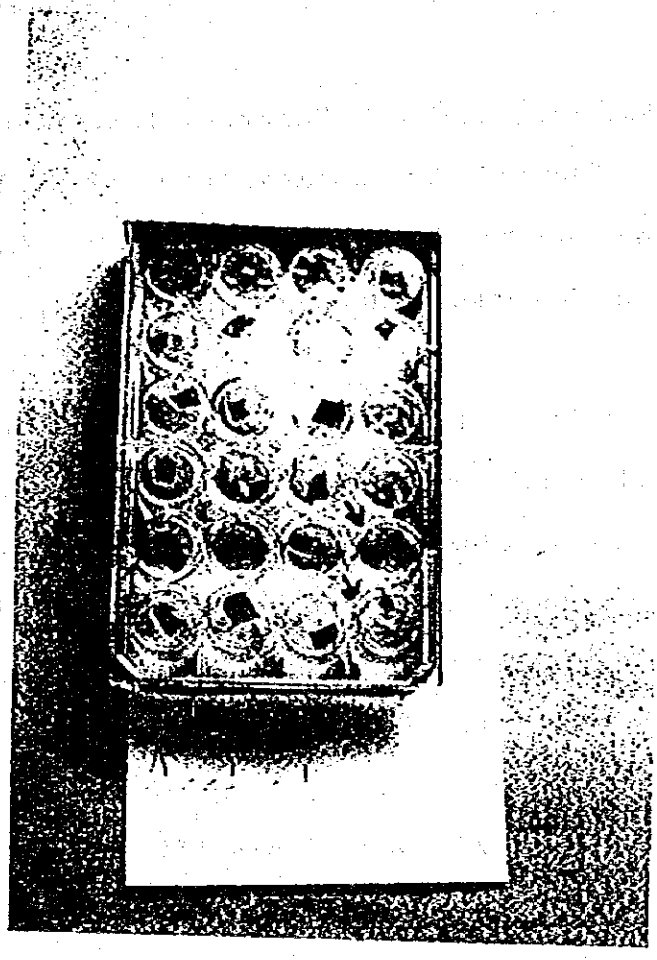


Figure 8 Histochemical GUS staining leaf disc under kanamycin selection without acetosilingon.
concentration on leaf disc of sweet potato.

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2. GHANA

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GENETIC VARIATION OF LOW TEMPERATURE GERMINABILITY OF RICE (Oryza sativa)

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Submitted to fulfil the requirement as a JICA trainee in
The Group Training Course in Plant Genetic Resources

Genetic variation of low temperature germinability of rice (*Oryza sativa*)

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Abstract

Genetic variation of low temperature germinability at 15°C was investigated using a core collection of rice, which composed of 43 varieties from Indica varietal group and 51 from Japonica group. Differences of the germinabilty between the two varietal groups were also studied. A large genetic variation was found in the low temperature germinability among the varieties tested. The following varieties, Canabongbong, Ghuizhao 2, Guanluai 4, Bodat Mayang, Nepal No 1, Liuzhoubaoyazao, Toboshi, Hongmi, Xuanchami, IR 30, Surjamkhi, Dourado Precoce, R.M., Oba, Norin No 1 and Yamayutaka had very good low temperature germinability. There was no significant difference of the germinability between the two varietal groups. Germination at 15°C of the tropical Japonica was relatively lower than that of the temperate Japonica.

Introduction

The genus Oryza belongs to the tribe Oryzeae in the family Gramineae. There are twenty wild species and two cultivated species in the genus (Chang 1976). Rice is the most important staple crop and it is evaluated to be the most nutritious among cereals. This crop is widely cultivated with various methods in the tropics, subtropics, temperate as well as some subarctic regions.

Compared with the transplanting method the direct sowing method involves less cost and labour relating to transplanting is rather more (Yasunobu, et al. 1994). However rice varieties applicable to the method need a sufficient and fast germination under low temperature conditions.

Omrod and Bunter (1961) reporting on minimum temperature for germination found that of Indica varieties higher than that of Japonica varieties. Sasaki (1974) reported that varieties from Hokkaido, northern part of Japan have higher germinability under low temperature conditions. Nakaghara (1985) in Japan noted that Boro rice from India have good germinability at low temperatures than Japanese varieties.

This ability is a very important character for seedling establishment in the fields at high altitudinal areas in the subtropics and tropics as well as well as in high latitudinal countries. Since the information on the variation of low temperature germinability among rice

varieties and varietal groups were not sufficient, an investigation was made on the genetic variation of the characters using a core collection of rice.

Here we report the germinability variation under low temperature conditions of the characters among rice varieties and the varietal group differences.

Materials and Methods

Rice varieties of 94 accessions from various countries, which is a core collection of the Plant Genetic Diversity Laboratory in National Institute of Agrobiological Resources were used in the experiment. They comprised of 51 varieties of Japonica varietal group and 43 Indica group. Plastic boxes of 15 cm x 8 cm x 2.5 cm with two filter papers in each of them were used in the germination test. The test was conducted according to method of the International Seed Testing Association. After the filter paper was adequately moist 100 seeds for each of the varieties were set on the papers. Germination of the seeds was recorded until eighteenth day after setting the experiment. Weak germination, abnormal germination showing only root or shoot elongation as well as hard seeds and fungi infested seeds were also observed.

To check germinability at room temperature condition of the varieties germination was investigated in an incubator at 25°C with a randomized complete block design of three replications.

The low temperature germinability was tested in an incubator of 15°C by the same method as that of the 25°C experiment.

Results and Discussion

High germination percentages above 85% were scored for all the varieties at 25°C (Appendix 1 & 2). T-test analysis showed no significant variation among the varieties (Table 1). Fig. 1 shows the germinability of some of the varieties at 25°C. Germination was not delayed, by the fifth day most of the seeds had germinated. We concluded that the various varieties used had good germinability.

At 15°C a large variation on germination percentage among varieties was found. The variance of germinability at 15°C was significantly large (Table 3). Variation in the germination percentage in the rice varieties at 25°C and 15°C is shown in fig. 3. The following varieties , Canabongbong, Ghuizhao 2, Guanluai 4, Bodat Mayang, Nepal No. 1, Liuzhoubaoyazao, Toboshi, Hongmi, Xuanchangmi, IR30, Surjamkhi, Dourado Precoce, and R.M., Oba, Norin No. 1,and Yamayutaka, had very high germinability above 95%, while varieties such as Ladang, Deng Mak Tek, Moroberekan, Haogang, Tiazhongyu 204 had very low germinability below 40% (appendix 3 & 4). There was no significant difference between Japonica and Indica varietal group at 15°C (Table 2).

Omrod and Bunter (1961) found that Japonica varieties have better germinability at low temperature. Our results however indicated that majority of the varieties with high percentage of germination at 15°C were Indica but not Japonica varieties.Our results subscribes to the finding that Japanese cultivars and genetic testers differ appreciably in the rate at which seed germination proceed at 15°C (Sasaki et al. 1974).

Acknowledgment

I express my sincere thanks and appreciation to Japan International Co-operation Agency (JICA) for accepting me as a participant to its 1995 course in Plant Genetic Resources as a result of which the writing of this report is made possible. I am grateful to the Head, Dr T. Nagamine and the staff of the Plant Conservation Laboratory who supervised my work and assisted me by divers ways to produce this report. I am thankful to the head and staff of the genebank at National Institute of Agrobiological Resources (NIAR) for their assistance to me during the laboratory work.

The council for Scientific and Industrial Research and the Plant Genetic Resources Centre of Ghana gave me a study leave, I am grateful to them.

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Table 1 T-test of germinability at 25°C between Indica and Japonica

	Japonica		Indica
Average	95.503		96.597
Variance	14.484		13.521
No of varieties	51		43
T-value		-1.414	
Probability		0.803	

Table 2 T-test of germinability at 15°C between Indica and Japonica

	Japonica		Indica
Average	80.901		86.627
Variance	390.925		195.879
No of varieties	51		43
T-value		-1.638	
Probability		0.0524	

Table 3 T-test of germinability for 94 varieties between 15°C and 25°C

	15°C		25°C
Average	83.521		96.003
Variance	306.861		14.182
No of varieties	94		94
T-value		-6.754	
Probability		4.654	

Table 4 F-test of variance of germination between 15°C and 25°C

	15°C		25°C
Average	83.521		96.003
Variance	306.861		14.183
No. of Var.	94		94
Degree of Freedom	93		93
F-value		21.64	
Probability		5.42	

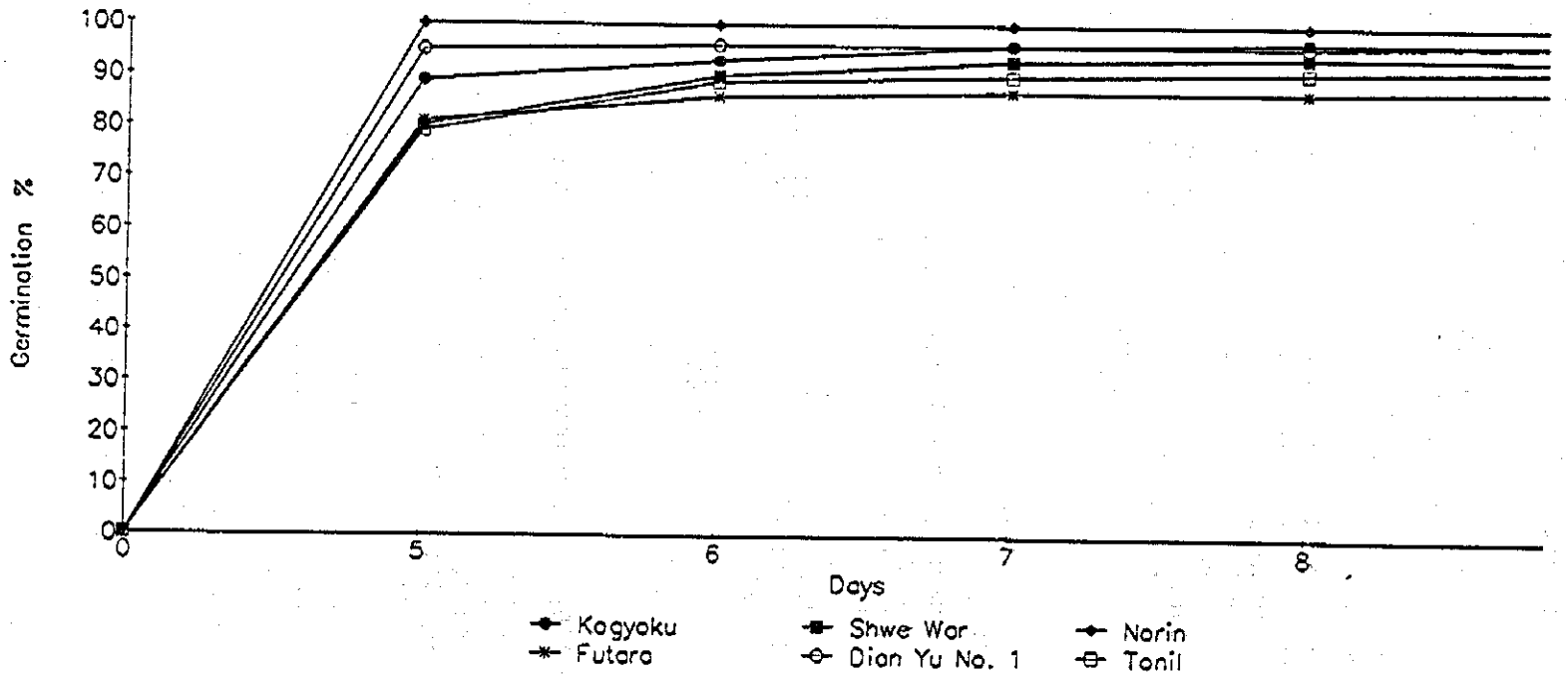

Fig. 1. Changes of germination percentage at 25°C.

