

**ANDROGENIC DOUBLED HAPLOIDS IN INDICA RICE :
IN VITRO INDUCTION, PLANT REGENERATION
AND EVALUATION**

**A Thesis Submitted for the Degree of
DOCTOR OF PHILOSOPHY**

By

Rabindra Nath Bhuyan



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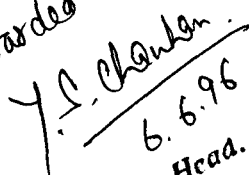
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INTRODUCTION

Rice, the major source of calories for 40% of the global population, is ranked as the world's most important food crop. Consequently, it is one of the most intensively investigated crop plant. The accumulated knowledge-base has been of considerable value in enhancing rice productivity and, in turn, in the betterment of millions of people who depend on it as a source of food and income.

As in the case of other crop plants, rice breeders have mainly pursued the exploitation of natural variation for tailoring cultivars suitable for the demands of agriculture. The process of hybridization and selection have been the main vehicles of this delivery system. The achievements in this regard have been impressive. Taichung Native1 (TN 1) and IR series starting from IR8 to IR74 lines/varieties released by the International Rice Institute, Manila, the Philippines and Institution of National Agriculture Research System are known for their impact on enhancing productivity per unit area and time.

The increase in rice production has helped alleviate widespread hunger and famine by keeping pace with ever growing human population. With the rice-consuming population increasing at an alarming rate of hundred million per year, by 2020 A.D. the rice requirement of Asia would exceed 760 million tonnes. The situation in India, the largest rice growing country, is also not stable. Although we are self sufficient at present, our rice requirement is estimated to increase at a rate of 25 to 30 million tones every decade. This means that we should achieve the target of producing 100 million tonnes per year (presently 75 million tonnes) by the year 2000 A.D. This requirement would increase

to 160 million tonnes by 2020 A.D. Accordingly, the production of rice has to be doubled in next 25 years. It has to be recognised here that plant breeding is a slow and painstaking process that has severe biological limitations, and that such ^{demand} ~~estimated~~ increase in yield and productivity cannot be sustained indefinitely. It is in this context that rapidly emerging techniques of plant cell and molecular biology have attracted much attention for they provide a powerful and novel means to supplement and complement the traditional methods of plant improvement.

Since 1930's, plant tissue culture has progressed mostly on dicotyledonous plants, and studies on monocotyledonous plants lagged behind. The *in vitro* culture of rice started with the organ culture, when Fujiwara and Ojima (1955) cultured excised roots. The culture conditions for various stages of immature embryos were examined by Amemiya et al. (1956) and obtained seedlings from even five day old cultured embryos. F1 hybrids from otherwise unsuccessful rice crosses were subsequently obtained by Bourhamont (1961), Iyer and Govila (1964). Culture of nodes and young seedlings on Heller's medium containing vitamins and auxins was reported by Furuhashi and Yatazawa in 1964. This was followed by plant regeneration from seed derived callus (Tamura, 1968; Nishi et. al., 1968 and Maeda ^{et al.}, 1968). Plants

were also regenerated from root callus (Kawata and Ishihara, 1968) as well as pollen derived callus (Niizeki and Oono, 1968; Cho and Zapata, 1988).

The demonstration of Guha and Maheshwari (1964, 1966, 1967) that immature anthers of *Datura innoxia* when cultured in an appropriate nutrient medium can give rise to plantlets with a haploid number of chromosomes, opened up a new approach to the induction of haploidy in plants generally known as androgenesis. Soon it was found that this technique can be effectively employed in *Oryza* (Niizeki and Oono, 1968; Guha et al., 1970), *Nicotiana* (Bourgin and Nitsch, 1967; Nitsch et al., 1969; Mikata and Tanaka, 1968; Nitsch and Nitsch, 1969; Sunderland and Wricks, 1969; Melchers and Labib, 1970), *Brassica* (Kameya and Hinata, 1970) and *Hordeum* and *Lolium* (Clapham, 1971). In *Oryza sativa*, varietal response to anther callusing were reported by Guha et al. (1970). The underlying principle of androgenesis is to direct the development of the microspores whose fate is normally to become gametes and to force its development directly into a plant or callus. This abnormal pathway is possible if the pollen cell is provided with certain specific conditions.

Utilization of anther culture technique in the breeding programmes has been in practice, mostly in China, for more than

20 years. Since the release for cultivation of Hua-Yu No. 1 and Hua-Yu No. 2, the rice varieties developed through anther culture breeding in 1976, Chinese researchers ~~have reported to~~ have developed over 100 varieties and lines with objectives like earliness, yield, resistance to disease and grain quality (Shen et al., 1983). Researchers in China, have also successfully used anther culture for several other purposes like, solving sterility problems in indica-japonica hybrids. Outside China, utilization of anther culture in rice breeding programme has been comparatively of a lesser magnitude. In Korea, the technique has been mainly used for developing blast resistance and cold tolerant varieties (Gun, 1982). In Taiwan, application of anther culture was mainly to produce inter-subspecific and inter-specific F1 hybrids (Woo and Chen, 1982). International Rice Research Institute, Manila's programme of anther culture has been focussed on production of varieties for cold tolerance using F1 hybrids of *japonica-indica* and *indica-japonica* crosses. Researchers in Nepal (Agrawal et al., 1994) have also regenerated plants from anthers of cold tolerant rice. The biggest programme outside China is at CIAT (Pulver and Jennings, 1986) as for example, 20,000 diploid R2 lines are reported to have been raised from approximately 100 triple crosses for obtaining R1 lines for blast resistance, Fe-tolerance and white belly determination.

Factors affecting androgenesis in rice have been reviewed by Chen et al. (1991). Cold or heat shock for short period is known to enhance the yield of pollen embryos and green plants remarkably. This beneficial effect of cold pretreatment was first reported by Nitsch and Norreel (1973) in *Datura innoxia*. It has also been shown to increase the ratio of responding anthers in many species, such as *Nicotiana tabacum* (Nitsch, 1974), *Datura innoxia*, *Hyoscyamus niger*, *Hordeum vulgare* (Sunderland and Roberts, 1977) and *Oryza sativa* (Chaleff et al., 1975). In the ~~procedure~~ of rice anther culture, this treatment has been an essential step and broadly adopted by many researchers. Callus induction frequency of Keng (*Oryza sativa* Subsp. *japonica*) or Hsien (*Oryza sativa* Subsp. *indica*) rice can be raised from two to more than ten times (Ying, 1986) via an appropriate cold treatment.

The time needed for pretreatment depends on the temperature used. In general, lower the temperature (as low as 3°- 5°C) shorter the time needed, and higher the temperature (as high as 10°- 15°C) longer the period of treatment required. Through the experiment by Wang and his associates (1974) in indica rice, 10 day pretreatment at 3° - 5°C, 10 to 15 day at 6° - 8°C, or 15 to 20 day at 9° - 10°C was optimum for

the enhancement of callus induction frequency. When the period of cold pretreatment is too long, the frequency of green plantlet differentiated from callus decreased dramatically while ^{that} of albinos increased greatly. The appropriate cold pretreatment benefits the differentiation of green plantlets and raises the differentiation frequency and formation of green plantlets (Zhou and Chang, 1981; Zahu, 1983; Gu and Chen, 1983a). SP ?

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Physiological investigation has revealed that cold pretreatment could reduce the respiration intensity of anther and the consumption of materials, and could prolong the lifespan of anther. In addition, it was found that the weight of anther also increased during cold pretreatment. All these effects would be advantageous to the initiation and development of pollen grains inoculated (Zhu, 1983). Gu and Chen, (1983 b) demonstrated that the cold pretreatment supported the survival of most of the grains and completion of the induction process for androgenesis at the same time, and therefore led to a much higher induction frequency of callus than that of the control.

Other treatments such as heat shock (Keller and Armstrong 1978, 1979), placing the detached tillers in water at room temperature (Wilson et al. 1978) and putting the excised anthers in a water saturated atmosphere (Dunwell, 1981) have

also been found beneficial to production of embryos and plants.

Formation of embryo or callus depends greatly on the appropriate nutrients in the culture medium (Nitsch, 1969; Wang et al., 1974). Clapham (1973) discovered that high concentration of ammonium ions in LS medium (Linsmaier and Skoog, 1965) was disadvantageous for callus formation in barley microspores. Subsequently, Chu et al. (1975) developed N_6 medium which is characterised by having a low concentration of $(NH_4)_2SO_4$ and high KNO_3 . This medium proved to be very efficient for rice anther culture (Chu, 1978; Genovesi and Magill, 1979; Chen et al. 1982; Tsay et al., 1982). Specially in ^{the} Graminae, a high sucrose concentration has been found to be beneficial for plant production through anther culture (Clapham, 1973; Ouyang et al., 1973; Ono and Larter, 1976; Miao et al., 1978). Chen (1978) reported that in rice 6% sucrose in callusing medium and 3% sucrose in regenerating medium resulted in highest frequency of callus and green plant regeneration ~~capability~~ in rice. But some investigators have reported that 6% sucrose is too high and 5% was recommended (Huang et al., 1978, ^L Chu, 1978; Chaleff and Stolarz, 1981). It has been observed that high concentration of sucrose is responsible for maintaining osmotic pressure of the nutrient medium (Wang et al., 1974; Chaleff and Stolarz, 1981). Plant growth regulators like auxins and cytokinins play a crucial

role in the process of androgenesis. In earlier days, 2,4-dichloro phenoxyacetic acid (2,4-D) was thought to be the only hormone essential for androgenesis and it was always added to the culture medium with or without cytokinin (usually kinetin) and/or other auxins (Niizeki and Onono, 1968; Harn, 1969; Nishi and Mitsuoka, 1969; Guha- Mukharjee, 1973; Wang et al., 1974). The inhibitory effect of 2,4-D, and that it can be replaced by α - naphthylacetic acid (NAA), was first reported by Niizeki and Onono, (1971). After wards it was also found out that for obtaining callus of high morphogenic potential cytokinin is also needed (Chen, 1977; Chaleff and Stolarz, 1981; Lee and Chen, 1982). However, this conclusion of hormonal requirement may not be applicable to all cultivars as genotypic differences in hormone requirement has been reported (Liang, 1978; Cornejo - Martin and Primo - Millo, 1981).

Light and temperature play an important role in androgenesis (Maheshwari et al., 1980). The light conditions varied from complete darkness (Niizeki and Onono, 1968; Harn, 1969; Woo and Tung, 1972; Guha - Mukharjee, 1973) to continuous illumination (Nishi and Mitsuoka, 1969; Niizeki and Onono, 1971). Darkness is favourable for initiation of callus (Cornejo - Martin and Primo - Millo, 1981). For plant regeneration high intensity of light is essential.

Other factors that influence anther response are composition of the gas mixture in the culture vessel (Dunwell, 1979; Johansson et al., 1982) and the density of anther inoculated in the culture medium (Bajaj et al., 1977; Sunderland et al., 1981).

The genotype of the donor plants has a strong effect on pollen plant formation. There are several reports on genotypic variation in anther response of rice (Guha et al., 1970; Iyer and Raina, 1972; Wang et al., 1974; Raina and Iyer, 1974; Tsai and Lin, 1977; Chen and Lin, 1976; Chu, 1982; Rush and Shao 1982; Ding et al 1983; Zapata, 1985; Hu, 1985; Miah et al., 1985; Karim et al., 1985; Boyajiev and Kuong, 1986; Guiderdoni et al., 1986; Mikami and Kinoshita, 1988). In the initial study of Iyer and Raina (1972), out of 15 cultivars only 5 showed callusing and of these only one (IARI - 5788) regenerated roots and shoots. Genotypic variation is now seen mostly in the extent of response, rather than in the existence of some responsive genotypes and some unresponsive genotypes (Raina, 1989). In general, japonica rice varieties are known to respond better than indicas (Chen and Lin, 1976; Hu, 1978; Rush and Shao, 1982; Chu 1982; Wood and Chen, 1982; Gun, 1982; Zapata 1985). Hu (1984, 1985) reported

that the induction frequency of green plants was more than 10% in japonica cultivars, whereas it was only 1% in indica rice.

The reason that this technique of androgenesis has attracted such wide attention is the possibility of obtaining haploid cells, tissues and complete organisms, which has a single dose of gametic information. Instant homozygous lines could then be obtained by doubling the chromosome complement in these haploids without having to resort to a lengthy and time consuming programme of inbreeding, which is indispensable for obtaining homozygosity by conventional methods. In addition, recessive mutants can be readily discovered because of presence of only one set of chromosomes, and hence only one copy of each gene.

The other attraction of anther culture is the possibility of early fixation of recombinants. Pollen plant population derived from anther culture of F1 hybrids will express segregation patterns otherwise expressed in the F2 or F3 generation. Diploidization of haploids will render them homozygous, resulting in the rapid establishment of pure lines that otherwise would require several generations of selfing. The saving of time and resources would be far greater in long duration compared to short duration cultivars, in which homozygosity can be achieved rather speedily through conventional

methods such as single seed descent, by growing two or three crops per year. In rice, therefore, anther culture breeding would be more advantageous in situations involving temperate environment, photosensitivity or environmental stresses because in these cases only one generation can be raised in a year.

Increased selection efficiency is the other advantage of anther culture breeding, especially when dominance variation is significant. In conventional breeding, early generation lines (F2 - F4) show phenotypic differences to which both additive and dominance effects contribute. In contrast, double haploid lines derived from F1 anther culture will show only additive variance and, therefore, high heritability due to elimination of dominance effects. Thus, compared to the F2 population, fewer double haploid plants will be required for the purposes of selection of the desired recombinant(s). To elaborate this point, Baenziger and Schaeffer, (1983) cited the hypothetical example of a cross segregating for three recessive genes and for three dominant genes. In the case of three recessive genes, 1/8 of the double haploids derived from such a cross would be selected as compared to 1/64 of the F2. In the case of three dominant genes, whereas 1/8 of the double haploids, all of which would breed true, 27/64 of F2 plants would be selected, on the basis of phenotype, but only 1/64 would be true breeding. Chinese scientists have

estimated that about 150 pollen plants derived from F1 anthers would be enough instead of 4 - 5,000 F2 plants, for the purpose of selection of desirable genotypes (Shen et. al., 1983). Double haploid breeding would be particularly useful in situation in which extensive recombination is not important (Chaleff, 1979; Chu, 1982). In rice, efforts to combine the desirable traits of indica and japonica cultivars have often been frustrated by the high degree of sterility of the hybrids and vigorous segregation toward the parental types in F2 and subsequent generations. Double haploids produced through anther culture of the F1 hybrid, or of a promising segregant, would ensure stabilization and thereby produce and arrest segregation in successive generations. Use of anther culture for solving the problems arising from inter-subspecific and inter-specific hybridization can be regarded as the most important area of its application in rice improvement.

Another area of use of double haploid is the selection of the races of pathogen of insect biotypes (Rush and Shao, 1982). Use of 'pure line' varieties or breeding lines may cause considerable experimental error due to residual heterozygosity of the genotypes investigated. Anther culture could be used to obtain homozygous lines for utilization in such situations, as well as in screening for resistance to herbicides, physiological

studies, and in purification of A, B and R lines for hybrid rice programmes.

The present investigation is aimed at standardization of conditions for anther culture-derived callus induction and plant regeneration from cold tolerant rice varieties grown in high altitude areas of North Eastern Hill of India. All these varieties are redkerneled, possess cold tolerance and they are poor yielders. Having standardized the conditions for androgenesis, we produced several double haploid lines through anther culture of F1 derived hybrids between IR 70, an elite IRRI breeding line and Khonorullo, a local cold tolerant line. The doubled haploid lines were evaluated for agronomic characters under medium altitude (950 m. msl) vallyland^e condition and promising ones with high yield and white kernel colour were identified for further evaluation.

MATERIALS AND METHODS

2.1 MATERIALS

The following locally adapted varieties of rice (*Oryza sativa* L). were used initially to assess the response to callus induction during anther culture:

1. Khonorullo
2. RCPL 1 - 1C (Megha Rice 2)
3. RCPL 1 - 2C
4. RCPL 1 - 3C
5. DR 92 and
6. Pusa 33



They were grown in the experimental fields of Plant Breeding Division, Indian Council of Agricultural Research for North Eastern Hills Region, Barapani, Meghalaya, with normal fertilizer and crop management.

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In addition, IR 65, IR 68, IR 70, Khonorullo, Yamuk, Pusa 33 and DR 92 (Subhadra) were grown in pots, with normal fertilizers for crossing. One cross i.e., IR70 X Khonorullo was made and attempted for double haploid production.

2.2 METHODS

2.2.1 Time of collection of panicles:

Panicles were collected while still inside the sheath, mostly in the mornings (between 8.30 AM - 10.30 AM) between middle of August to the end of September i.e, in the flowering period. They were wrapped in polythene bags with a little water and stored at 8 - 10°C for 1 to 16 days.

2.2.2 Preparation of staining solutions and staining procedure

2.2.2.1 Acetocarmine stain:

2 gm of carmine was dissolved in 100 ml of 45% acetic acid and was kept overnight. Next day the stain was filtered and used for staging of microspores. A few anthers were squashed in a drop of stain and observed under a research microscope.

2.2.2.2 Feulgen stain:

Feulgen reagent was prepared by adding 1 gm basic fuschin, 2 gm potassium metabisulphite, 5 ml 1N HCl, 85 ml water and 1 gm activated charcoal (Nitsch 1981). The solution was allowed to stand overnight in fudge.

The anthers were dissected out from the spikelets and fixed in acetic alcohol (1 : 3) for a period ranging between overnight to 24 hour. They were brought to water level through alcohol grades and hydrolyzed with 1 N HCl at 60°C for 5 to 7 minutes. After the hydrolysis the vials were immediately put into ice cold water to stop further action of HCl. After repeated washing with water the anthers were squashed in a drop of the staining solution and observed under the microscope after 5 minutes.

2.2.2.3 Iron alum Haematoxylin stain:

A stock solution of 4% haematoxylin in 45% acetic acid was prepared and 1 gm of iron alum was added to it. The solution was allowed to stand for 24 hour. To 10 ml of this stock solution, 4 gm of chloral hydrate was added and after complete dissolution, it was used (Chang et al., 1978)

Anthers were squashed in a drop of the staining solution under a cover glass. After 2 to 5 minutes, the slides

were observed under a research microscope.