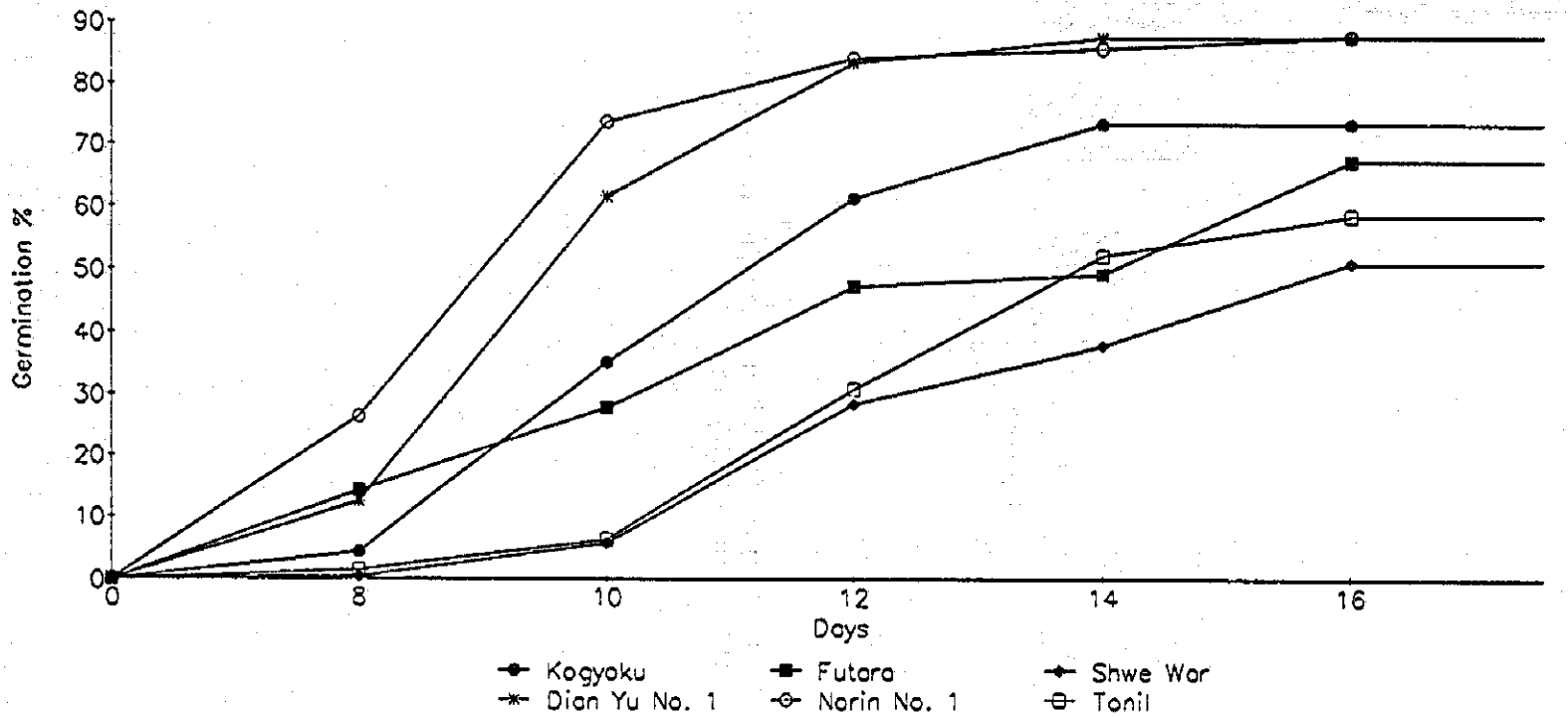

Fig. 2. Changes of germination percentage at 15°C

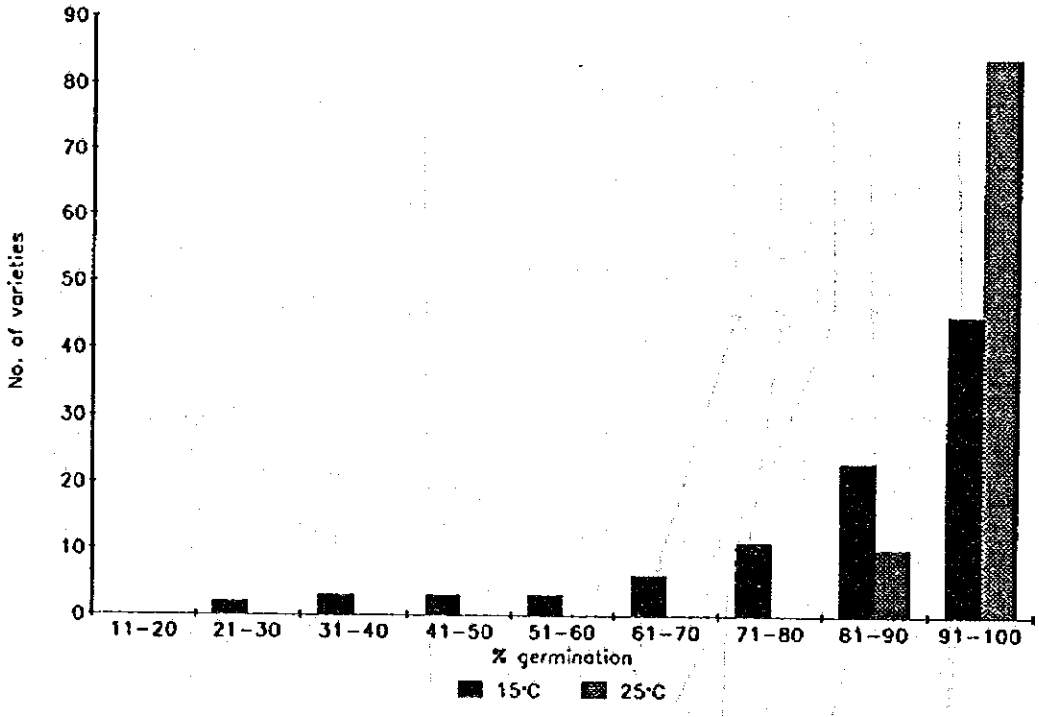

Fig. 3 Variation of germination percentage of rice varieties at 25/15° C .

Appendix 1. Germinability of Japonica Varieties at 25°C

Acc. No.	Varietal Name	Varietal Group	Rep. 1	Rep. 2	Rep. 3	Total	Mean
7048	Dian Yu No. 1	JP	99	94	94	287	95.67
7049	Nan Jing Xiang Dao	JV	98	97	97	292	97.33
7050	Simedel	JV	96	92	92	280	93.33
7051	Siampang	JV	94	94	94	282	94.00
7052	Ladang	JV	95	84	84	263	87.67
7053	In Sitt	JV	96	99	99	294	98.00
7054	Khauk Yoe	JV	100	100	100	300	100.00
7055	Canabongbong	JV	100	100	100	300	100.00
7056	Mack Kheua	JV	94	94	94	282	94.00
7057	Vista	JV	100	100	100	300	100.00
7061	Tambo	JV	95	95	96	286	95.33
7063	Bodat Mayang	JV	95	97	98	290	96.67
7064	Masumikir	JV	91	95	93	279	93.00
7065	Padi Kemikir Puti	JV	91	92	94	277	92.33
7066	KU 70-1	JV	96	97	97	290	96.67
7067	Basilanon	JV	96	86	99	281	93.67
7068	Dourado Precoce	JV	95	88	94	277	92.33
7069	Geraldine	JV	92	92	97	281	93.67
7071	Deng Mack Tek	JV	85	85	93	263	87.67
7072	Dam Ngo	JV	91	93	96	280	93.33
7074	Moroberekan	JV	79	93	85	257	85.67
7076	Hao Gang	JV	87	86	83	256	85.33
7078	Afgha WYR-5088	JV	97	97	98	292	97.33
7079	Shwe War	JV	96	93	95	284	94.67
7080	CS-S4	JV	92	91	97	280	93.33
7082	R.M	JP	95	100	99	294	98.00
7083	Wzbeuskij 2	JP	98	98	99	295	98.33
7084	Romeo	JP	98	97	97	292	97.33
7085	K78	JP	83	91	90	264	88.00
7086	Sinaba	JP	87	97	99	283	94.33
7087	Huang Gu	JP	95	100	97	292	97.33
7089	Li Zhi Hong	JP	98	98	98	294	98.00
7090	Kogyoku	JP	100	97	94	291	97.00
7092	Akage	JP	96	97	97	290	96.67
7093	Shirakawa	JP	97	98	98	293	97.67
7094	Shinshukaneko	JP	96	100	98	294	98.00
7095	Shinshu	JP	96	95	95	286	95.33
7098	Oba	JP	98	98	99	295	98.33
7101	Jikkoku	JP	99	99	99	297	99.00
7102	Ginbozu	JP	98	96	97	291	97.00
7103	Banzai	JP	99	95	98	292	97.33
7104	Kameji	JP	99	96	98	293	97.67
7105	Shinriki	JP	99	99	93	291	97.00
7107	Norin No. 1	JP	100	100	100	300	100.00
7108	Norin No. 8	JP	96	98	98	292	97.33
7109	Futara	JP	88	88	86	262	87.33
7110	Kinmaze	JP	98	95	99	292	97.33
7111	Taichu	JP	99	99	99	297	99.00
7114	Kitakihari	JP	99	98	98	295	98.33
7115	Yamayutaka	JP	99	100	96	295	98.33
7120	Reimei	JP	100	99	100	299	99.67
Mean			95.29	95.33	95.88		95.50

HS = Hsien = Chinese Indica
JV = Javanica = Tropical type Japonica
JP = Japonica
IN = Indica

Appendix 2. Germinability of Indica Varieties at 25°C

Acc. No.	Varietal Name	Varietal Group	Rep. 1	Rep. 2	Rep. 3	Total	Mean
7001	Muha	IN	99	96	96	291	97.00
7002	Dakanalo	IN	97	94	98	289	96.33
7003	Surjamuki	IN	100	98	100	298	99.33
7004	Dular	IN	98	94	93	285	95.00
7005	Pusur	IN	92	93	97	282	94.00
7007	Kasalath	IN	97	99	98	294	98.00
7008	Chinsurah Boro II	IN	100	98	99	297	99.00
7010	Co 13	IN	91	96	97	284	94.67
7011	Nepal No. 1	IN	98	100	91	289	96.33
7013	Nepal No. 18	IN	99	100	100	299	99.67
7014	Nepal No.555	IN	97	99	99	295	98.33
7017	Kinandang Puti	IN	98	100	100	298	99.33
7018	IR24	IN	97	97	95	289	96.33
7020	IR29	IN	99	100	100	299	99.67
7021	IR30	IN	100	99	100	299	99.67
7022	IR2061-214-3	IN	97	100	99	296	98.67
7023	Miliang 23	IN	90	91	94	275	91.67
7024	Xuanchangmi	HS	99	100	100	299	99.67
7025	Liuzhoubaoyazao	HS	99	100	100	299	99.67
7026	Xiligu	HS	98	99	100	297	99.00
7028	Hong Mi	HS	99	99	100	298	99.33
7029	Chishiendao	HS	100	100	100	300	100.00
7030	Hongxuenuo	HS	98	100	99	297	99.00
7031	Hunanhsien	HS	97	95	99	291	97.00
7033	Daorenqiao	HS	99	99	100	298	99.33
7034	Duanguanghualuo	HS	99	99	100	298	99.33
7035	Bayenuo	HS	95	93	92	280	93.33
7037	Hongcheuhzhai	HS	99	99	99	297	99.00
7039	Wuguhualu	HS	97	98	98	293	97.67
7041	Deng Pao Zhai	HS	98	99	100	297	99.00
7042	Toboshi	HS	100	99	100	299	99.67
7043	Tadukan	HS	91	91	100	282	94.00
7045	China 830	HS	93	96	97	286	95.33
7046	Zaiyeqing	HS	98	100	98	296	98.67
7047	Ghuizhao 2	HS	97	97	100	294	98.00
7128	Hokuriku	IN	95	96	100	291	97.00
7154	Kanto 146	IN	91	84	87	262	87.33
7155	Guangluai 4	HS	97	99	97	293	97.67
7157	Taizhogyu 204	HS	91	89	87	267	89.00
7158	Nanjing	HS	89	85	94	268	89.33
7159	Taizhongzailai 1	HS	96	99	99	294	98.00
7160	Suweon 258	IN	96	86	79	261	87.00
7161	Tonil	IN	85	90	90	265	88.33
Mean			96.40	96.40	97.00	289.79	96.60

Appendix 3. Germinability of Japonica at 15°C

Acc. No.	Varietal Name	Varietal Group	Rep. 1	Rep. 2	Rep. 3	Total	Mean
7048	Dian Yu No. 1	JP	97	76	88	261	87.00
7049	Nan Jing Xiang Dao	JV	92	97	99	288	96.00
7050	Simedel	JV	85	69	91	245	81.67
7051	Siampang	JV	92	91	95	278	92.67
7052	Ladang	JV	10	26	18	54	18.00
7053	In Sitt	JV	99	97	95	291	97.00
7054	Khauk Yoe	JV	89	98	94	281	93.67
7055	Canabongbong	JV	97	98	99	294	98.00
7056	Mack Kheua	JV	90	88	95	273	91.00
7057	Vista	JV	74	93	95	262	87.33
7061	Tambo	JV	88	92	94	274	91.33
7063	Bodat Mayang	JV	98	98	99	295	98.33
7064	Masumikir	JV	89	80	81	250	83.33
7065	Padi Kemikir Puti	JV	65	53	48	166	55.33
7066	KU 70-1	JV	63	93	86	242	80.67
7067	Basilanon	JV	83	83	72	238	79.33
7068	Dourado Precoce	JV	97	97	95	289	96.33
7069	Geraldine	JV	77	92	58	227	75.67
7071	Deng Mack Tek	JV	24	52	36	112	37.33
7072	Dam Ngo	JV	47	78	51	176	58.67
7074	Moroberekan	JV	36	50	22	108	36.00
7076	Hao Gang	JV	15	25	35	75	25.00
7078	Afgha WYR-5088	JV	85	91	37	213	71.00
7079	Shwe War	JV	41	38	73	152	50.67
7080	CS-54	JV	56	51	49	156	52.00
7082	R.M	JP	100	98	97	295	98.33
7083	Wzbeuskij 2	JP	94	96	92	282	94.00
7084	Romeo	JP	89	93	83	265	88.33
7085	K78	JP	76	68	84	228	76.00
7086	Sinaba	JP	94	92	99	285	95.00
7087	Huang Gu	JP	83	96	80	259	86.33
7089	Li Zhi Hong	JP	88	72	95	255	85.00
7090	Kogyoku	JP	80	65	74	219	73.00
7092	Akage	JP	95	92	92	279	93.00
7093	Shirakawa	JP	94	98	90	282	94.00
7094	Shinshukaneko	JP	93	97	92	282	94.00
7095	Shinshu	JP	85	63	86	234	78.00
7098	Oba	JP	96	95	94	285	95.00
7101	Jikkoku	JP	86	72	88	246	82.00
7102	Ginbozu	JP	94	93	97	284	94.67
7103	Banzai	JP	86	83	74	243	81.00
7104	Kameji	JP	89	88	81	258	86.00
7105	Shinriki	JP	57	66	61	184	61.33
7107	Norin No. 1	JP	98	100	94	292	97.33
7108	Norin No. 8	JP	87	83	81	251	83.67
7109	Futara	JP	69	64	68	201	67.00
7110	Kinmaze	JP	93	97	90	280	93.33
7111	Taichung No. 65	JP	85	95	97	277	92.33
7114	Kitahikari	JP	90	95	95	280	93.33
7115	Yamayutaka	JP	99	98	96	293	97.67
7120	Reimei	JP	75	99	100	274	91.33
Mean			79.69	81.65	80.10	80.48	

ASSESSING GENETIC PURITY OF *Vigna unguiculata* BY SEED PROTEIN ELECTROPHORESIS

Amaning TWUM

Abstract

The genetic purity of cowpea (*Vigna unguiculata*), varietal name Sanji collected from the northern region of Ghana was studied using seed albumin by polyacrylamide gel electrophoresis. All the electrophoregrams from the fifty seeds used in the study had the same band pattern. From the above it was ascertained that the seeds of the variety studied has a high purity of 100% and this makes them suitable and reliable for breeding work.

Introduction

One of the major areas by which germplasm can be utilized is to make them available for breeding work. Breeding work however requires genetic materials which are identifiable and of high genetic purity if misleading results are not to be obtained.

Cowpea is the most important legume in Ghana. It is used in many kinds of dishes and often cooked together with rice as a meal to provide protein. It is also eaten alone as a meal after it has been cooked and fried with tomato and onion.

Cooked cowpea may also be eaten with other kinds of food such as gari(a processed cassava food) and fried plantain.

With such a standard of popularity and importance as a food legume in Ghana it is not surprising that the crop has attracted the attention of many scientists especially plant geneticists and breeders. A project commissioned by Canadian International Development Agency (CIDA) with the Crops Research Institute of Ghana at Kumasi works more on the breeding of this legume than any other legume. At the Plant Genetic Resources Centre at Bunso, Ghana, over eighty varieties are being conserved and it is the legume with the highest number of varieties conserved at the Centre.