2.2.3 ANTHOR CULTURE

2.2.3.1 Methodology

Panicles of the varieties mentioned above were surface sterilized. Since individual panicles contain microspores at different stages of development at different places, the sheath was opened at five places and one floret was taken out from each opening. Anthers of these florets were examined under the microscope for ascertaining the nuclear stage. Portions containing microspores at mid-uninucleate stage were taken out and anthers were removed from it. The anthers were then passed through 70% ethanol for a few seconds, and then sterilized in 0.1% mercuric chloride for 7 to 8 minutes and rinsed 3 times with sterile distilled water. They were then inoculated in the following semisolid/liquid medium.

2.2.3.2 List of media and their composition used for anther callusing

Medium No. 1	G ₅	(Semisolid)
Medium No. 2	E ₂₄	(Semisolid)
Medium No. 3	Minimal	(Semisolid)
Medium No. 4	LB	(Semisolid)

Medium No. 5 MP2 (Semisolid)
 Medium No. 6 E₁₀G (Liquid)
 Medium No. 7 Modified N6 (Liquid)
 Medium No. 8 E10 YK (Liquid)

Medium No. 1 G₅ (Semisolid):

Blaydes medium: NaH₂PO₄ (150 mg/l), (NH₄)₂SO₄ (134 mg/l), KNO₃ (2500 mg/l), MgSO₄ · 7H₂O (250 mg/l), CaCl₂ · 2H₂O (150 mg/l), MnSO₄ · 4H₂O (10 mg/l), ZnSO₄ · 7H₂O (2 mg/l), H₃BO₃ (3 mg/l), KI (0.75 mg/l), Na₂MoO₄ (0.25 mg/l), CuSO₄ · 5H₂O (0.025 mg/l), CoCl₂ (0.025 mg/l), [Na₂EDTA (745 mg), FeSO₄ · 7H₂O (557 mg) - dissolved in 100 ml of water and 5 ml of this solution is used for 1 litre], Sucrose (50 gm/l), Agar (8 gm/l), fortified with 2,4-D (2 mg/l), Nicotinic acid (0.5 mg/l), Pyridoxin HCl (0.5 mg/l), Thiamine HCl (1.0 mg/l), Glycine (2.0 mg/l) and Inositol (100 mg/l).

Medium No. 2 E24 (semisolid):

G₅ basic salts, fortified with 2,4-D (1 mg/l), BAP (0.5 mg/l), IAA (0.5 mg/l) and Inositol (160 mg/l).

Medium No. 3 Minimal (semisolid):

Medium No. 12 of Song et. al (1978): NH₄NO₃ (360

mg/l), KNO_3 (100 mg/l), KH_2PO_4 (300 mg/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (200 mg/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (110 mg/l), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (10 mg/l), [Na_2EDTA (745 mg), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (557 mg) dissolved in 100 ml of water and 5 ml of this solution was used for 1 litre], Sucrose (30 gm/l) Agar (0.8%) fortified with 2,4-D (2 mg/l), Nicotinic acid (0.5 mg/l), Pyridoxin HCl (0.1 mg/l), Thiamine HCl (0.1 mg/l) and Glycine (2 mg/l).

Medium No.4 LB (Semisolid):

KNO_3 (3000 mg/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (185 mg/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (150 mg/l), KH_2PO_4 (540 mg/l), [$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (557 mg), Na_2EDTA (745 mg/l) dissolved in 100 ml of water and 5 ml of this solution was used for 1 litre], $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (22.30 mg/l), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mg/l), KI (1.0mg/l), H_3BO_3 (6.0 mg/l), Nicotinic acid (3.0 mg/l), Thiamine HCl (2.5 mg/l), Pyridoxin HCl (5.0 mg/l), Inositol (100 mg/l), Glycine (2.0 mg/l) and Lactalbumin hydrolysate or Casein hydrolysate (300 mg/l), Kinetin (1.0 mg/l), Sucrose (50 g/l) and Agar (6 g/l).

Medium No. 5 E_{10G} (Liquid):

Potato extract 10% aqueous (for 1 litre of medium, 100 gm of fresh potato tubers were weighed out and cleaned with water, the sprouts on the tubers if any were removed. The tubers

were cut into small pieces, boiled in a given volume of distilled water for 20-30 minutes and filtered through two layers of cheese cloth. The residual potato pieces were extracted once again in the same manner. Finally, the two filtered liquids were mixed for immediate use), KNO_3 (100 mg/l), $(\text{NH}_4)_2\text{SO}_4$ (100 mg/l), $\text{Ca}(\text{NO}_3)_2$ (100 mg/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (125 mg/l), KH_2PO_4 (200 mg/l), KCl (35 mg/l), Sucrose (100 gm/l) and Agar (8 gm/l). *no growth regulator*

Medium No. 6 E₁₀G (Liquid):

G₅ medium without agar and fortified with 2, 4-D (1 mg/l), BAP (0.5 mg/l), IAA (0.5 mg/l), Glucose (5 gm/l), Nicotinic acid (0.5 mg/l), Pyridoxin HCl (0.5 mg/l, Thiamine HCl (1 mg/l), Glycine (2 mg/l), Inositol (100 mg/10) and Glutamine (47 mg/l).

Medium No. 7 Modified N₆ (Liquid):

$(\text{NH}_4)_2\text{SO}_4$ (463 mg/l), KNO_3 (2830 mg/l), KH_2PO_4 (400 mg/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (185 mg/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (166 mg/l), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (4.4 mg/l), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.5 mg/l), H_3BO_3 (1.6 mg/l), KI (0.8 mg/l), $[\text{Na}_2\text{EDTA}$ (745 mg), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (557 mg) - dissolved in 100 ml of water and 5 ml of this solution was taken for 1 litre of medium], fortified with 2,4-D (4 mg/l), Nicotinic acid (0.5 mg/l), Pyridoxin HCl (0.5 mg/l), Thiamine HCl (1.0 mg/l) and

Glycine (2 mg/l) Sucrose (60 g/l).

Medium No. 8 E₁₀YK (liquid):

G₅ salts without agar and fortified with 2,4-D (1.0 mg/l), BAP (0.5 mg/l), IAA (0.5 mg/l), Glucose (5 mg/l), Nicotinic acid (0.5 mg/l), Pyridoxin HCl (0.5mg/l), Thiamine HCl (1.0 mg/l), Glycine (2.0 mg/l), Myo-inositol (100 mg/l), Yeast extract (3g/l) and Kinetin (1.0 mg/l).

The media pH was adjusted to 5.8 in all cases and they were autoclaved at 1.06 kg/cm² for 20 minutes.

2.2.3.2.1 Callus proliferation and regeneration:

Small calli were transferred to proliferation media viz. N-19 and J-19 on a paper boat. The composition of J-19 and N-19 media are given below.

J - 19 (Liquid):

B₅ basic salts without agar and fortified with sucrose (20 gm/l), Inositol (160 mg/l), Kinetin (1mg/l) and NAA (1 or 2 mg/l).

N - 19 (Liquid):

MS (Murashige, ¹⁹⁷⁴) basic salts without agar and fortified with sucrose (30 gm/l), NAA (1 or 2 mg/l), Kinetin

(1 mg/l) and Inositol (100 mg/l).

Calli thus obtained were transferred to different regenerating media. Ten media i.e., MSA, MSB, MSI, MSBT, MSC, MSD, MSBS, RGA, RG₂ and N₆ without hormones were tried for regeneration. The composition of the media are given in Table 1:

Table 1: Different regenerating media with their composition:

Media		Concentration of Auxins, Cytokinins, Vitamins and Sucrose.				
		NAA (mg/l)	BAP (mg/l)	Kinetin (mg/l)	Thiamine HCL (mg/l)	Sucrose (gm/l)
MSA	MS+	1.0	1.0	0.5	-	30
MSB	MS+	0.5	-	3.0	-	30
MSI	MS+	2.0	1.0	2.0	-	30
MSBT	MS+	0.5	3.0	10.0	10.0	30
MSC	MS+	1.0	0.5	0.6	-	30
MSD	MS+	1.0	0.1	3.0	-	30
MSBS	MS+	0.5	-	3.0	-	60
RGA	MS+	1.0	2.0	0.6	-	60
RG ₂	MS+	0.5	5.0	-	-	30

and N₆ without hormone.

2.2.3.2.2 Ploidy Status

Excised roots of about 1 cm long were pretreated with 2 mM ^{no} hydroxyquinoline at room temperature for 2-3 hours to arrest/accumulate cells at metaphase. These were fixed in Carnoy's fixative (6:3:1 - absolute ethanol : chloroform : glacial acetic acid ; with two changes). The roots were stored at 4°C for at least 2 days which can be extended upto 60 days.

The fixed roots were washed in distilled water for 2-3 times (5-6 minutes each) and then in ice cold 0.01M citrate buffer (0.01M citric acid monohydrate in 0.01M sodium citrate, pH 4.6) 2-3 times (2-3 minutes each). Root tips (2-3 mm) were cut and transferred to an ependroff tube containing an enzyme solution [2-6% Cellulase (Onozuka R-10) and 1-2% Pectolyase (Yakult Y-23) in citrate buffer] held at 37°C in a water bath. The root tips were digested for 45-75 minutes and washed in 0.01M citrate buffer 2-3 times (10 minutes each) and then in sterile distilled water twice (5-10 minutes each) to remove the enzyme solution (Islam and Stich, 1989).

Root tips were transferred singly to an acid cleaned and ethanol washed slide. Excess water was removed without disturbing the slide. A drop of 3 : 1 absolute ethanol: glacial acetic acid was placed onto the root tip and squashed immediately with a needle. Another drop of 3 : 1 fixative was added to and

the material was spread~~ed~~ over the slide. Slides were air dried and stored in a desiccator^c overnight.
A

Slides were examined under phase contrast microscope. For better contrast the chromosomes were stained by placing a drop of 1% acetocarmine or 1% propionic carmine over the air dried cells and covered with a cover slip and heated briefly over a spirit lamp. Excess stain was removed before applying light pressure to the cover slip.

2.2.3.3 Production of promising double haploids:

2.2.3.3.1 Selection and hybridization

After standardizing the media and environmental conditions for callus induction and plant regeneration, crosses were made between IR70 and Khonorullo. The crossings were carried out by following standard procedures of emasculation in the evening and pollination next morning.

2.2.3.3.2 Culturing of anthers from F1, regeneration and production of double haploid lines

In the next season, F1 derived seeds obtained from the crosses were grown separately. The anthers obtained from them¹⁹ were cultured for callusing in different media as mentioned earlier. The resultant calli were transferred to regeneration

medium.

Regenerated plantlets were transferred singly to pots where they matured. The data on agrobotanic characters of F1's were recorded and seeds were harvested from fertile plants.

During the next year, seeds of different double haploid (DH 1) lines were multiplied in upland, lowland and in pots and double haploid (DH 2) lines thus obtained were evaluated for yield and yield contributing characters under medium altitude (950 m. msl) lowland condition.

2.2.4 Field evaluation

The experiment was conducted on the farm of the ICAR Research Complex for NEH Region, Barapani in the wet season of 1994. The test entries, 9 DH lines (line no. 1, 6, 7, 10, 13, 15, 16, 19 and 21) and 2 parents (IR70 and Khonorullo) were evaluated in a 3m X 1m plot in ^A randomized block design^L with five replications. Each plot contained five rows with 30 plants/row. Single seedling was transplanted/hill at 20cm X 10cm spacing. Recommended dose of NPK fertilizer was applied.

2.2.4.1 Data collection

Observations were recorded on plants in the three middle rows (excluding border plants) for following characters:

Panicle length: Average length from five plants/plot measured in cm at maturity.

Panicle weight: Average weight from five plants /plot weighed as gm at maturity.

Percent sound grain: Calculated as percentage of filled grains from total number of spikelets/panicle of five plants/plot.

Plant height: In cm, from the ground to the tip of the main panicle at maturity.

Ear bearing tillers: Average number of ear bearing tillers from five plants/plot at maturity.

Grain weight/panicle: Average weight of grain from five panicles/plot at maturity.

Thousand grain weight: Recorded from evenly oven dried seeds of five plants/plot.

Number of spikelet/panicle: Average number of spikelets from five panicles/plot.

Grain yield: Weight of clean and dry grains harvested from the whole plot (excluding border plants) computed at 14% moisture content in t/ha.

2.2.4.2 Data analysis

In order to have the basic information on the extent of genetic variability, statistical analysis^e were carried out on analysis of variance, coefficient of variation and heritability.

2.2.4.3 Analysis of variance

The analysis of variance was carried out to test the genetic difference between the genotypes under investigation following the method suggested by Gomez and Gomez (1984).

The analysis of variance was based on the assumptions underlying linear model. A simple additive model is given below:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \epsilon_{ijk}$$

$$Y_{ijk} = \mu + q_{ij} + b_k + e_{ijk}$$

where $i = j = 1 \dots n$
 $k = 1 \dots r$

$i = 1 \dots n$
 $j = 1 \dots n$
 $k = 1 \dots r$

Y_{ijk} = the phenotypic value of genotype ij grown in k^{th} block.

μ = the general mean

q_{ij} = the generation effect of the ij^{th} genotype

b_k = the k^{th} effect

e_{ijk} = the environmental effect associated with each observation.

The μ, q_{ij} and b_k are assumed to be fixed unknown parameters. The e_{ijk} 's are assumed to be normally and independently distributed with mean zero and variance (σ^2) . The analysis of variance table is given below:

Source of variation	df	Mean sum of square	Expected mean square
Block	$r-1$	$\frac{1}{g} \frac{\sum_j x_j^2}{j}$	$\frac{\sum_j x_j^2}{r g}$
Genotype	$g-1$	$\frac{1}{r} \frac{\sum_i x_i^2}{i}$	$\frac{\sum_i x_i^2}{r g} + r \sigma^2$
Error	$(r-1)(g-1)$	By subtraction	

$$\text{Total} \quad rg-1 \quad \times \quad \sum_i \sum_j x_{ij}^2 - \frac{x^2}{rg} \dots$$

Various mean squares were tested against error variance by using F - test. The standard error of mean and difference of means were calculated as $(EMS/r)^{1/2}$ and $(2 EMS (s/r))^{1/2}$ respectively where EMS is the error mean square.

2.2.4.4 Coefficient of variation

For comparing the extent of variability between different characters, coefficient of variation was calculated at phenotypic and genotypic levels using the methodology of Burton (1952).

Phenotypic coefficient of variation (PCV):

$$PCV = \frac{\sqrt{\frac{\sum (x_i - \bar{x})^2}{n}}}{\bar{x}} \times 100$$

σ_p^2 not σ^2

Genotypic coefficient of variation (GCV)

$$GCV = \frac{\sqrt{\frac{\sum (x_i - \bar{x})^2}{n}}}{\bar{x}} \times 100$$

σ_g^2

Where σ^2_p and σ^2_g are phenotypic and genotypic variances obtained from the expectation of mean squares in the analysis of variance tables and \bar{X} is the general mean of the populations.

2.2.4.5 Heritability

Heritability (H) in a broad sense was calculated by the method described by Allard (1960) and was defined in percentage where,

→ formula

h^2 = Heritability in percentage

σ^2_p = Phenotypic variance

σ^2_g = Genotypic variance.

2.2.4.6 Genetic advance

It is calculated as

$$GA = \frac{V_g}{\sqrt{V_p}} \bar{X} \cdot K$$

Where K is the selection differential based on phenotypic deviation ($K = 2.06$ at 5% selection intensity level)

Genetic advance as percentage of mean is calculated as

$$GA\% = \frac{GA}{\text{mean}} \times 100$$

2.2.4.7 Correlation

Correlation coefficient (r) between various characters studied were worked out according to the method suggested by Robinson et al. (1951). The form of analysis of covariance is given below:

Table Analysis of covariance			
Source	df	Mean sum of Products	Expectation of mean Sum of products
Replication	r-1	r12	ee12 + ger12
Genotype	g-1	t12	ee12 + reg12
Error	(r-1)(g-1)	e12	ee12

Also, different simple correlation coefficient among different characters were worked out:

Phenotypic coefficient of correlation (rp12):

$$r_{P12} = \sigma^{-1} \sqrt{\frac{\sigma^2_{P12}}{\sigma^2_{P1} \times \sigma^2_{P2}}}$$

Where,

r_{P12} = Correlation between phenotypic values of character 1 and 2.

$\frac{\sigma^2}{P1}$ = Variation of phenotypic values of character 1

$$\sigma^2 = \text{Variation of phenotypic values of character 2}$$

$$\sigma_{12} = \text{Covariance between 1 and 2}$$

The significance of phenotypic coefficient of correlation was tested against "r" values and error degrees of freedom at $p=0.05$ and $p= 0.01$ levels.

2.2.4.8 Path coefficient analysis

Path coefficient is simply a standardised partial regression coefficient and as such measures the direct influence of ~~the~~ one variable upon another. Path coefficient analysis was done to partition the total correlation into direct and indirect effects by using the concepts of Wright (1921). The phenotypic correlation among different character combinations were utilized for this purpose. The path coefficient analysis was performed in accordance with the method given by Dewey and Lu (1959). The basic theory for the calculation of path coefficient is as follows:

$$r_{Y1} = P_{Y1} + P_{Y2}r_{12} + P_{Y3}r_{13} + \dots + P_{Yx}r_{1n}$$

$$r_{Y2} = P_{Y1}r_{Y2} + P_{Y2} + P_{Y2}r_{23} \dots + P_{Yr}en$$

$$\vdots$$

$$\vdots$$

$$r_{Yx} + P_{Y1}r_{1n} + P_{Y2}r_{2n} + P_{Y3}r_{2n} + \dots + P_{Yn}$$

Where,

r_{yi} is the total correlation between x_i 's (independent variable) and Y (dependent variable)

P_{Yi} is the direct effect of variance x_i on Y

$P_{Yn} e_{In}$ is the indirect effect of variable x_i on Y through variable x_n

Then the equation in matrix form are as under:

$$\begin{matrix} r_{12} & r_{13} & \dots & r_{1n} & & P_{Y1} & r_{Y1} \\ r_{21} & r_{23} & \dots & r_{2n} & \times & P_{Y2} & r_{Y2} \\ r_{n1} & r_{n2} & r_{n3} & & & P_{Yn} & r_{Yn} \end{matrix}$$

$$\begin{matrix} r_{n1} & r_{n2} & r_{n3} \\ (R)_{r \times n} & \times & P_{yi} = r_{yi} \end{matrix}$$

$$\text{Hence } P_{yi} = (R)^{-1} r \times n \times r_{yi}$$

Where,

$(R)_{r \times n}$ = Correlation matrix

P_{yi} = Column vector of path coefficient

r_{yi} = Column vector correlation coefficient of individual trait with dependent variable

It $(R)^{-1} r \times n = (D) r \times n$ the path coefficient are calculated as under :

Direct effects:

$$P_{1y} = C_{11} r_{1y} + C_{12} r_{2y} + C_{13} r_{3y} + \dots$$

$$P_{2y} = C_{21} r_{1y} + C_{22} r_{2y} + C_{23} r_{3y} + \dots$$

$$P_{ny} = C_{n1} r_{1y} + C_{n2} r_{2y} + C_{n3} r_{3y} + \dots$$

Indirect effect of n^{th} variable (X_n) via variable (X_i) were worked out as $P_{iy} r_{ni}$.