The variety sanji used in this experiment is one of the varieties being conserved at Bunso. It was collected by the Plant Genetic Resources Centre at Bunso and their counterparts from Japan in 1993. Since seed protein electrophoresis method is useful and recognized for resolving taxonomic and evolutionary problems (Ladizinsky and Hymowitz 1979), we use this method to test for the genetic purity of the variety for utilization in breeding work.

Here we report the result of genetic purity of cowpea as detected by polyacrylamide gel eleotrophoresis (PAGE).

Materials and Methods

Materials

Vigna unguiculata seeds, varietal name Sanji was sampled

from a seed lot stored at National Institute of Agrobiological Resources (NIAR) Japan. This was weighed and was inspected for seeds of other species and inert particles. The seeds were found to conform to pure seeds by International Seed Testing Association Standard. These seeds were counted and seed protein (albumin) was extracted from individual seeds for electrophoresis to test for genetic purity. A total of 50 seed albumin samples were collected.

Protein extraction

Individual seeds were ground separately and 10mg of each was weighed into a separate eppendorf tube. 300 micro liters of 0.02M Tris Hcl (Ph 8.0) was added to the ground seed in each tube to extract albumin at room temperature for two hours. Thereafter it was centrifuged at 14,000 rpm for three minutes. 150 micro liters of the supernatant liquid was pipetted out into another microtube and 15 micro liters of 1M sodium acetate buffer (pH4.5) was added then centrifuged at 14,000 rpm for 3 minutes. To the supernatant liquid was added 300 of micro liters acetone and this mixture was kept in a deep freezer at -20°C overnight.

In the following morning the supernatant was poured off leaving only the extracted albumin in the microtube. To the extracted albumin was added sample buffer (10 micro liters of 4% SDS) and a drop of mercapt ethanol to get the albumin dissolved.

Electrophoresis

Albumin samples extracted from individual seeds were analyzed by PAGE. The electrophoresis was conducted at 110 volts for the first 15 minutes and thereafter at 160 volts for 45 minutes. The electrophoresis gel was stained with 0.1% coomasie brilliant blue dissolved in a mixture of ethanol and acetic acid and was destained with a mixture of methanol and acetic acid.

The analysis of the banding pattern was performed with one electrophoregram to score genetic purity each seed.

Results and Discussion

Physical purity of seeds

When the bulk of seeds sampled from the experiment were inspected for inert particles and seeds of other species none was found. All other materials which could mar the seed purity of the variety that was being studied were clearly absent.

The formula for seed purity below :

$$\frac{\text{weight of pure seed sample}}{\text{total weight of seed}} \times 100$$

gave 100% for seed purity.

Genetic purity

All the 50 electrophoregrams for the fifty individual seeds showed the same band pattern without any difference. This also showed that the genetic constitution of the seeds was the same and therefore genetic purity was 100% for the variety. Fig. 1 shows the electrophoregrams from ten of the seeds.

The variety is therefore recommended as a reliable pure line for breeding work.

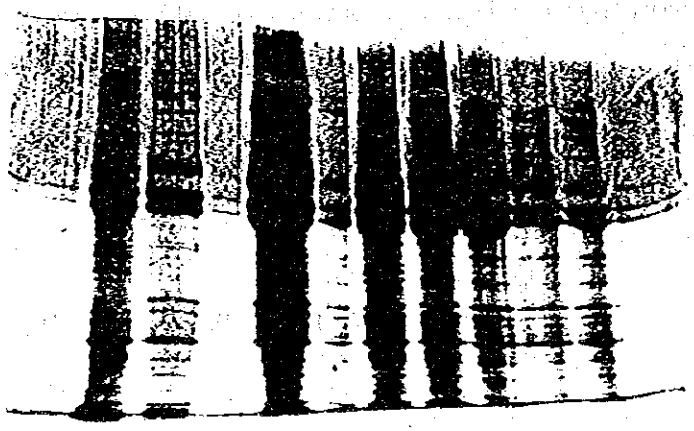

Fig. 1. Electrophoregrams of cowpea seeds

Acknowledgment

I am thankful to Japan International Cooperation Agency (JICA) who offered me the opportunity to participate in its group training course in Plant Genetic Resources for 1995, the result of which made the production of this report possible.

I express my thanks to the Head, Dr. T. Nagamine and the staff of the Plant Conservation Laboratory who supervised and trained me when this report was being produced.

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3. LAOS

Vandy PHETPASEUT

Studies on blast resistance and polymorphism of isozyme variation in some Lao rice varieties

VANDY PHETPASEUT

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Introduction

Blast is one of the most widely distributed rice diseases. The Commonwealth Mycological Institute reported that blast has been found in 85 countries (CMI Distribution Maps of Plant Diseases,51,ed.6,1981). It may be said that pblast is present practically everywhere that rice is grown commercially on a large scale, althrough it has not been reported in California and perhaps has not caused much damage in a few other rice-growing areas. Its adaptability to various environmental conditions is remarkable. In the middle East, where rice is grown under conditions of very high temperature and very low relative humidity and is irrigated by water from underground springs or from rivers, the fungus is found to infect the node just above the field water level, though there is no lesion on the leaves or on any other parts of the plants. This was referred to as "shara" disease in Iraq (Ou 1985).

The blast is controlled by some combination of host plant resistance, cultural practices, and fungicides. Host plant resistance appears to be the most promising method for controlling blast. There is a long history of the development of resistant varieties to blast. It seems very likely that, in the future, there will be increased

emphasis on using genetic resistance because it will not pollute the environment (Zeigler et al 1986).

Isozyme polymorphism in Asian cultivated rice (*Oryza sativa* L.) has received much attention in recent years. A major input was the demonstration by Second and Trouslot (1980) that considerable variation can be revealed by starch gel electrophoresis. Some extensive studies have involved up to 25 genes expressed at tillering and flowering (Second 1982), and 21 genes in coleoptiles a few days after germination (Glaszmann 1985a). At present, at least 36 polymorphic loci can be surveyed during the development of the rice plant. Location of isozyme loci on the rice chromosomes has progressed simultaneously, by linkage analysis (Glaszmann 1985b, Nakagahra and Hayashi 1976, Pai et al 1975, Sano and Barbier 1985, Sano and Morishima 1984) and by trisomic analysis (Ishikawa et al 1986, Ranjhan et al 1986, Wu et al 1987). The former has resulted in the precise mapping of 3 loci, while the latter has associated 16 genes with their respective chromosomes.

Knowledge of the extent of polymorphism and the chromosomal location of genes encoding isozymes makes them helpful as genetic markers in rice. These genes present various advantages (Tanksley and Rick 1980), such as stable expression in a wide range of environments; the absence of epistatic interrelationships, which permits surveying many genes simultaneously; and usually, codominance, which permits determining the exact genotype. The use of tissues of young plants simplifies the

Table 1. Test for true resistance to blast

Entry No.	Variety	Gene	001.0*	101.0 001.1	003.2 002.2	033.1 003.1	006.0 007.0	007.0	031.1 030.1	037.3	073.1 042.0	043.0	002.1 043.0	047.0	047.1 046.0	071.1	073.1	077.1 076.1	077.1	075.0 077.1
1	Shin 2	*Pi-k*^{s}, *sh*	S	MR	R	MS	S	S	R	S	R	MS	S	S	R	-	-	R	S	S
2	Aichiasahi	*Pi-a*	R	R	S	MS	S	S	R	S	S	S	S	S	S	R	S	S	S	S
3	Yamabiko	*Pi-a*	R	R	S	MS	S	S	R	S	S	S	S	S	S	R	S	S	S	S
4	Fujisaka 5	*Pi-i*	R	R	R	R	S	S	R(b)**	MS	R	R	R(b)	S	S	R	R	S	S	S
5	Inabawase	*Pi-i*	R	R	R	R	S	S	R(b)	MS	R(b)	R	R(b)	S	S	R	-	S	S	S
6	Kusabue	*Pi-k*	R	R	R	R	R	R	S	MS	R	R	R	R	R	S	S	S	S	S
7	Tatsumimochi	*Pi-k*	R	MR	R	R	R	R	S	S	R	R	R	R	R	-	-	S	S	S
8	Tsuyuake	*Pi-k*^{m}	R	R	R	R	R	R	S	MS	MR	R	R	R	R	S	S	MS	S	S
9	Fukunishiki	*Pi-z*	R	R	-	R	R	R	MR	R	S	MS	S	S	S	S	S	S	S	S
10	Yashiromochi	*Pi-ta*	R	R	R(b)	MR	R	R(b)	R(b)	R	S	R	R(b)	R(b)	R	R	R	R	R	R(b)
11	Pi-No4	*Pi-ta*^{2}	R	-	R	MR	R	R	R	R	R	R	R	R	R	-	R	R	R	R(b)
12	Toride 1	*Pi-z*^{t}	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
13	K 60	*Pi-k*^{p}	R	S	R	S	R	R(b)	S	MS	R	R	MR	R	R	R	S	S	S	S
14	BL-1	*Pi-b*	R	R	-	R	R	R	R	S	R	R	MR	R	R	R	-	R	R	R
15	K 59	*Pi-t*	R	R	R	R	R(b)	R(b)	R(b)	R	R(b)	R	R(b)	-	R(b)	R(b)	R(b)	R(b)	R(b)	R(b)
16	Gaya		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
17	Mangoku		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
18	Kenbaiwai		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
19	Kanousen 41		R	R	R	R	R	R(b)	R	R	R	R	R	R	R	R	R	R	R	R
20	H90-58		R	R	R	R	R	R(b)	R	R	R	R	R	R	R	R	R	R	R	R(b)
21	H92-42		R	R	R	MR	R	R	R	-	R(b)	R	R	R	MS	R	R	R(b)	S	R
22	H93-61		R	R	R	R	MR	MR	R	-	MS	R	MR	R	S	-	-	R	MS	R
23	H94-6		R	-	R	R	R	R	R	-	R	R	R	R	R	-	-	R	R	R
24	IR66		R	-	R	R	R	R	R	-	R	R	R	R	R	-	-	R	R	R
25	IR72		R	R	R	R	R	R	R	-	R	R	R	R	R	R	R	R	R	R
26	Do Nang Nouane		R	S	R(b)	R	R	R	R(b)	-	R(b)	R	R	R	R(b)	R	R	R(b)	R(b)	R
27	Khan The		R	R	R	R	R	R	R	R	R	R	R(b)	R	R	R	R	R	R	R
28	Dam Do		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
29	Luang Keo		R	R	R(b)	R	R	R	R(b)	R	R	R	R	R	R	R	R	R	R(b)	R
30	Me Hay		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
31	Luang Koy		R	-	R	R	R	R	R	R	R	R	R	R	R	-	-	R	R	R
32	Do Nang Nouane A		R	R	R	R	R	R	R	R	R	R	R	R	R(b)	R	R	R	R	R
33	Mak Yom		R	MS	MS	R	S	MS	MS	MS	MS	MS	MS	MS	MS	-	MS	MS	MS	MS
34	Pa Lat		R	R	R	R	R	R	R	R	R	R	R	R	R	R	-	R	R	R
35	Chao Lepnok		R	R	R	R	R	R	R	R	R	R	R	R	R(b)	-	-	R	R	R
36	Do In Tok		R	R	R	R	MR	R(b)	R	R	R	R	R(b)	R(b)	R	R	R	R	R	R(b)
37	Dong Dok May		R	R	R	R	R	R	R	-	R	R	R	R	R	R	R	R	R	R
38	Kh. Khi Tom		R	R	R	R	R	R	R(b)	R	R	R	R(b)	R(b)	R(b)	R	R	R	R	R
39	Kh. Mak Hing		R	R	R	R	R	R	R	R	R	R	R	R	R(b)	R	R	R	R	R
40	Samsy		R(b)	R	R(b)	R(b)	R	R	R(b)	R	MR	MR	MR	R	R(b)	MR	R(b)	R	R	R

* code number of blast race; upper row is original code, lower row is rearranged code.
** (b);brown spot.

manipulation of the materials, permits early determination of their isozymatic characters, and thus makes the possible associated screening procedures more efficient.

There are mainly three research objectives in this study as follows; 1) to know how to test for blast resistance of rice varieties, 2) to know which blast resistance genes are distributed in the Lao.PDR., and 3) to know the genetic variation in the Lao rice varieties using isozyme analysis.

A. Blast Resistance

1. True resistance to blast

The two kinds of resistance, true resistance and field resistance, are known against rice blast.

True resistance is a qualitative, race specific, and is characterized by a hypersentivity to the pathogen.

Materials and Methods

A total of forty varieties were tested for true resistance to blast (Table 1). They were fifteen Lao varieties(entry No. 26 to 40), ten varieties and breeding lines from IRRI(24, 25), Korea(16, 17), Taiwan(19), China(18) and Japan(20 to 23), with fifteen Japanese differentials(1 to 15).

Seeds of each entry were sown on the soil in seedling boxes(30x60cm). Twenty entries are sown in one box. Each batch consisted of two boxes. After seeding and then watering, the seedling boxes were put into an incubator to

accelarate germination for three days. Then the seedling boxes were transferred into a glasshouse.

Inoculation: The concentration of inoculum is adjusted about 15-26 spores in microscope field (x150 magnification). Inoculation is carried out by spraying on seedlings older than 3rd-4th leaf stage. After inoculation the seedling boxes are put in an incubator at 24-26 °C for twenty four hours (Ikeda 1994).

The experiment was designed to use 25 races of blast. However, I could not got any results in this experiment because the inoculation was not effective. The temperature in the greenhouse might be too high to grow the blast fungi in the rice plants. I tried again the test using the ratoon plants of same materials. The test materials were cut down at about 5 cm from the soil surface. Two days before cutting about 10g of ammonium sulfate were supplied on the seedling boxes to help the growth of new leaves. Seven to ten days after cutting the ratoon plants were inoculated again.

Results and Discussion

Six varieties, Toride 1, Gaya, Mangoku, Kenbaiwai, Dam Do and Me Hay showed resistance to all blast races. Two varieties are from Lao(Dam Do, Me Hay) two are from Korea(Gaya, Mangoku), one each is from China(Kenbaiwai) and Japan(Toride 1).

Six varieties were also resistant but could not get complete data because they were died against some races.

Two varieties are from IRRI(IR 66, IR 72), three are from Lao(Luang Koy, Pa Lat, Dong Dok May) and one is from Japan(H 94-6).

Six varieties were resistant but they had brown spots to some races. One variety is from Japan(H 94-6) and five varieties are from Lao.