$P_{1y} r_{12}$ is the indirect effect of X_1 on Y via X_2 :

$P_{2y} r_{23}$ is the indirect effect of X_2 on Y via X_3 :

and similarly all the indirect effects were calculated.

Residual effect:

The variation in Y may be due to some other variables not studied, effect of pseudo - variables which have no correlation with any other independent variable etc. It is termed as residual effect.

3.RESULTS

3.1 Anther culture- callus induction and plant regeneration from local cold tolerant cultivars

Microspores of rice during anther culture are known to give rise single or multiple calli which can be regenerated into plants. In order to apply anther culture technique in rice breeding for compressing the breeding cycle, anther culturability of the local varieties was first tested. Apart from *in vitro* responsiveness, various physical parameters and suitable culture conditions are also needed to be standardized. Therefore,

experiments were first undertaken to work out suitable conditions for induction of callusing in the anthers.

3.1.1 Standardization of conditions for callusing of microspore

3.1.1.1 Time of collection of panicles

The panicles collected in the morning hours gave better callusing response compared to the panicles collected at different times though the difference was not significant. The experiments were done with panicles collected in the forenoon (between 8 AM to 10.30 AM).

3.1.1.2 Environmental conditions at the time of collection of panicles

A significant difference in frequency of callusing was observed in the panicles collected on different days. In order to find out optimum conditions, panicles were collected between 25th August to 30th September and kept in cold at different temperatures for cold pretreatment. The range of temperature, humidity and sunshine hour_l during the period when anthers were collected vis-a-vis frequency of callusing is presented in Fig 1 and Table 1 .

Perusal of Fig 1 indicates that callusing frequency was maximum (78%) in the anthers taken from the panicles collected ~~in~~ 16th ⁹September when the temperature ranged from

Table 1 : Frequency of callusing in the anthers collected on different dates (from 25.8.92 to 30.9.92)

Date of collection	No. of anther inoculated	No. of callusing anthers	% callusing	Temperature		Humidity (%)	Total Sunshine hour
				Max.	Min (in °c)		
25.8.92	30	NIL	NIL	29.8	21.4	83	8.52
26.8.92	400	63	15.7	27.2	20.5	88	2.23
28.8.92	1600	150	9.3	28.2	20.0	95	4.77
30.8.92	390	42	10.7	27.2	20.8	79	8.25
5.9.92	320	112	35.0	28.4	18.5	90	9.50
6.9.92	970	226	23.2	28.0	19.6	74	9.20
8.9.92	420	77	18.3	27.0	19.6	90	3.62
9.9.92	710	163	22.9	28.3	19.6	93	7.55
11.9.92	300	52	17.3	27.3	19.3	87	6.23
13.9.92	200	68	34.0	26.7	19.0	78	5.70
14.9.92	200	96	48.0	26.5	18.6	81	6.20
15.9.92	1600	290	18.1	24.8	18.0	93	4.75
16.9.92	205	160	78.0	26.3	17.2	83	3.10
17.9.92	1700	340	20.0	24.6	17.8	100	2.50
19.9.92	2500	270	10.8	26.6	17.2	77	8.70
23.9.92	80	4	5.0	28.8	19.6	89	5.00
30.9.92	600	20	3.3	24.4	18.0	88	2.60

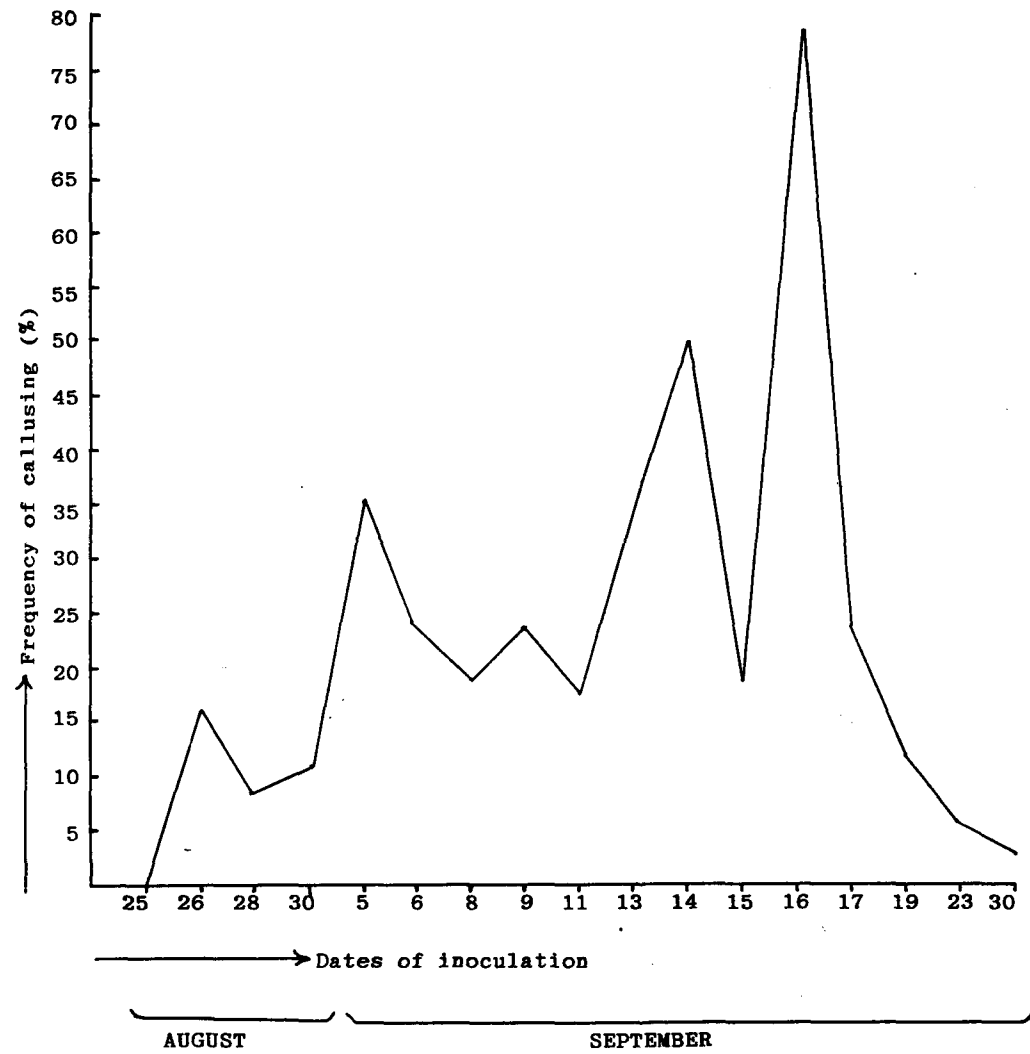


Fig. 1 Frequency of callusing of different dates of inoculation
(from 25-8'92 to 30-9'92)

17.2-26.3°C, humidity 83%- 100% and sunshine, 3.10 hour/day. In addition, Fig.1 also shows frequency of callusing ranging from 9.3 - 48% during August 28th to September 14th.

3.1.1.3 Length of cold pretreatment

Cold pretreatment is known to exert profound effect on anther callusing. In the present investigation it was observed that frequency of callusing increased with the period of cold pretreatment (at 8-10°C), in both the media viz; G₅ and E₂₄ which were tested. Two to 4 % callusing was observed in the anthers which were treated for 1 day. Thereafter, frequency of callusing increased with increase in days of cold pretreatment and reached maximum (43.3% in G₅ and 16% in E₂₄) after 10 days of pre-treatment. After prolonged pre-treatment beyond 10 days, the frequency of callusing decreased abruptly from 43.2% to 12.3% in G₅ medium after 12 days. In case of E₂₄ medium, it slowly decreased with increase in days of cold pretreatment and reached 4% after 26 days of cold pre treatment. None of the anther callused in G₅ medium after 26 days of cold pre treatment (Fig.2).

The present study, thus, indicates that cold pretreatment at 8°C for 10 days to the anthers before inoculation, is optimum for obtaining maximum callusing in local rice cultivars.

3.1.1.4 Staging of microspores

Culture of anthers containing microspores at various developmental stages ~~are~~ known to influence the frequency of callusing. Therefore, in order to inoculate microspores at ~~various~~ developmental stages, an attempt was first made to find out a suitable stain to ascertain the exact developmental stage of the microspores. Anthers from different parts of panicle were stained with various stains and the results obtained are presented below:

3.1.1.4.1 Staging with acetocarmine stain

Microspore nucleus could not be seen distinctly in ~~a~~ squash preparations of 2% acetocarmine. Even the addition of mordants did not help in distinguishing nucleus from the cytoplasm (Fig. 3). Therefore, this stain was not found suitable for staging of rice microspores.