One variety from Lao, Mak Yom, showed susceptible to most of races.

These results shows that most of Lao varieties have unknown resistance gene to blast. So we can not identify the resistance gene in such Lao varieties by using Japanese differentials.

2. Field resistance to leaf blast

Field resistance is a quantitative, non-race specific resistance. Field resistance to leaf blast is usually tested at the seedling stage on the upland nursery. The upland nursery is more favorable for evaluating the field resistance in rice than the flooded field.

Many resistant rice varieties have been developed against blast. But as in disease resistant varieties of other cereals, their usefulness was short-lived, as their resistance was lost or "broke down" because of the development of new races or pathotypes of the pathogen. Many works have searched for more stable types of resistance such as field resistance or general resistance.

Materials and Methods

Table 2. Scores of resistance to leaf blast in nursery

Score	General description of symtoms	% of infexted leaf area
0	No susceptible lesiions of leaf blast	0
1	Few susceptible lesions	1
2	A few susceptible lesions	2
3	Several susceptible lesions	5
4	Many suscptible lesions	10
5	A large number of susceptible lesions with few dead leaves	20
6	A few dead leaves	40
7	Several dead leaves	60
8	Many dead leaves	80
9	Almost all leaves dead	90
10	All leaves and stems dead	100

Nineteen varieties with known resistance genes were tested for the field resistance to leaf blast with three replications. The 20kg and 10kg/10a of Nitrogen were applied on the upland nursery as basal and top dressing, respectively. Previously the seed of Koshihikari as a spreader were sown on the upland nursery of the NARC at May 26, 1995, then the isolate of blast ($037b^{+}$) was sprayed on the spreader at June 23. The test materials were sown on the adjacent seedbeds in the same nursery at June 15. One entry was sown in the row in the upland nursery. After seeding we set the birds net. Fourteen days after inoculation on spreader, the epidemic on the test materials (5th or leaf stage) became apparent. The number and size of susceptible lesions on the leaves in each row were observed and compared with the check varieties (Table 2). In this case, varieties with the same genotypes for true resistance must be compared with each other.

Results and Discussion

Norin 22, Norin 29, Nipponbare, Aichiasahi, Kinmaze and Yamabiko have same resistance gene *(Pi-a)*. These varieties showed no differnces of field resistance to leaf blast with each other.

Todorokiwase, Fujisaka 5 and Inabawase are the same genotypes *(Pi-i)* but Todokiwase is almost all leaves dead. Fujisaka 5 and Inabawase are all leaves and stems dead.

Table 3. Field resistance to leaf blast in the differentials

Variety	Gene	July 7	10	11	13
Koshihikari	Pi-k^s, sh	4.7	8	9	9.3
Norin 22	Pi-a	5	8	9	9.3
Yamabiko	Pi-a	4	8.3	9.3	9.7
Kinmaze	Pi-a	3.7	8.7	9.3	10
Aichiasahi	Pi-a	4	9	9.7	10
Norin 29	Pi-a	5	8	10	10
Nipponbare	Pi-a	5	8.7	10	10
Todorokiwase	Pi-i	2	4.7	6	8.7
Fujisaka 5	Pi-i	3	8.7	9.3	9.7
Inabawase	Pi-i	3.7	9	9.7	10
Tatsumimochi	Pi-k	1	2	2.7	4.3
Wangetsumochi	Pi-k	1	4	5.3	8.3
Kusabue	Pi-k	1.3	6	8	10
Tsuyuake	Pi-k^m	1	2	2.7	4.7
Fukunishiki	Pi-z	0	0	0.3	2.3
Yashiromochi	Pi-ta	0.7	1	1	3.3
Pi-No4	Pi-ta^2	0	0.3	0.3	2
Toride 1	Pi-z^t	0	0	0	1
BL 1	Pi-b	1	2	2.7	4.3

Tatsumimochi, Mangetsumochi and Kusabue are the same genotypes(*Pi-k*) but they were damaged of blast different level.

Tatsumimochi is several susceptible lesions(4.3).

Mangetsumochi is many dead leaves(8.3).

Kusabue is all leaves and stems dead.

Fukushiki(*Pi-z*), Yashirimochi(*Pi-ta*), Pi-No 4(*Pi-ta^2*) and Toride 1(*Pi-z^t*) were no lesions of leaf blast. (Table 3)

B. Polymorphism of isozyme genetic variation in some Lao rice varieties

The genetic variation in some Lao varieties were analyzed using two isozymes according to Glaszmann et al (1988).

Materials and Methods

Forty three Lao varieties were obtained from the gene bank, NIAR. They were collected from different ecological areas in the Lao. It is known that the variation of rice varieties in Lao. depends on the ecological conditions. Two varieties, IR 36 and Nipponbare, were used as the checks of *Indica* and *Japonica*, respectively.

1) Gel and sample preparation: A starch gel is prepared using the appropriate gel buffer for the specific enzymes under investigation (Table 4). A solution of hydrolyzed Starch (14%) and buffer is carefully homogenized in a 1 liter erlenmeyer flask and heated with continuous swirling on a magnetic stirrer hotplate until a clear, vigorously

boiling solution is obtained. The solutions then deaerated with a tap aspirator and poured into an accrylic gel mold (Fig.1) in which electrode strips have been sealed with masking tape. Solid particles and air bubbles can be quickly removed with forceps. The gel is allowed to cool and set for approximately 30 min at room temperature is then covered with a plastic film to prevent excessive dehydration. It is placed for 20 min in a freezer (-15 °C) or for 1 h in a refrigerator for final cooling before use.

The plumages and coleoptiles at 4 days after germination are used for enzyme extraction. They are placed on spot plates while the gel is cooling. When the gel is cold, a slit is prepared approximately 6 cm from its cathode end; a bromophenol blue solution is applied to serve as a tracking dye. The plant tissues are ground with a small amount of cold distilled water. Filter paper wicks (whatman no.3) are used to absorb the extracts. Their sizes can be adjusted to the specific requirements of the experiment. They are inserted in the slit so that they form a continuous arrangement, to avoid distortion on the sides of the papers and to facilitate comparison of migration distances among the bands produced. Intermixing between adjacent papers is avoided by removing excess extract with absorbent paper prior to insertion into the gel.

Adding a small amount of an antioxidant(e.g.,0.1% mercaptoethanol) to the water used for extraction can be satisfactorily investigated with pure water.

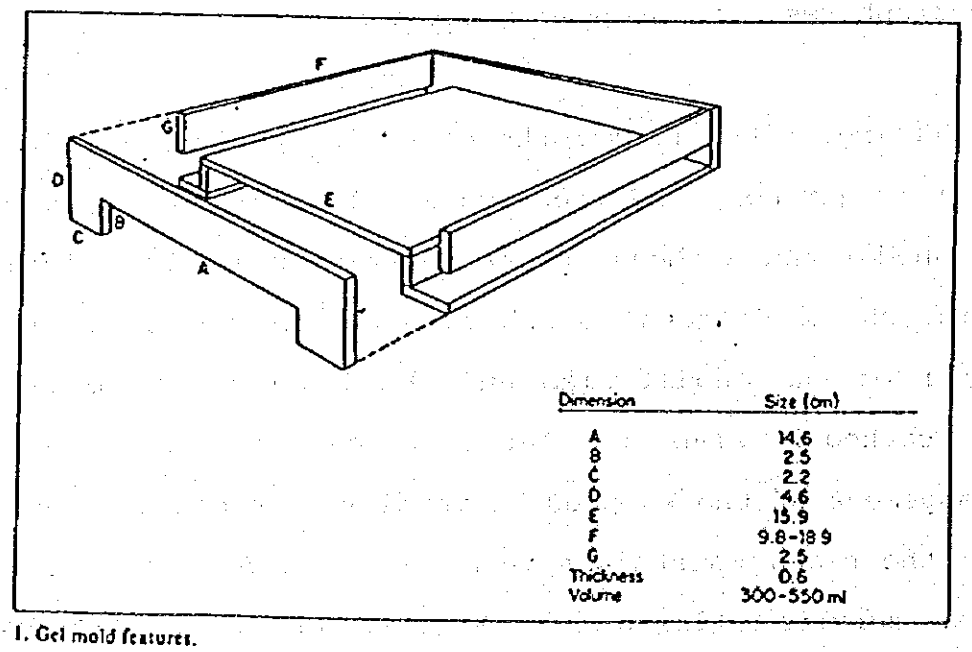
2) Electrophoresis: The plastic film is trimmed at the edges of the mold, and the masking tape is peeled off to expose the gel in the electrode strips. The gel is then mounted onto the electrode trays containing the appropriate tray buffer in a refrigerator at about 2 °C. The side where the samples were loaded is connected to the cathodal tray. A plastic bag of ice water is placed atop the gel to provide additional cooling. Platinum wire in the anodal tray and ordinary stainless steel wire in the cathodal tray serve as electrodes and are connected to a continuous-current power supply. A potential difference is applied through the gel. The constant parameter is the intensity, which is chosen so that the initial voltage will be about 10 volts/cm of length of the gel. The electrophoresis is stopped after 4 h.

3) Slicing: After electrophoresis, the gelis removed from the refrigerator, and rectangular slabs are prepared with the anodal and cathodal parts starting from the origin of migration. A diagonal slash is made on the upper right corner of the anodal slab and the lower right corner of the cathodal slab to later trace back the initial arrangement of the samples. Several slices can be prepared from the slabs to stain several enzymes. A slab is placed on an acrylic slicing bed (Fig.2), and a wire is drawn horizontally through the gel to cut a 1-mm slice. The upper part of the gel is then placed on another slicing bed, and the accessible slice is transferred to a stain

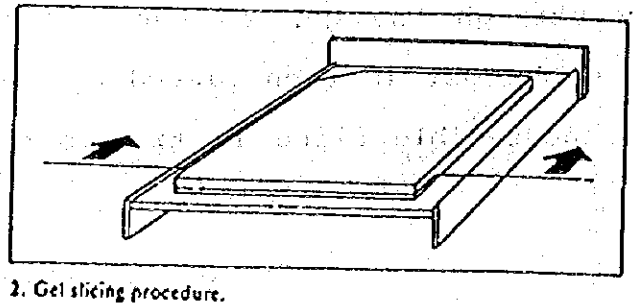
Table 4. Buffer system for electrophoresis

Enzyme: PGI, EST	
Buffer system: Gel	**Stock solutions**
Tris (0.009 M)	Trizma base (Tris) 10.40g
Histidine (0.005 M)	Histidine mono HCl 9.60g
pH 8.0	Completed with H_2O 0.3 l

PGI:	Buffer (0.1M Tris-HCl pH 8.0)	75 ml
	D-fructose 6-phosphate barium salt (F)	40 mg
	NADP+	1 ml
	MTT	1 ml
	PMS	1 ml
	G6PDH	10 unit
EST:	Buffer (0.1M Phosphate pH 6.5)	75 ml
	Fast garpet GBC salt (F)	22.5 mg
	α-naphthylacetate 75 mg/ml (1-propanol)	1 ml
	β-naphthylacetate 37.5 mg/ml (1-propanol)	1 ml



1. Gel mold features.



2. Gel slicing procedure.

Table 5. Polymorphism of isozyme variation in Lao varieties

No	Acc. No	Var. Names	PGI		EST	
			Pgi-1.	*Pgi-2.*	*Est-9.*	*Est-2*
1	9310001	Do Nang Nouane	2	2	2	2
2	9310002	Khan The	1	1	2	2
3	9310003	Dam Do	1	1	2	2
4	9310004	Luang Keo	2	2	2	2
5	9310005	Me Hay	1	1	2	2
6	9310006	Luang Koy	2	1	1	0
7	9130007	Do Nang Nouane A	2	2	2	2
8	9310032	Mak Yom	2	2	2	2
9	9310034	Khitom	1	2	2	2
10	9310036-2	Khao Vieng	1	2	2	2
11	9310037	Makkok	1	2	2	2
12	9310041	Nam Ang	1	1	2	2
13	9310043	Nam Manh	1	2	2	2
14	9310053	Y Thai	1	2	2	2
15	9310055	Pa Kheng	2	1	1	2
		IR 36	1	1	2	2
		Nipponbare	2	1	1	0
16	9310074	Pa Lat	1	1-2	2	2
17	9310122	Khao Sam Deuane	2	1	1	0
18	9310209	Kh. Khi Tom	1	2	2	2
19	9310221	Kh. Mak Hing	2	1	1	0
20	9310222-1	Samsy	2	2	2	2
21	9310237	Lep Heth	2	1	1	0
22	9310277	Pa Loth	2	2	2	2
23	9410061	Khao Namma	1	2	2	2
24	9410064	Khao Nga Xang Khao	2	1	1	0
25	9410074	Khao Pat Hinh	2	1	1	0
26	9410088	Khao Sam Neua 1	1	2	2	2
27	9410089	Khao Sam Neua 2	1	2	2	2
28	9410094	Khao Vanthong B	2	1	1	0
29	9410110	Chao Lepnok	1	1	2	2
		IR 36	1	1	2	2
		Nipponbare	2	1	1	0
30	9410122	Do In Tok	1	2	2	2
31	9410126-1	Do Khao	2	1	1	0
32	9410131	Dong Dok May	2	1	1	0
33	9410194	Khao Long Ma	1	2	2	2
34	9410198-2	Khao Mak Kam	1	1	2	0
35	9410201	Khao Mak Keua	2	1	1	0
36	9410205	Khao Manpet	1	2	2	2
37	9410207-2	Khao Mouang Sing	2	1	1	0
38	9410265	Khi Tom Deng	1	2	2	2
39	9410310	Pama	1	1	2	0
40	9410311	Pama Do	1	1	1	0
41	7600019	Khao Tong	2	1	1	0
42	7600022	Nock Kot	1	2	2	2
43	7500096	Mack Kham	1	2	2	2
		IR 36	1	1	-	-
		Nipponbare	2	1	-	-

Table 6. Classification of Lao varieties by polymorphism of Phosphoglucose isomerase

Group	Sample	% of sample
Pgi-1: 1 *Pgi-2: 1*	Khan The Dam Do Me Hay Nam Ang Pa Lat Chao Lepnok Mak Kham Pama Pama Do IR 36	20.93
Pgi-1: 2 *Pgi-2: 1*	Luang Koy Khao Sam Deuane Kh. Mak Hing Lep Heth Khao Nga Xang Khao Khao Pat Hinh Khao Vanthong B Do Khao Dong Dok May Khao Mak Kheua Khao Mouang Sing Khao Tong Nipponbare	27.90
Pgi-1: 1 *Pgi-2: 2*	Khitom Khao Vieng Makkok Nam Manh Y Thai Pa Lat Kh. Khi Tom Khao Namma Khao Sam Neua 1 Khao Sam Neua 2 Do In Tok Khao Long Ma Khao Manpet Khi Tom Deng Nock Kot Mack KHAM	37.20
Pgi-1: 2 *Pgi-2: 2*	Do Nang Nouane Do Nang Nouane A Luang Keo Mak Yom Samsy Pa Lot	13.95

box. This procedure is repeated until the desired number of slices is prepared.

4) Staining: Zones of enzymatic activity are revealed by immersing the gel slice into a stain assay (Table 4). The stain boxes chosen are only slightly larger than the slices so that 50 ml of the solution is sufficient to stain a slice. Some assays may have specific requirements such as total darkness, incubation at 40 °C, immediate scoring (catalase), or overnight staining (acid phosphates), which are summarized in Table 4.

In markedly disjointed activity zones, an individual slice can be used to reveal several enzymes: 1) by cutting several portions in the slice (e.g.,catalases from 0 to 4 cm, alanine aminopeptidases from 0 to 3 cm, and esterase from 3 to 8 cm), 2) by staining an enzyme (e.g., shikimate dehydrogenases followed by isocitrate dehydrogenases); or 3) by using the agar layer method for an enzyme and reusing the same slice by immersing in another assay solution (e.g., phosphoglucose isomerases followed by alcohol dehydrogenases).

For cases 1 and 2, the possibility of unexpected alleles must be in mind, requiring special attention.

Results and Discussion

Phosphogluscose isomerase genotypes have helped to clarify of some Lao rice varieties Table 5, Table 6 shows four different groups as follows:

Table 7. Classification of Lao varieties by Polymorphism of Esterase

Group	Samples	% of sample
Est-9: 2 *Est-2*: 2	Do Nang Nouane Khan The Dam Do Luang Keo Me Hay Do Nang Nouane A Mak Yom Khitom Khao Vieng Makkok Nam Ang Nam Manh Y Thai Pa Lat Kh. Khi Tom Samsy Pa Loth Khao Namma Khao Sam Neua 1 Khao Sam Neua 2 Chao Lepnok Do In Tok Khao long Ma Khao Manpet Khi Tom Deng Nock Kot Mack Kham IR 36	62.79
Est-9: 1 *Est-2*: 0	Luang Koy Khao Sam Deuane Kh. Mak Hinh Lep Heth Khao Nga Xang Khao Khao Pat Hinh Khao Vanthong B Do Khao Dong Dok May Khao Mak Kheua Khao Mouang Sing Khao Tong Nipponbare	27.90
Est-9: 2 *Est-2*: 0	Pama Pama Do Khao Mak Kham	6.97
Est-9: 1 *Est-2*: 2	Pa Kheng	2.32

Table 8. Classification of Lao varieties by Polymorphism of both *Pgi* and *Est.p*

Group	Sample	% of sample
Pgi-1: 1 *Pgi-2: 1* *Est-9: 2* *Est-2: 2*	1. Than The 2. Dam Do 3. Me Hay 4. Nam Ang 5. Pa Lat 6. Chao Lepnock IR 36	13.95
Pgi-1: 2 *Pgi-2: 1* *Est-9: 1* *Est-2: 0*	1. Luang Koy 2. Khao Sam Deuane 3. Khao Mak Hing 4. Lep Heth 5. Khao Nga Xang Khao 6. Khao Pat Hinh 7. Vanthong B 8. Do Khao 9. Dong Dok May 10. Khao Mak Kheua 11. Mouang Sing 12. Khao Tong Nipponbare	27.90
Pgi-1: 1 *Pgi-2: 2* *Est-9: 2* *Est-2: 2*	1. Khitom 2. Khao Vieng 3. Makkok 4. Nam Manh 5. Y Thai 6. Pa Lat 7. Khao Khi Tom 8. Khao Namma 9. Khao Sam Neua 1 10. Khao Sam Neua 2 11. Khao Long Ma 12. Khao Manpet 13. Khi Tom Deng 14. Nock Kot 15. Mack Kham	34.65
Pgi-1: 2 *Pgi-2: 2* *Est-9: 2* *Est-2: 2*	1. Do Nang Nouane 2. Do Nang Nouane A 3. Luang Keo 4. Mak Yom 5. Samsy 6. Pa Lot	13.95
Pgi-1: 1 *Pgi-2: 1* *Est-9: 2* *Est-2: 0*	1. Pama 2. Pama Do 3. Khao Mak Kham	6.97
Pgi-1: 2 *Pgi-2: 1* *Est-9: 1* *Est-2: 2*	1. Pa Kheng	2.32

Group 1 (*Pgi-1: 1, Pgi-2: 1*) includes nine varieties (20.93%) indicating the same genotypes of *Indica* variety IR 36,

Group 2 (*Pgi-1: 2, Pgi-2: 1*) includes twelve varieties (27.90%) indicating the same genotype of *Japonica* variety Nipponibare,

Group 3 (*Pgi-1: 1,Pgi-2:.2*) includes sixteen varieties (37.20%), and

Group 4 (*Pgi-1: 2,Pgi-2: 2*) includes six varieties (13.95%).

Table 7 shows four different groups as follows:

Group 1 (*Est-9: 2, Est-2: 2*) includes twenty seven varieties (62.79%) indicating the same genotype of *Indica* vriety IR 36,

Group 2 (*Est-9: 1, Est-2: 0*) includes twelve varieties (27.90%) indicating the same genotype *Japonica* variety Nipponbare,

Group 3 (*Est-9: 2, Est-2: 0*) includes three varieties (6.97%), and

Group 4 (*Est-9: 1, Est-2: 2*) includes one variety (2.32%).

Table 8. shows six different groups as follows:

Group 1 (*Pgi-1: 1, Pgi-2: 1; Est-9: 2, Est-2: 2*) includes six varieties (13.95%) indicating the same genotype of *Indica* variety IR 36,

Group 2 (*Pgi-1: 2, Pgi-2: 1; Est-9: 1, Est-2: 0*) includes twelve varieties (27.90%) indicating the same genotype of *Japonica* variety Nipponbare,

Group 3 (*Pgi-1: 1, Pgi-2: 2; Est-9: 2, Est-2: 2*) includes fifteen varieties (34.65%),

Table 9. Isozyme variation of Lao varieties from the different areas

Area	Variety	Pgi	Est	Total
Northern	Khao Kouang sing	2	2	2
	Khao Sam Neua 1	3	1	3
	Khao Sam Neua 2	3	1	3
	Khao Long Na	3	1	3
	Khao Namma	3	1	3
	khao Vieng	3	1	3
Central	Dong Dok Nay	2	2	2
	Khao Vanthong B	2	2	2
	Ne Hay	1	1	1
Southern	Pa Lat	3	1	3
	Khao Sam Deuane	2	2	2
	Do Nang Nouane	4	1	4
	Do Nang Nouane A	4	1	4
	Nak Yom	4	1	4
	Samsy	4	1	4
	Pama	1	3	5
	Pama Do	1	3	5
	Chao Lepnok	1	1	1
Check	IR36	1	1	1
	Nipponbare	2	2	2

Group 4 *(Pgi-1: 2, Pgi-2: 2; Est-9: 2, Est-2: 2)* includes six varieties (13.95%),

Group 5 *(Pgi-1: 1, Pgi-2: 1; Est-9: 2, Est-2: 0)* includes three varieties (6.97), and

Group 6 *(Pgi-1: 2, Pgi-2: 1; Est-9: 1, Est-2: 2)* include one variety (2.32%).

Eighteen out of 43 Lao varieties tested are known their cultivated area in Lao Table 9. The eighteen varieties are six from Northern area, there from Central area, and nine from Southern area of Lao.

In northern area, one variety showed the same pattern of *Japonica*, but the other five showed the different pattern of *Indica* and *Japonica*.

In Central area, two varieties showed the same pattern of *Japonica* and one variety showed the same pattern of *Indica*.

In Southern area one variety showed the same pattern *Indica* and but the other seven varieties showed the different pattern of *Indica* and *Japonica*.

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4. MONGORIA

Bayarsukh NOOV

TECHNICAL REPORT

GENETIC VARIATION OF *AEGILOPS* SPP. IN CAUCASUS USING RAPD ANALYSIS

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1995

Genetic variation of *Aegilops* ssp.in Caucasus using RAPD analysis

Abstract:

Intra and inter-specific variation in total cellular DNA of *Aegilops cylindrica* (genome constitution DDCC), *Aegilops squarrosa* (var.*typica* and *meyeri*; DD), *Aegilops biuncialis* (UUCC) and *Aegilops triuncialis* (UUCC) were studied by RAPD analysis.Polymorphic DNA from 61 accessions of the genus *Aegilops* was amplified with 12 different oligonucleotide primers. A total of 94 bands which showed polymorphism between accessions were scored. A Phenogram constructed using UPGMA method. Samples were divided into major two groups one group consisted of D genome species and the other of U genome species. *Ae.biuncialis* was more diverse than other species.

Introduction

The wild wheats are of great scientific interest. This has two major aspects : first classical taxonomic, cytological and evolutionary studies and second more pragmatic considerations such as the introduction of alien variation into cultivated forms by techniques of chromosome and genetic engineering (Kimber and Feldman 1987).

Linearus (1753) named seven genera in the tribe *Triticeae* including both *Triticum* and *Aegilops*. The genus *Triticum* contains species with cultivated forms while *Aegilops* encompassed wild relatives. Stebbins (1956), Bowden (1959) and Moris and Sears (1967) proposed a classification of including both *Aegilops* and *Triticum* in the one genus *Triticum*. More recently, Cordon et al. (1987) developed a new key in the identification of wild species of wheat.

Wheat group has 22 wild species having three ploidy levels form: diploid (with seven pairs chromosome), tetraploid (with 14 pairs) and hexaploid (with 21 pairs). Every diploid species has a distinct complement genome. Polyploid species of *Triticum* are a classical example of evolution through amphiploidy.

Genome analyses has indicated the origin of cultivated wheat as eincorn wheat (A genome), *Aegilops speltoides* (B genome). The putative diploid donor of the D genome of hexaploid wheat is *Aegilops squarrosa (Triticum tauschii)* (Kihara 1944; McFadden and Sears 1946; Rilley 1965).

As wild relatives of wheat can be crossed with cultivated wheat, they can serve as potential donors of desirable characters (Feldman and Sears 1988).

Evaluation of the molecular genetic diversity in the wild relatives of wheat is the first step in the use of this huge genepool of useful genes (Gill et al. 1987).

Different methodologies have been used to estimate the variation in wild wheat population. These include the morphological phenotypes (Jain et al.1975; Jaradat 1989; Poiakova and Blum 1983); protein electrophoresis especially

isozymes and storage proteins (Asins and Carbonell 1989; Nevo and Beiles 1989; Lugadah and Halloran 1988). These methods are of limited use for genetic diversity studies as there is much variation in different tissues, development stages and environmental factors.

Restriction Fragment Length Polymorphism (RFLP) method has been used for detection of genetic variation of many plants including the wheat group (Havey and Muchlbauer 1989; Keim et al.1989; Song et al. 1988; E.L.Lubbers et al 1990; N.Mori et al. 1994).

However, DNA polymorphism is not frequent in common wheat (Kim-Morgan, et al. 1989).

Williams et al. (1990) and Welsh and McClelland (1990) reported a new polymorphism assay based on the amplification of DNA sequences by the polymerase chain reaction using single oligonucleotide primers of arbitrary sequence. They have called the amplified product Random Amplified Polymorphic DNA (RAPD).

RAPD has been used to study genetic variation in various crop plants such as tomato (Klevin-Lankhorst et al. 1991); wheat (Devos and Gale 1992; He et al.1992; Vierling and Nguen. 1992; S.He et al. 1992); cabbage, cauliflower, broccoli and other cruciferious crops (Demek et al.1992); potato (Quiros et al. 1993; Singsit and Ozias-Akins 1993; Xu et al. 1993) and millets (Hilu 1994; H.K.M'Ribu and K.W.Hilu 1994).

RAPD markers as a source genetic markers were studied in wheat, including both wild and cultivated species and used for detecting the genetic diversity (K.M.Devos and M.G.Gale 1991; Richard A.Vierling and Henry T.Nguen 1992; He, Ohm and Mackenzie 1991). Results indicate that RAPD analysis is a powerful tool for determining the extent of genetic diversity among wheat genotypes.

This study deals with the analysis of inter and intra-specific diversity of *Aegilops* species by the RAPD method.

Materials and Methods

Plant material

Totally 61 accessions of *Aegilops* consisting of 23 of *Ae.cylindrica*, 6 of *Ae.squarrosa* var.*typica*, 6 of *Ae.squarrosa* var.*meyeri*, 16 of *Ae.biuncialis* and 10 of *Ae.triuncialis* were studied. Japanese bread wheat variety Norin-61 was used as a standard (table 1).

All samples of Aegilops used in this study were collected during collaborative mission between NIAR and Vavilov Research Institute for Plant Industry (VIR).

Samples were grown in the field of the National Agriculture Research Center (NARC), Tsukuba and leaves were sampled for DNA analysis.(The multiple collection from the same site and probable duplicates are not included).