3.1.1.4.2 Staging with Feulgen stain

The nuclei were seen distinctly using this stain (Fig. 4). They appeared magenta coloured while cytoplasm remained colourless. But this technique was time-consuming and required minimum 2 to 5 hours. Therefore, it had limited application, especially when large number of anthers were to be inoculated everyday.

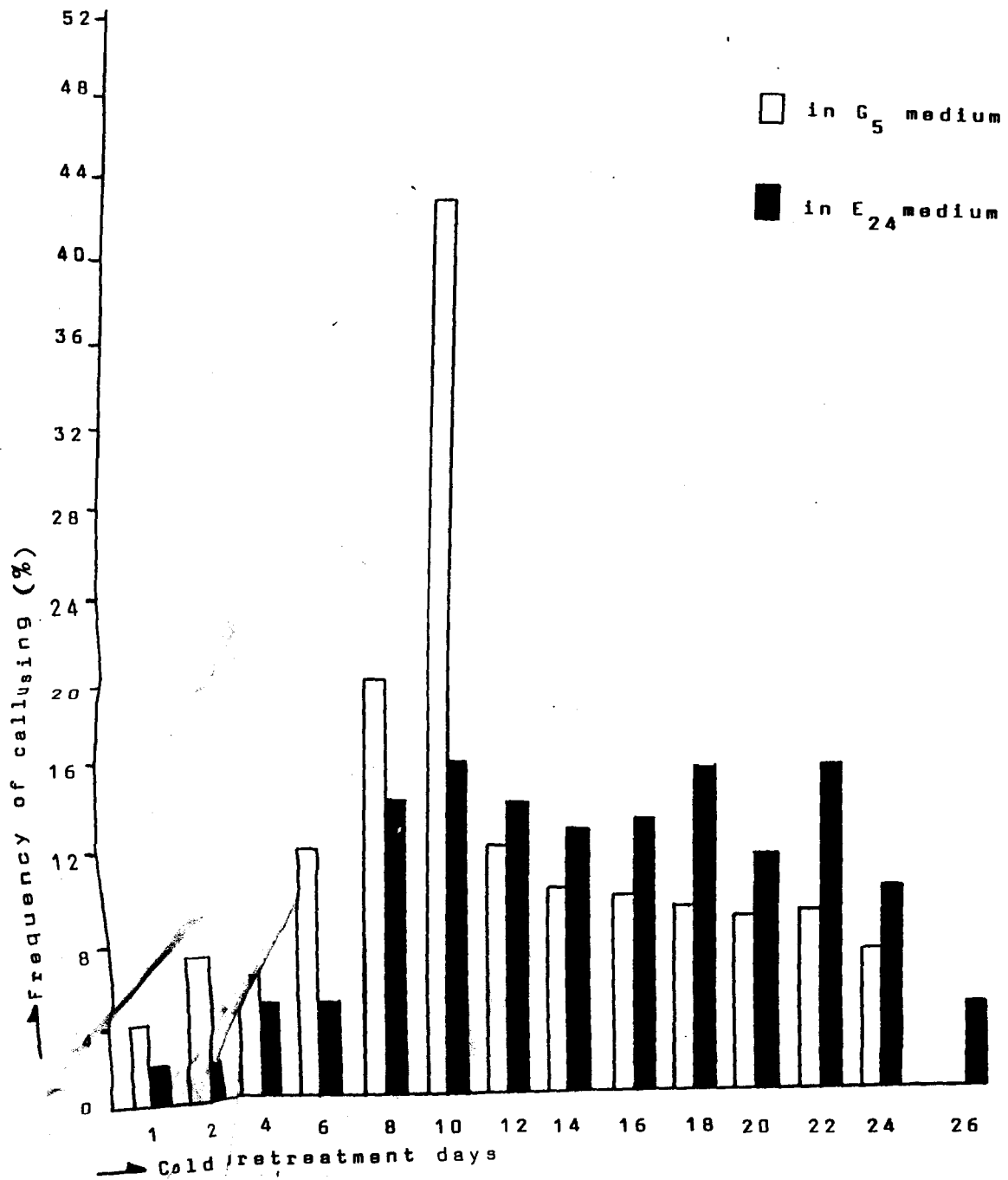


Fig. 1: Effect of days of cold pretreatment on frequency of callusing

3.1.1.4.3 Staging with iron alum - haematoxylin stain

Haematoxylin with iron alum stained the microspore nuclei very distinctly. Nuclei were stained deep brown against colourless cytoplasm (Fig. 5). Using this staining procedure, large number of spikelets could be staged within a short period because microspores could be stained within 2-5 minutes and thus this stain was used routinely.

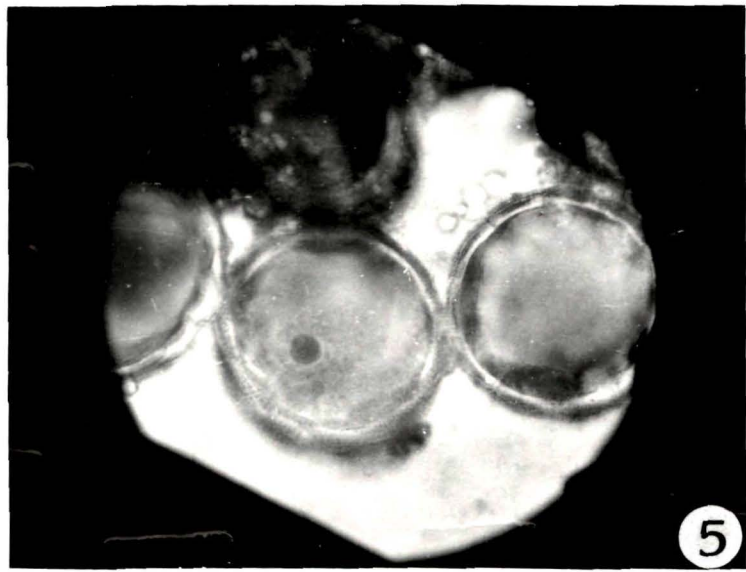
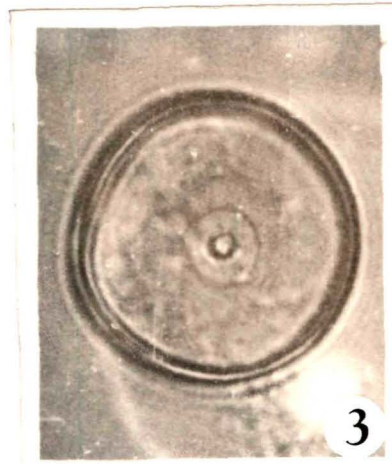
3.1.2 Effect of different culture media on callusing frequency and callus proliferation

Altogether 12 semisolid and 10 liquid media were tried ~~to check~~ the induction of callusing in the local cultivars. Preliminary screening revealed callus induction in all the media but, there was wide variation in the frequency. In order to narrow down the number of media and to find out the responsive one (s), only those media were tested which induced 5% or more callusing. Three semisolid media viz., G₅, E₂₄ & minimal and two liquid media viz., N₆ and E_{10G} were found promising and thus these were used for further study. Anthers of Khonorullo, RCPL 1-1C, RCPL 1-2C, RCPL 1-3C, Pusa 33 and DR 92 were inoculated in semisolid G₅, E₂₄ (both containing B₅ basic salts) & minimal, and liquid E_{10G} (containing B₅ basic salts) & modified N₆ media.

Fig. 3 : Microspore stained with 2% acetocarmine stain
(X 750)

Fig. 4 : Microspore stained with feulgin stain (X 750)

Fig. 5 : Microspore stained with iron-alum haematoxylin
stain (X 750)



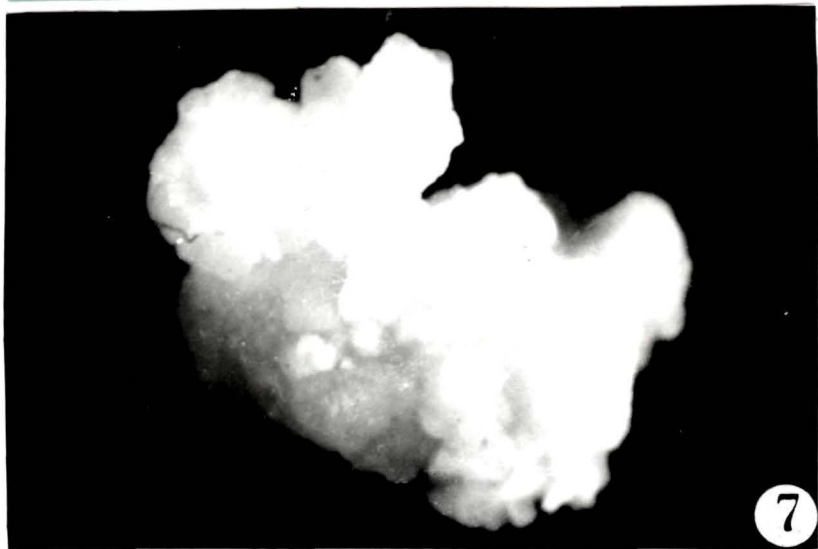
It was observed that in case of semisolid medium, the anther started browning during first three to four weeks of inoculation. Subsequently, callus mass bursted out of the anthers and the anthers turned deep brown after 40-45 days of inoculation (Fig. 6 & 7). The calli were cream coloured with organised sections which appeared as embryoids.