Table 1. Accessions used in this study

Aegilops cylindrica:

Acc No	Location	Altitude
K1	Belorechensk	100
K2	Ivanovskaya	60
K3	Temryuk, Azovsky sea	100
K4	Azovsky sea	0
K5	Temryuk, Azovsky sea	20
K6	Azovsky sea	0
K7	Anapa	40
K8	Anapa	80
K9	Anapa	70
K13	Anapa	10
K14	Novorossisk	0
K18	Tuapse	0
K20	Nevinnomyssk	360
K22	Stavropol	700
K25	Pyatigorsk	380
K26	Pyatigorsk	530
K27	Agoy beach	0
K29	Tuapse	100
K31	Tuapse	40
K33	Tuapse	20
K36	Pendjik	570
K37	Maraga	540
K40	Machmudkent	350
K41	Makhachkala	70
K42	Karabudakent	160
K45	Makachkala	270

Aegilops squarrosa :

Acc No	Location	Altitude
var. *typica*		
K46	Dagestanskie Ogni	35
K48	Maraga	600
K50	Maraga	540
K53	S. Verhnii Larag	650
K54	Zele Gun	580
K55	Frig	90
var. *meyeri*		
K56	Stavropol	340
K58	Maraga	600
K59	Magaramkent	440
K61	Machmudkent	350
K62	Makhachkala	120
K65	Makhachkala	270

Aegilops biuncialis:

Acc No	Location	Altitude
K66	Tamanj	20
K69	Anapa	70
K71	Anapa	30
K72	Novorossisk	0
K74	Novorossisk	120
K76	Dagestanskie Ogni	35
K77	Gegjoh	95
K78	Maraga	600
K80	S. Horel	620
K81	S. Verhnii Jarag	650
K83	Machmudkent	350
K84	Machmudkent	120
K85	Karabudakent	160
K86	Pervomask	90
K108	Novorossisk	0
K109	Novorossisk	10

Aegilops triuncialis:

Acc No	Location	Altitude
K88	Azovsky sea	40
K90	Anapa	10
K94	Tuapse	70
K95	Dagestanskie Ogni	35
K97	Maraga	540
K98	S. Horel	620
K99	S. Verhnii Jarag	650
K100	Machmudkent	350
K101	Makhachkala	120
K103	Makhachkala	270

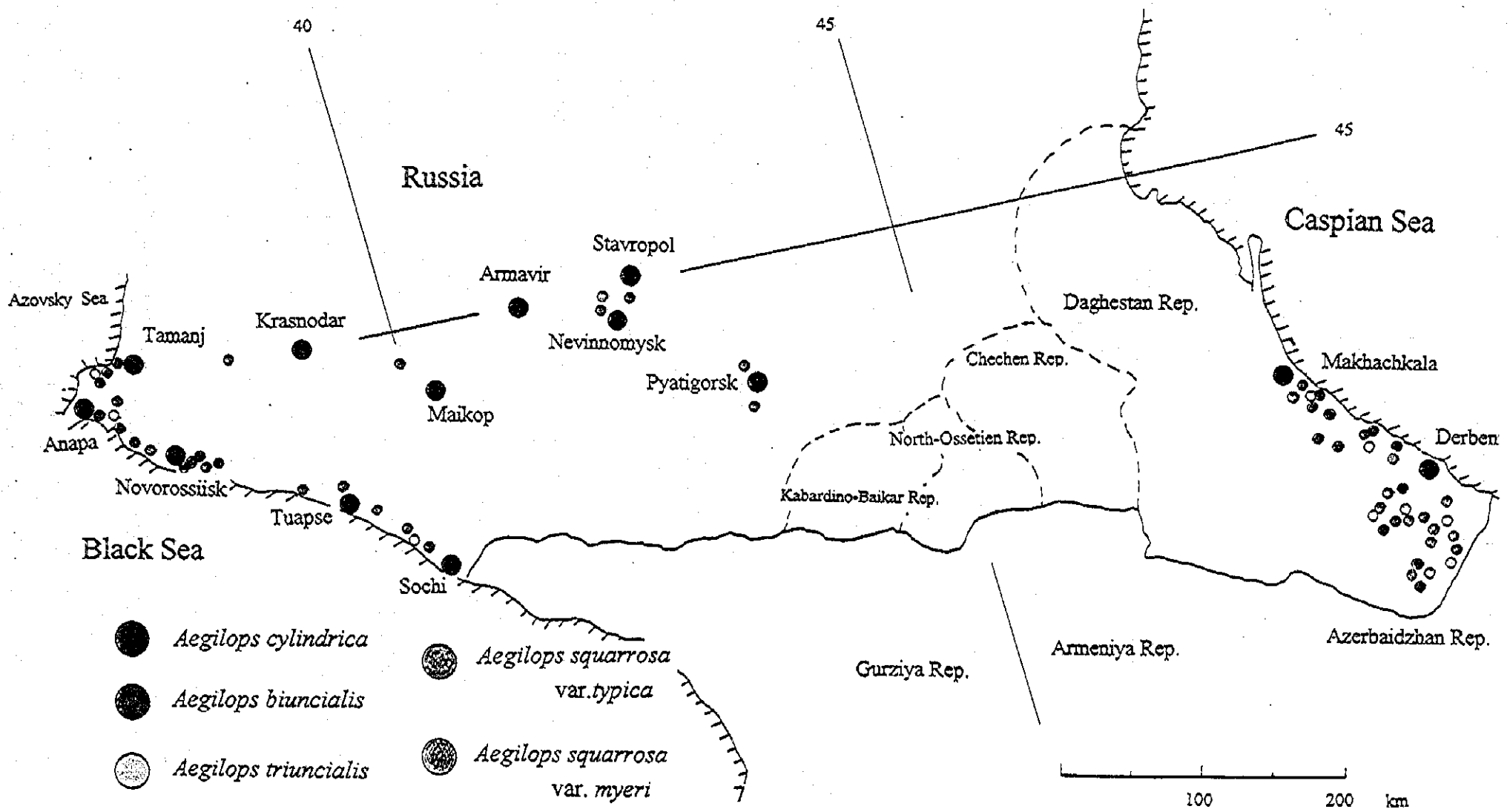

Figure 1. Collection sites of 61 *Aegilops* accessions used in this experiment.

Template DNA extraction and quantification

DNA was isolated in larger quantities based on the method of Murray and Thompson (1980).

Tender leaves about 6-8g of fresh weight were collected from one (several) plants put in an ice conteiner, cut into small pieces and frozen in liquid nitrogen and stored in deep freezer at -80°C.

Samples were freeze dried for 48 hours and approximately 5-6g of tissue was ground into fine powder using a mill grinder. Sample powder suspended in 25 ml of 1xCTAB extraction buffer (1% cetyltrimethyl ammonium bromide, 0.05M Tris-HCl (pH-8), 0.01M EDTA, 0.7M NaCl) with addition 25ul (microlitre) B-mercaptoethanol. The mixture was incubated in waterbath at 57°C for 30 minutes. The 15ml of Chloroform-Isoamyl alcohol (24:1) was added, centrifuged at 2500 rpm for 20 minutes.

Proteins were separated into heavier lower phase. The supernatent was precipitated with an equivalent volume of precipitation buffer (1%CTAB, 0.05M Tris -HCl, pH-8, 0.01M EDTA) and recovered by centrifugation at 2500 rpm for 20 minutes. The pellet was completely dissolved in 5ml HS-TE buffer (1M NaCl, 0.01M Tris-HCl, 1mM EDTA) with addition of 1*ul* RNAse at 36°C in incubator for 48 hours. DNA reprecipitated with an equal volume of isopropanol after centrifugation at 2500 rpm for 5 minutes and dissolved in 1xTE (0.01M Tris-HCl, 1mM EDTA) with 1*ul* RNAse again at 36°C for over night.

The 3.5 volume of ammonium acetate and ethanol (1:6) was added to the DNA solution and precipitated DNA was transferred carefully into 1.5ml microtest tube using disposal pipette. Pellets were washed 2-3 times with 70% ethanol, air dried and finally stored at 4°C in 200-300ul of 0.1xTE buffer.

DNA concentration was estimated with the Spectrophoto meter DU-7400 and agarose gel in comparison with lambda -DNA of known concentrations and adjusted to 5ng/*ul* with 0.1xTE and 1ng/*ul* with sterilized distilled water (SDW).

Primer

Out of 48 primers of ten base oligonucleotides, 12 primers which produce numerous distinct bands were selected for PCR amplification of *Aegilops* species. DNA was amplified twice using each of primers.

PCR condition

Polymerase Chain Reaction (PCR) was performed in the 96 well polycarbonate microplate using 62 wells per run in total volume of 10.1*ul* containing 2*ul* of DNA sample and 8.1*ul* premix.

The 2ul of 1ng/ul template DNA was first placed at the bottom of the well and subsequently 8.1ul premix added and finally overlaid with a drop of mineral oil.

The premix composition for PCR contained:

Sterilized Distilled Water (SDW)	5.4ul
10xPCR buffer	1.0ul
25mM MgCl	0.8ul
10mM dNTPs	0.2ul
20uM 10 mer oligonucleotide primer	0.5ul
Taq DNA polymerase (Thermus aquaticus)	0.2ul

The thermocycler (PHC-3) was programmed for 2 major cycles: the first one for 45 cycles each consisting of 93°C for 1', 35°C for 2' and 72°C for 3'; second cycle with 5°C for 1'.

Three different concentrations (5ng/ul; 1ng/ul; 0.1ng/ul) of template DNA have been attempted for PCR. Some accessions failed to producing amplified products with 5ng/ul templates. When accessions diluted into 1ng/ul and 5ng/ul the templates produced consistent, visible banding patterns and consequently 1ng/ul was used for the analysis.

Electrophoresis

The amplified DNA products were loaded on the 1.6% agarose gel in TAE (Tris-acetate EDTA) buffer containing ethidium bromide (Et-Br) and electrophoresis conducted at 70V for 3-4 hours. 7ul of lambda (0.24-4.4kb) was used as a standard size marker. DNA was stained with Et-Br and photographed under an ultraviolet light.

Also, 3% of Nusieve 3:1 agarose gel was used to separate PCR products of smaller size.

Data analysis

All accessions were scored for the presence or absence of RAPD band. Bands of low intensity but characteristic for certain species were scored as being present.
Polymorphic bands were scored "1" for presence or "0" for absence.

Genetic diversity (Nei and Li, 1979) between two entries was computed as:

$$\text{Dissimilarity index} = 1 - [2N/(Ni+Nj)]$$

where N is the number of shared bands and Ni and Nj are a total number of bands in entry i and j. The index was used to construct a cluster diagram by UPGMA (average linkage) method (Philip 3.5, procedure Neighbor, Felsentein 1993).

Results

94 scorable RAPD bands in size between 3.5-0.24 kbp were generated with 12 primers and 5 bands revealed across all accessions.

Primers used were varied by the number and size of amplified DNA fragments.

Most of bands with the molecular weight less than 2.2 kbp were reproducible. Primer P31 produced only one band with higher molecular weight more than 2.8 kbp in *Aegilops squarrosa* and *Aegilops cylindrica*. Average about 8 fragments per primer were detected.

5 DNA bands appeared in all species whereas others were unique to certain species or genotypes. 97%, 82%, 86%, 36%, and 57% of the total bands were not polymorphic in *Ae. cylindrica*, *Ae. squarrosa* var.*typica*, *Ae. squarrosa* var. *meyeri*, *Ae biuncialis*, *Ae. triuncialis*, respectively (table 2).
(examples of typical analysis are illustrated in fig.2 and fig.3).

Table 2. Number of RAPD markers observed in *Aegilops* species.

Species	Number of accessions	Number of markers detected within species	Number of polymorphic markers	%
Ae.cylindrica	23	33	1	3
Ae.squarrosa var.*typica*	6	33	6	18.2
Ae.squarrosa var.*meyeri*	6	37	5	13.5
Ae.biuncialis	16	44	28	63.6
Ae.triuncialis	10	40	17	42.5

The samples were classified into two clusters. One included *Ae. cylindrica*, *Ae. squarrosa* and common wheat with D genome. The other cluster included *Ae. biuncialis* and *Ae. triuncialis* with U and C genomes.
Using UPGMA method all accessions were classified into 4 groups (Fig 4). Each group (A,B,C,D) represented all the samples of *Ae. cylindrica*, *Ae. squarrosa*, *Ae. biuncialis* and *Ae. triuncialis*, respectively.

Discussion

Inter-specific variation

A large amount of polymorphism was not revealed in *Aegilops*, all species were distinct in their RAPD band profiles. The average dissimilarity index between species was 0.3.

Species *Ae. cylindrica* and *Ae. squarrosa* were most closely related (genetic distance 0.06) than others as well as to the common wheat (*Triticum aestivum* variety Norin 61) and dissimilarity index was 0.2. This relative position of

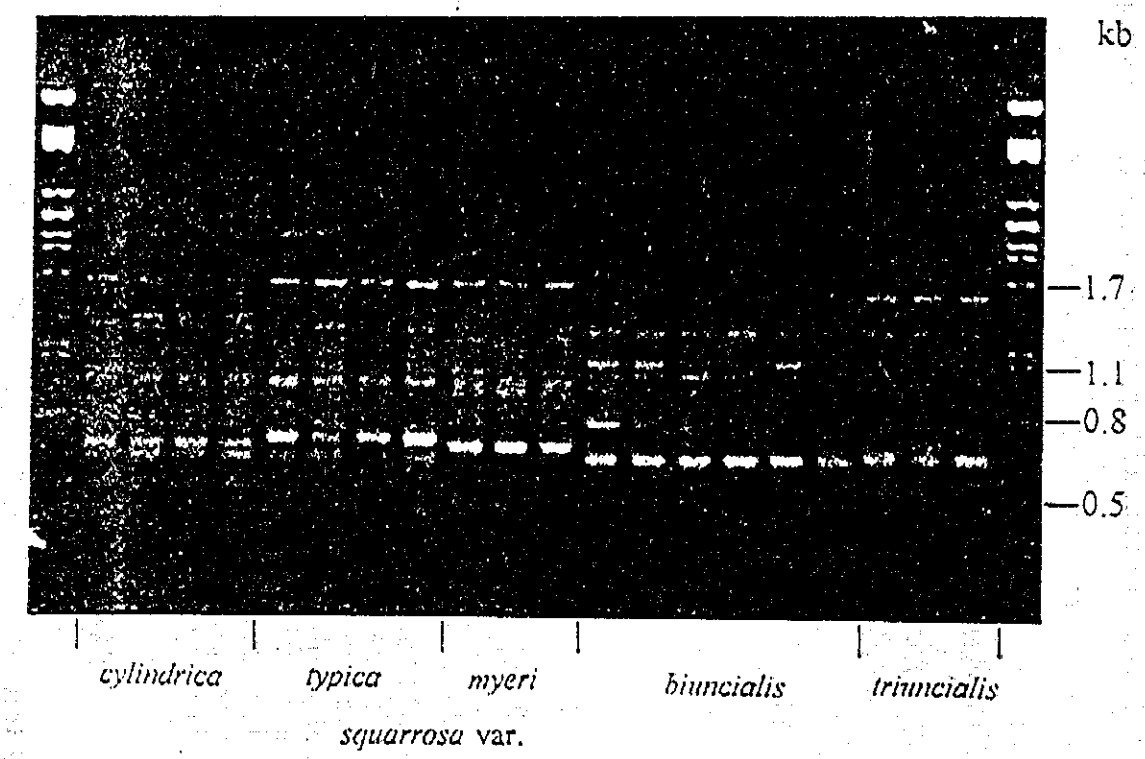

Figure 2. Amplification products of primer P21.

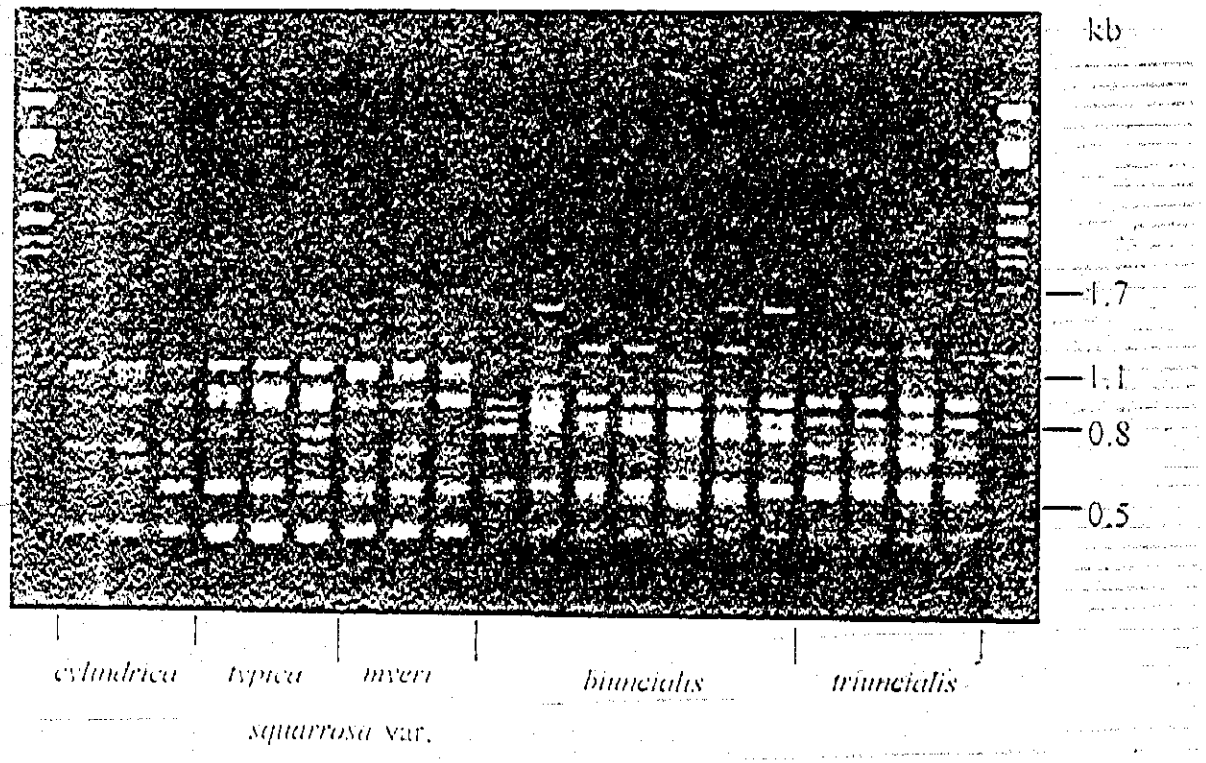

Figure 3. Amplification products of primer P30.

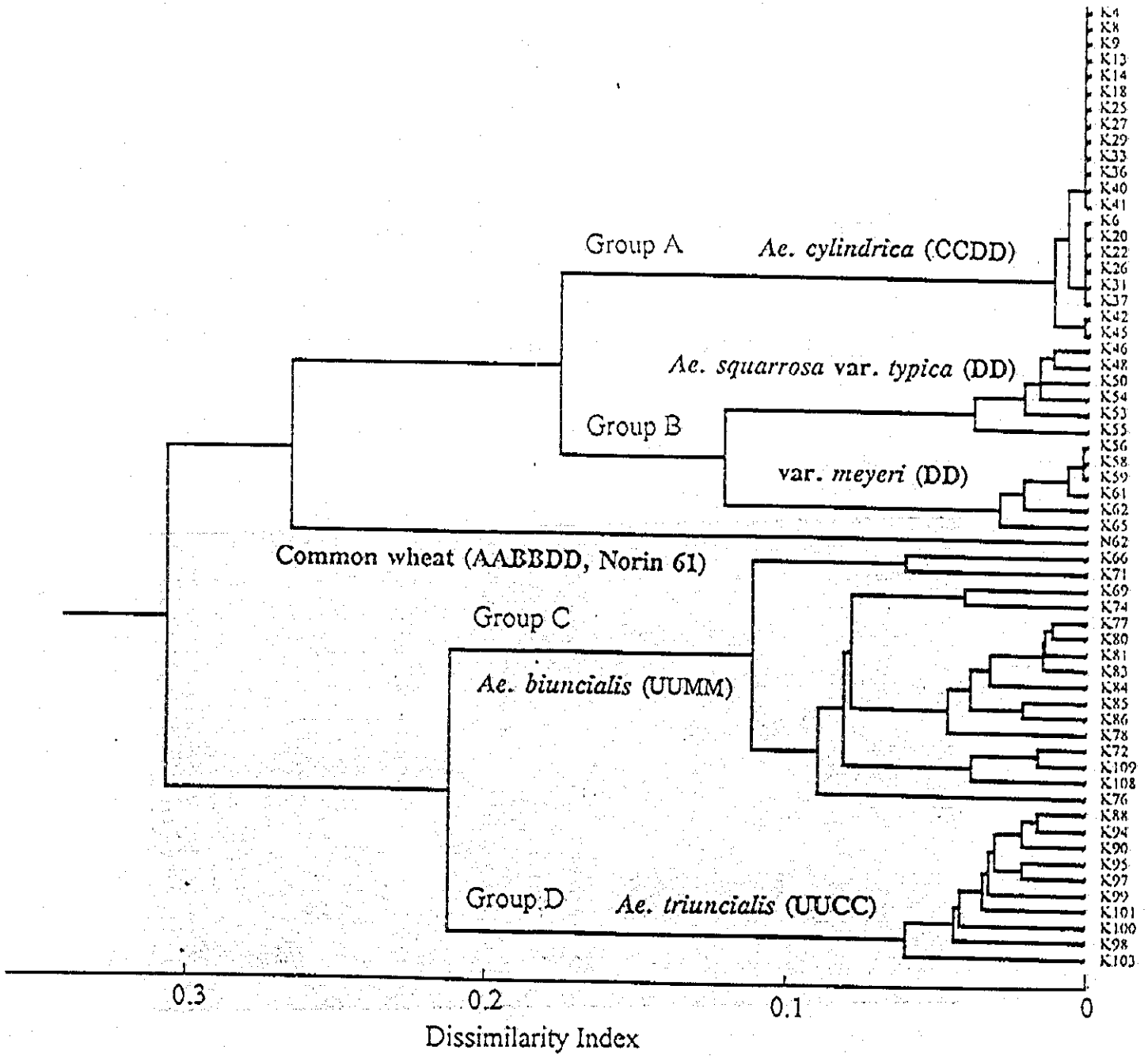

Figure 4. Phenogram of 61 *Aegilops* accessions based on the RAPD data using UPGMA cluster analysis.

T.aestivum and *Ae.squarrosa* (D genome donor to common wheat) cluster is in agreement with results of genome analysis (Kihara 1944, McFadden and Sears 1946, Riley 1965) and HMW electrophoresis analysis on relation between D genome donor and common wheat (Lagudah and Halloran 1987).

The close relation between group C and D due to that shearing a common genome U.

Intra-specific variation

All accessions in each group were closely linked, particularly group A has no variation. Group B subdivided into two different groups which corresponded to 2 varieties of *Ae. squarrosa* var. *typica* and *Ae. squarrosa* var. *meyeri*, respectively.

Group A and B were least polymorphic. Only 2 and 7 polymorphic bands were revealed for group A and B, whereas in other groups C and D have 28 and 17 polymorphic bands, respectively.

Especially, group C (*Ae. biuncialis*) has produced more polymorphic bands than others 64% of total bands were polymorphic. Also, 4 accessions collected along the Caspian sea showed a close relation with one other.

No significant genetic differentiation were found for the geographical distribution.

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Appendix

Species description (Kimber.G and Feldman.M. 1987).

Aegilops squarrosa: (synonym *Triticum.tauschii*). Annual. Diploid, D genome. The donor of the D genome to T.aestivum, the bread wheat of commerce to which it conferred baking quality and cold hardiness. Also the pivotal genome to a group of poliploid species.

Aegilops cylindrica: (synonym *Triticum.cylindricum*). Annual. Tetraploid, genomically CD, cytoplasm similar to that T.tauschii. Frequent natural hybrids with cultivated wheats. Many artificial hybrids.

Aegilops biuncialis: (synonym *Triticum macrochaetum*). Annual.Relatively limited morphological variation involving mainly spike shape, color, hairiness and awn development. Tetraploid, genomically UM.

Aegilops triuncialis: (synonym *Triticum triuncialis*). Annual.Tetraploid, genomically UC.Many artificial hybri

5. VIETNAM

NGUYEN Dung Tien

Variation of Grain Traits in Vietnamese Landraces of Rice

(Report of JICA Group Training Course on Plant Genetic Resources)

Nguyen Dung Tien

University of Forestry and Agriculture of Hue
Socialist Republic of Vietnam

1 November 1995

Variation of Grain Traits in Vietnamese Landraces in Rice

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Abstract

A total of 437 landraces of rice from Vietnam were analyzed for total seed protein by SDS-PAGE, and phenol reaction. Three different types of glutelin α subunits were detected. The level of *Wx* protein with 60kDa molecular weight was divided into 3 groups, corresponding to non-glutinous, intermediate and glutinous starch types. Based on the variation in seed storage protein and *Wx* protein, landraces were classified into 7 groups. Frequency distribution of types A and B of glutelin α subunits changed with the latitude at which rice landraces were collected. Geographical cline for phenol reaction was detected.

Key Words: rice, Vietnamese landraces, seed protein, *Wx* protein, phenol reaction

Introduction

Vietnam shares common borders with China, Laos and Cambodia, and is included in the Indo-Malayan center of diversity for many crops. Northern Vietnam which is adjacent to Yunnan province in China is the east edge of a center of diversity for cultivated rice and many ethnic groups are there.

Since 1994, exploration missions in Vietnam have been conducted in collaboration between the National Institute of Agrobiological Resources (NIAR), Japan, the International Plant Genetic Resources Institute (IPGRI) and the Department of Agricultural Science and Technologies (DAST), Vietnam. Missions were undertaken to

collect landraces of rice in northern, central and southern Vietnam in 1994. Esterase isozyme analysis revealed that 11 esterase genotypes out of 12 possible genotypes could be detected in the landraces from northwestern Vietnam which still has diverse rice germplasm (Ha *et al.* unpublished data).

This report deals with variation in seed protein, *Wx* protein and phenol reaction of Vietnamese rice landraces.

Materials and Methods

A total of 437 landraces collected by IPGRI/NIAR/DAST collaborative exploration missions were used in this study. These samples were collected from 18 provinces in 5 regions of Vietnam (Fig.1).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of crude protein, including *Wx* protein extracted from seed samples was carried out according to the method of Laemmli (1970).

Three seeds of each landrace were soaked in 1.5% phenol solution at 30 C for 2 days and were then evaluated for positive (intensity +++, ++ and +) and negative (-) reactions.

Results and Discussion

Electrophoretic variation in seed storage protein and Wx protein

Variation in electrophoregrams of seed protein was observed in glutelin α subunits and *Wx* protein with 60kDa molecular weight (Fig.2). Based on the variation in glutelin α subunits (α-1, α-2, α-3), landraces were classified into 3 different types. Type A has all the subunits and α-3 subunit with slightly higher molecular weight than type B. Both α-1 and α-2 subunits were found in types A and B. Type C has no α-1 subunit and the same pattern of α-2 and α-3 subunits as type B. Kagawa *et al.* (1988) reported 2 types of glutelin subunits in rice and one of these showed deletion of α-3 subunit in *indica* varieties. An α-1 subunit deficient type C was found for the first time in this study.

All landraces could be classified into 3 types based on the level of *Wx* protein; non-glutinous, glutinous and intermediate types (Fig.1). More than 60 % of samples from northwestern Vietnam were glutinous, whereas about 70% of samples from central and

southern Vietnam were non-glutinous or intermediate types.

Considering the results of both glutelin α subunits patterns and *Wx* protein, landraces can be classified into 7 groups; type A1 (α-1, α-2, α-3-higher, non-glutinous), type A2 (α-1, α-2, α-3-higher, intermediate), type A3 (α-1, α-2, α-3-higher, glutinous), type B1 (α-1, α-2, α-3-lower, non-glutinous), type B2 (α-1, α-2, α-3-lower, intermediate), type B3 (α-1, α-2, α-3-lower, glutinous) and type C1 (α-2, α-3-lower, non-glutinous).

Geographical distribution of the above 7 types is shown (Table 1 and 2, Fig.3). Landraces having type A is widespread thoughout central and southern Vietnam, while landraces with type B predominated in the northwest. Frequency distribution of types A and B changed with the latitude at which rice landraces were collected. The difference between the two different types is the molecular weight of glutelin α subunit. This latitudal difference in molecular weight of α subunit may be due to mutation of the structural gene which encodes glutelin α subunit during the domestication of rice landraces.

Wx protein is a gene product of *wx* locus on chromosome 6 of rice and is resposible for amylose production in endosperms and pollen grains. Intermediate type having lower content of amylose have been found in rice, foxtail millet and grain amaranth. A regulatory mechanism of intermediate type may be due to a trans-acting like element, *du* gene (Okuno *et al.* 1983, Okuno 1985) or *Wx* alleles at *wx* locus (Sano 1984). The genetic mechanism for intermediate level of *Wx* protein and low amylose content should be studied.

Variation in phenol reaction

Landraces were divided two major groups, positive and negative, and also subdivided 3 groups based on the reaction intensity. About 60% of landraces from the northwest showed negative reaction to phenol, whereas about 50% of landraces from central and about 90% of landraces from the south reacted positively. The difference in phenol reaction was very clear between northern and southern landraces. Geographical cline for phenol reaction was found (Fig.4).

In addition to negative phenol reaction, landraces with esterase genotype found in *japonica* rice predominated among the samples collected in northwestern Vietnam (Ha *et al.* unpublished data).