In most of the cases microspores started callusing after four weeks of inoculation and continued upto four months. The percent callusing was 13, 46, 33, 8 and 0.4% in 1st, 2nd, 3rd, 4th and 5th months, respectively, after inoculation. Maximum callusing was, however, observed between 40 to 50 days of inoculation.

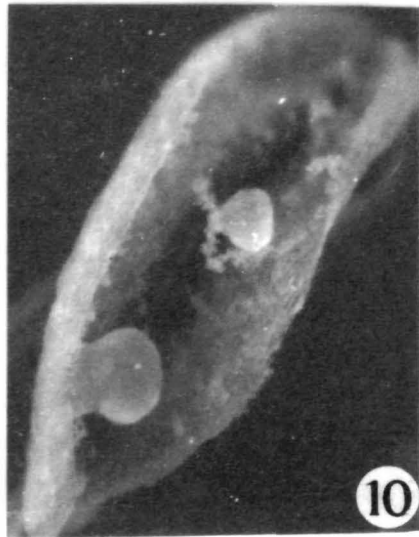
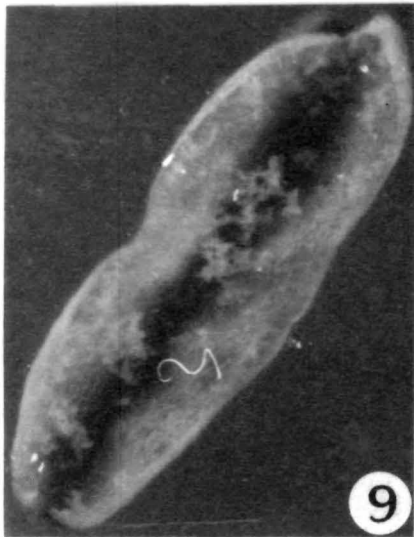
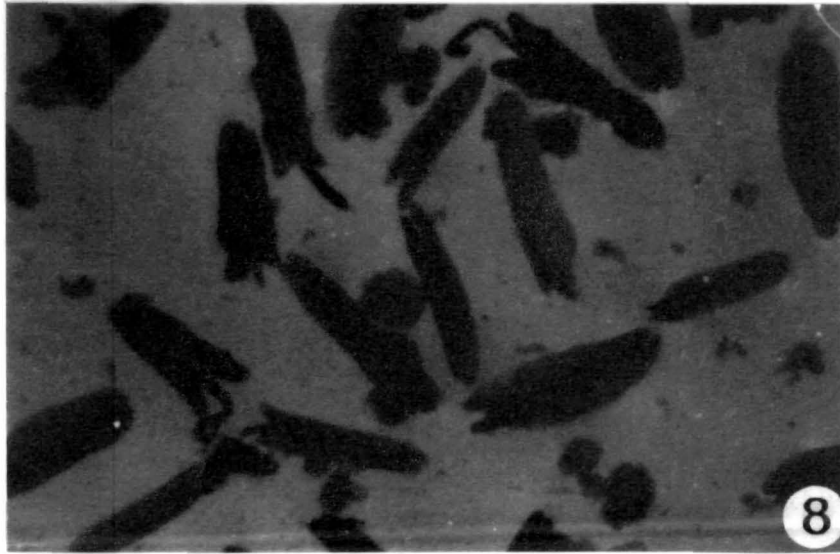
In case of liquid media (Fig. 8), anthers were found to split longitudinally thereby releasing microspores which were seen as aggregates (Fig. 9). The microspores gave rise to calli which remained attached to the anther wall in the initial stage (Fig. 10) and were released afterwards.

It was noticed that in most cases 200-300 microspores were released from each anther after splitting and the maximum release was recorded between 4th to 6th day of culture. Three types of microspores viz., highly elongated- empty; medium elongated- densely cytoplasmic and small unenlarged, in the ratio of 5:4:1, respectively, were observed. Nuclear

- 70
- Figs. 6-7
- 1 : Callusing in semisolid medium
 - 6 : Anther showing calli bursted out of the anther wall
 - 7 : Calli ready to be transferred to regeneration medium



- Figs. 8-10** : Callusing in liquid medium
- 8** : Calli released in the medium (single arrow)
(X 750)
- 9** : Longitudinal split anthers showing aggregates
of microspores (double arrow) (X 750)
- 10** : Anthers showing attached calli (X 750)



divisions occurred mostly in the medium-elongated and densely cytoplasmic microspores after 3rd day of culture and it continued upto 16th day with wall formation (Fig. 11-13). Further divisions and wall formation resulted in the formation of enlarged multicellular ~~pollen grain~~ generally composed of 50-60 cells (Figs 14 & 15). The exine of the multicellular structure eventually bursted out and around 30th day, small calli were released in the liquid media. Globular and heart shaped calli were observed after 55 days of culture (Figs. 16 & 17). ?

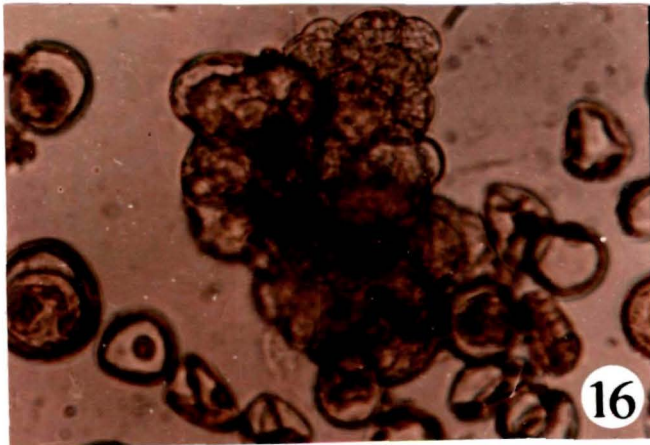
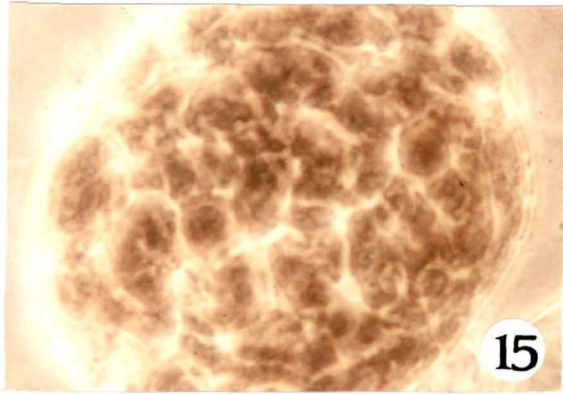
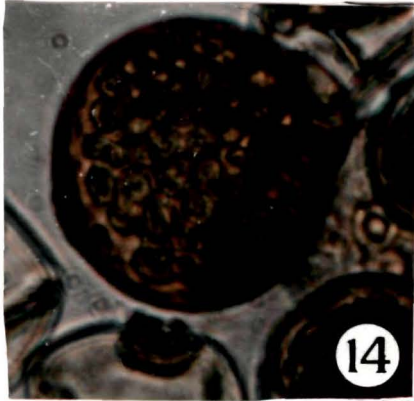
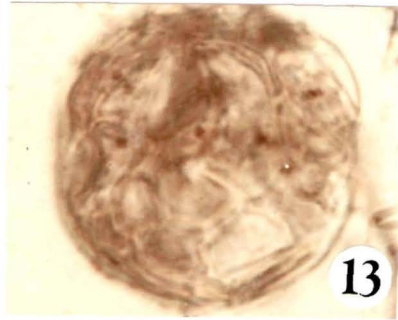
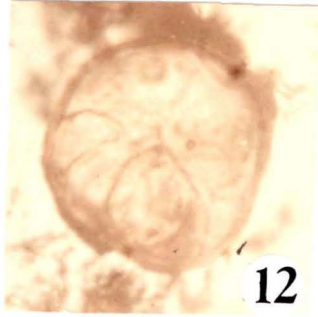
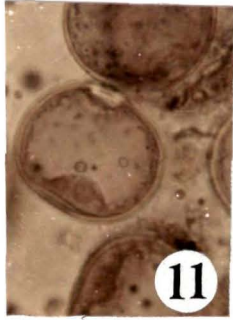
The overall frequency of callusing ranged from 4.5 to 77%. In case of Khonorullo, the frequency of callusing was generally good in almost all the media. However, liquid N₆ medium was the best where 57% callusing was observed. In case of semisolid medium, 50% callusing was found in G₅ medium (Fig. 18). In some tubes callusing frequency was as high as 98% in G₅ medium (Fig. 19).

In case of RCPL 1-1C, it was observed that liquid medium, E₁₀G was the best, where highest callusing (77%) was observed. However, in semisolid medium, highest frequency of 32.4% callusing was observed in E24 medium.