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Table 1 Geographical variation in electrophoregrams of total seed protein of Vietnamese rice landraces

Province	Electrophoregram pattern							
	A1	A2	A3	B1	B2	B3	C1	Total
Thua Thien Hue	1	1	0	2	0	0	0	4
Da Nang	5	2	0	2	0	0	1	10
Quang Ngai	4	0	0	1	1	0	0	6
Binh Dinh	3	2	2	0	0	0	0	7
Tuy Hoa	2	6	1	0	2	0	0	11
Lam Dong	0	0	0	5	2	0	0	7
An Giang	15	0	0	12	1	1	0	29
Ha Tien	1	3	0	3	1	0	0	8
Rach Gia	14	3	1	2	0	0	0	20
Dong Thap	7	2	0	0	0	0	0	9
Tay Ninh	6	5	0	12	0	0	0	23
Song Be	24	4	7	3	0	2	0	40
Dac Lac	20	19	2	8	5	1	0	65
Gia Lai	2	3	1	0	0	0	0	6
Kon Tum	1	2	1	0	0	0	0	4
Son La	6	7	2	0	14	3	0	32
Lai Chau	11	22	10	16	46	24	0	129
Lao Cai	5	3	0	1	3	10	0	22
Total	127	84	27	67	75	41	1	422

Table 2 Regional variation in electrophoregrams of total seed protein of Vietnamese rice landraces

Region	Electrophoregrams							
	A1	A3	A2	B1	B3	B2	C1	Total
Northwest	22	12	32	17	37	63	0	183
Central	15	3	11	10	0	5	1	45
Highland	23	4	24	8	1	5	0	65
South	37	1	8	17	1	2	0	66
Southeast	30	7	9	15	2	0	0	63
Total	127	27	84	67	41	75	1	422

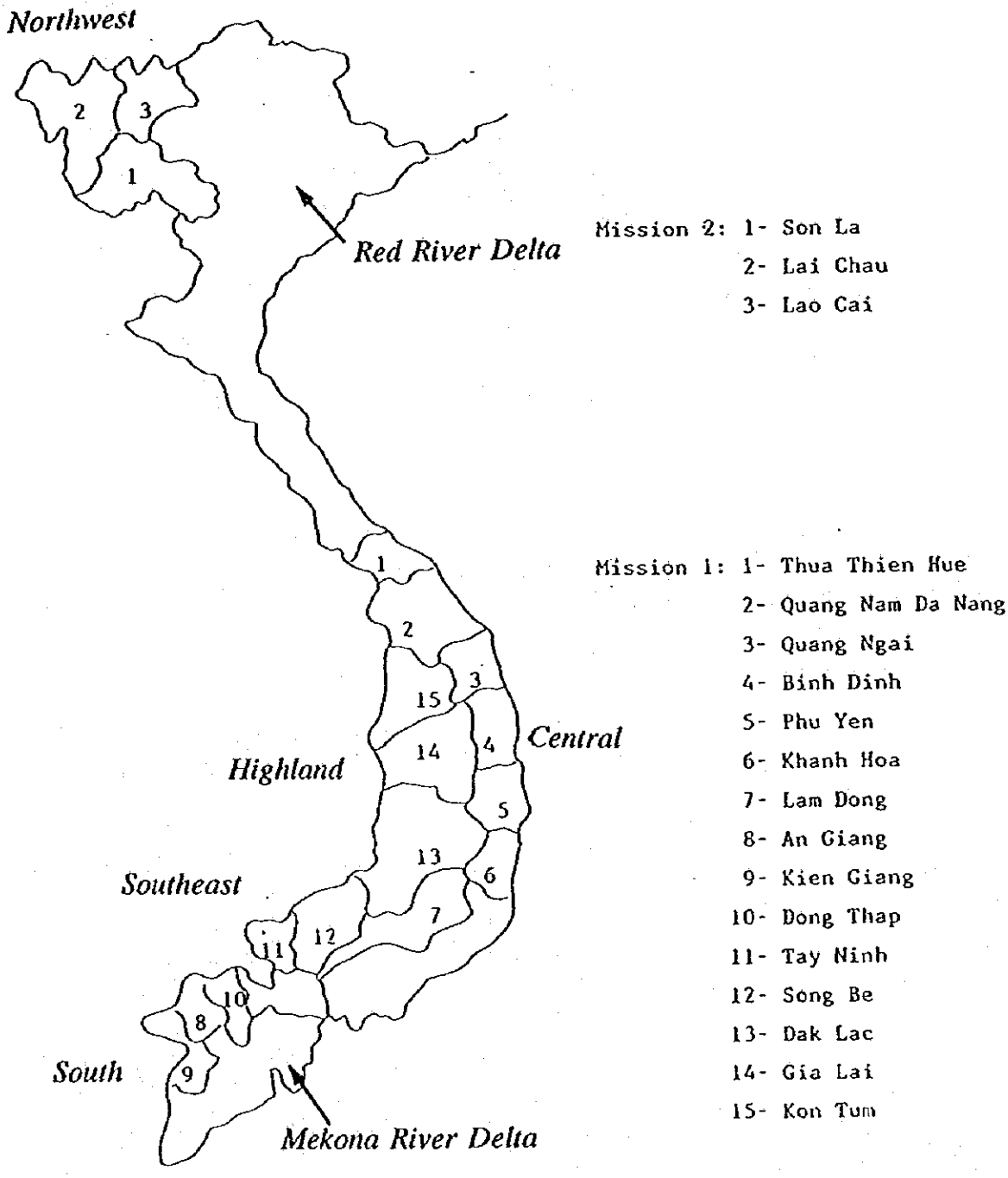

Fig.1 Regions and provinces in Vietnam which rice landraces were collected.

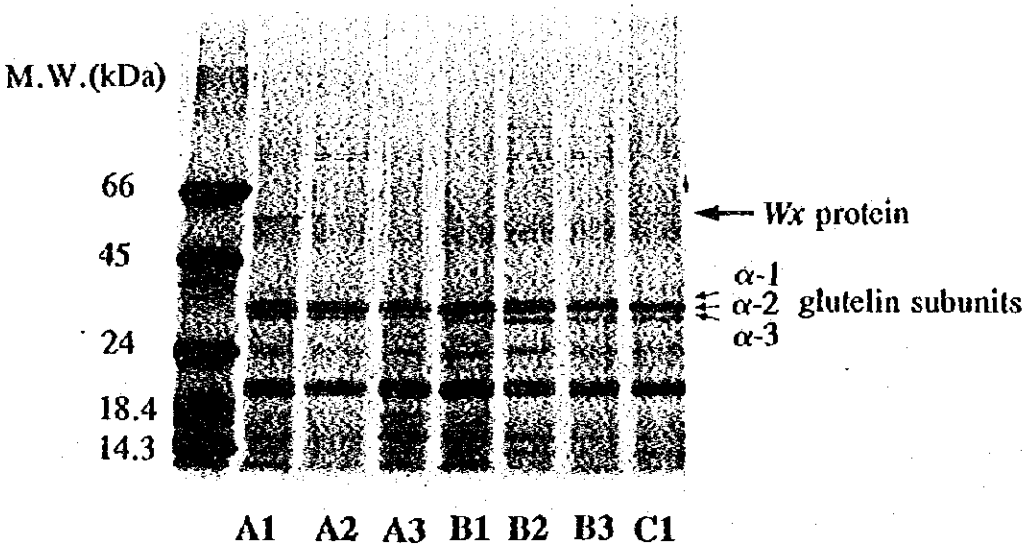

Fig.2 Variation in electrophoregrams of seed protein from Vietnamese rice landraces.

A1: α-1, α-2, α-3 (higher), non-glutinous
A2: α-1, α-2, α-3 (higher), intermediate
A3: α-1, α-2, α-3 (higher), glutinous
B1: α-1, α-2, α-3 (lower), non-glutinous
B2: α-1, α-2, α-3 (lower), intermediate
B3: α-1, α-2, α-3 (lower), glutinous
C1: α-2, α-3 (lower), non-glutinous

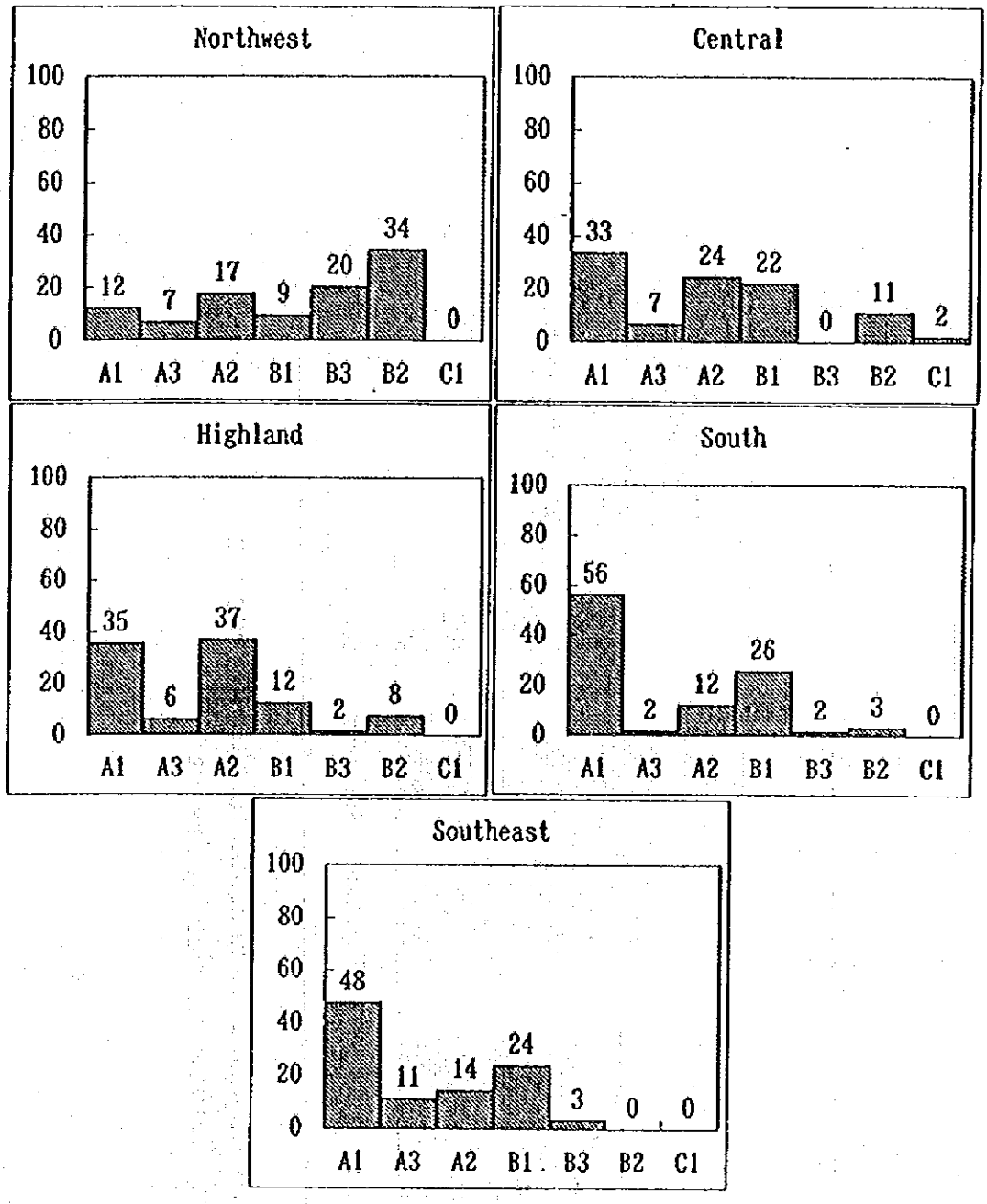

Fig.3 Geographical variation in glutelin α subunits and *Wx* protein of Vietnamese rice landraces.

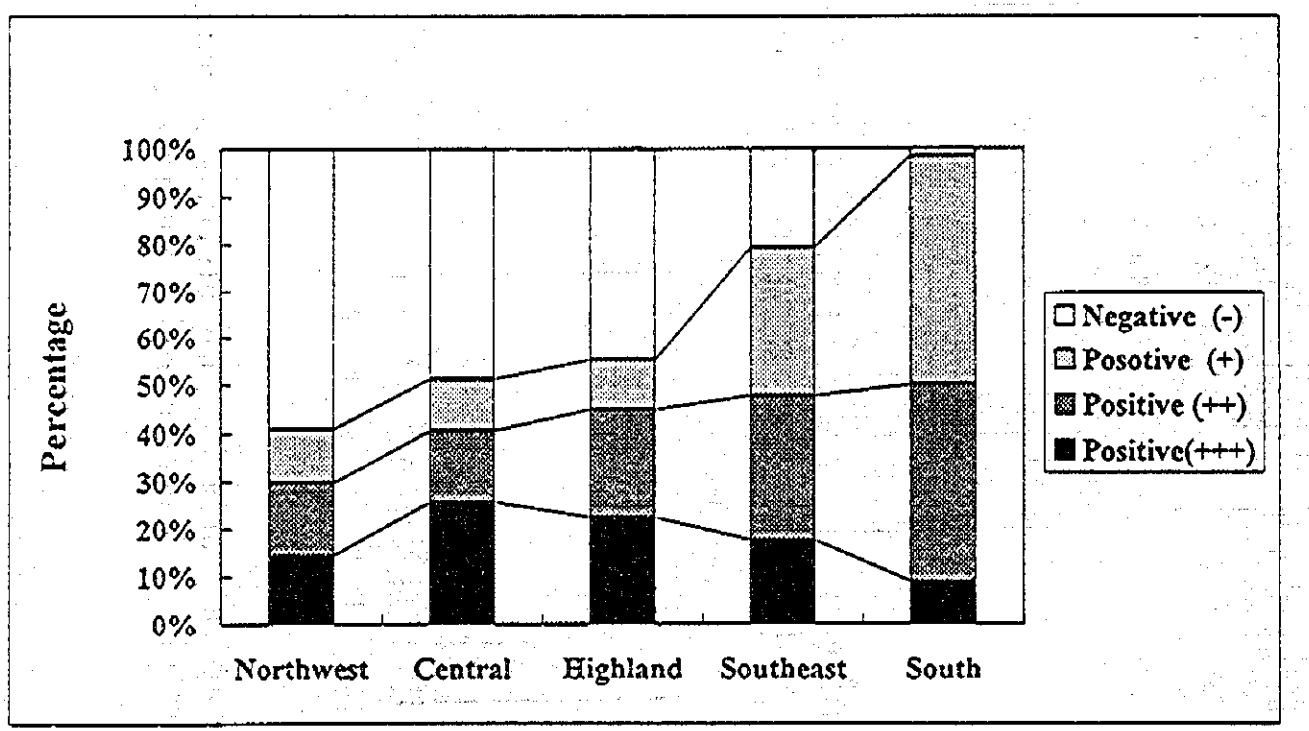

Figure 4. Geographical variation in phenol reaction of Vietnamese rice landraces.