In RCPL 1-2C, the highest callusing was recorded in semisolid media where it was 45.5% in G₅, 35.4% in E24 and 30.6% in minimal medium. In liquid N₆ and E10G medium, the

- Figs. 11-17** : Various developmental stages of the microspores in liquid medium
- 11 : Uninucleate microspore (X 750)
 - 12 : Microspore showing initial division (X 750)
 - 13 : Multicellular pollen (X 750)
 - 14 : Further divisions give rise to multicellular structure (X 750)
 - 15 : Individual multicellular pollen grains enclosed to show the densely staining cells (X 750)
 - 16 : Microspore exine bursts out releasing microcalli (X 750)
 - 17 : Microcalli ready for transfer to regeneration medium

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frequency of callusing was 22.0 and 20.0% respectively.

In RCPL 1-3C, callusing was equally good in both semisolid and liquid medium. Fiftyfour percent callusing was observed in E₂₄ semisolid (Fig. 20) and 58% in E106 liquid medium. However, only 5% callusing was observed in G₅ medium which was generally good for other varieties.

In general, anthers of Pusa 33 did not respond well in almost all the media and the frequency of callusing in both semisolid and in liquid media ranged from 6.1% to 15% only.

DR92 anthers responded well in G₅ semisolid (48%) and N₆ (31%) liquid medium. In minimal medium they did not respond and the frequency dropped to 4.5%. Data on the frequency of callusing in anthers of six varieties in semisolid and liquid media are collated in Table 2.

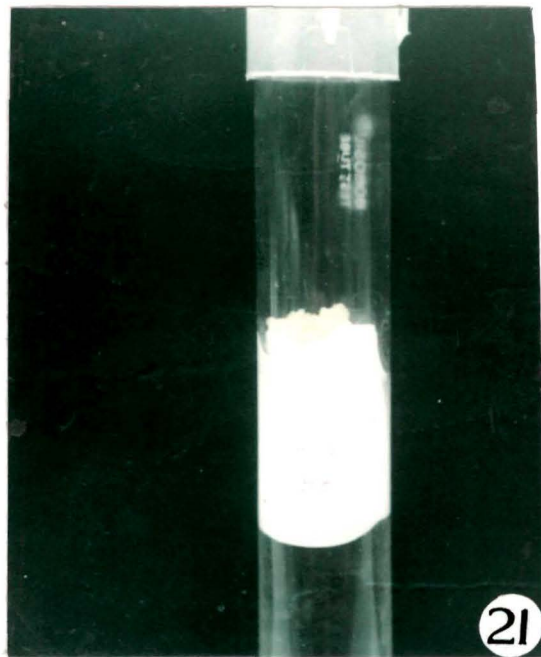
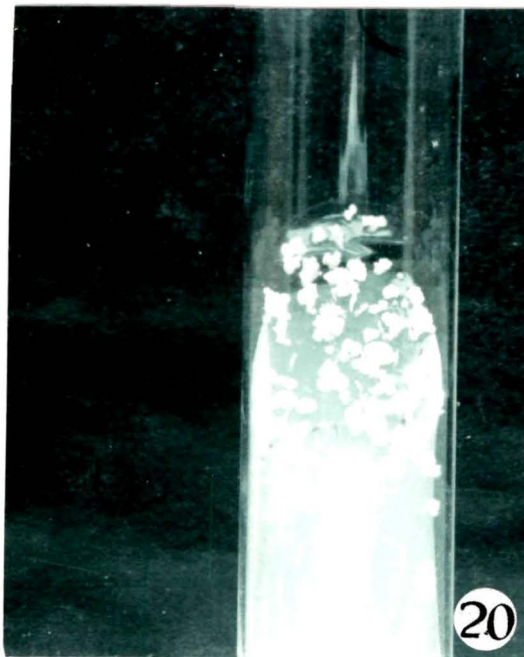
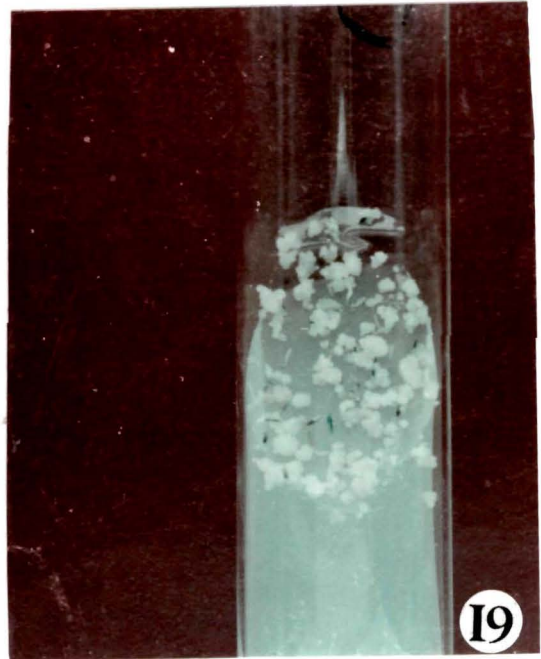
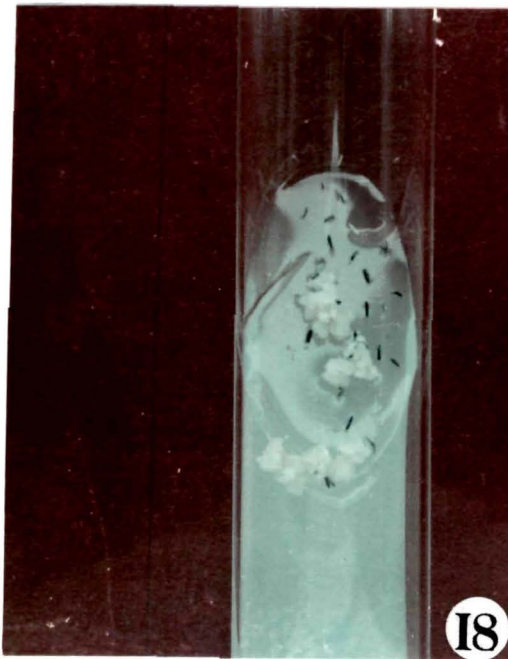
Perusal of data on callusing of anthers in six varieties (including parents and their progeny sister lines) indicated a strong genotypic effect. In general, it was observed that together with genotypic effect, media containing B₅ salts like G₅, E₂₄ and E106 responded well to callusing of anthers in local cultivars, except in Pusa 33 where highest frequency of callusing was observed in N₆ medium. Khonorullo was *in vitro* responsive variety whereas Pusa 33 was a recalcitrant one with low frequency of callus induction in almost all the media.

Therefore, apart from presence /availability of appropriate nutrients, genotype of the donor plant exerts a strong influence on callusing. Liquid media were relatively better than semisolid ones.

Proliferation of calli

Small pieces of calli (weighing 10 to 15 mg) were transferred to N-19 and J-19 liquid medium for proliferation. The tiny callus grew into a big mass of 2 to 3 g in 10 to 15 days (Fig.21). Both the media were found to be equally good for supporting proliferation, but addition of 2 mg/l NAA in both the media increased the rate of callus growth in some cases. Frequency of regeneration was however, affected. Proliferation in dark or in low intensity light, was found better for regeneration.

- Figs. 18-20** : Callus induction on semisolid media
- 18** : Callusing in Khonorullo on semisolid G₅ medium
 - 19** : Very high frequency of callusing in Khonorullo on semisolid G₅ medium
 - 20** : Callusing in RCPL 1-3C on E₂₄ medium
 - 21** : Callus proliferation in J-19 medium



(70)

Table 2 : Frequency of callusing of anthers from local cultivars in different semisolid and liquid media *.

Cultivars	Media				
	Semisolid		Minimal	Liquid	
	G5	E24		N6	E10G
Khonorullo	50.0	11.7	31.6	57.0	32.0
RCPL 1-1C	20.4	32.4	9.0	5.6	77.0
RCPL 1-2C	45.5	35.4	30.6	22.0	20.0
RCPL 1-3C	5.0	54.0	41.0	12.0	58.0
Pusa 33	12.0	9.0	6.1	15.0	9.0
DR 92	48.0	26.0	4.5	31.0	6.2

* Average of 3 experiments consisting of at least 500 anthers in each

** RCPL lines are sister lines selected from a cross between Pusa 33 and Khonorullo

3.1.3: Regeneration and confirmation of ploidy status

Anther - derived calli were transferred to regeneration media for differentiation. Regenerability of calli was tested by transferring them to regeneration media at various intervals after their first appearance. Ten to 15 days old calli gave higher regeneration frequency than older calli. Calli on transfer to regeneration media started differentiating within 10 days. Green pigments were first observed followed by appearance of shoots and roots (Figs 22 - 26).

It was also observed that proliferated calli tended to lose their regeneration capability as compared to directly transferred ones. In Khonorullo and RCPL 1-2C, small calli were proliferated in both J-19 and N-19 media and after 10-15 days they were transferred to MSB regeneration medium together with 10-15 days old primary calli. Out of 10 proliferating calli, only one regenerated in MSB media, whereas from the primary calli directly transferred to MSB medium, around 50% showed regeneration and produced green plantlets. Therefore, in further experiments, only primary calli were used for regeneration. Regeneration of calli obtained from different varieties in various regeneration media is presented in table 3.

Table 3: Regeneration percentage of anther-derived calli on different regeneration media

	Khonorullo									
	Media									
	MSI	MSA	MSB	MSBT	MSC	MSD	MSBS	RGA	RG2	N6-H
Total no. of tubes with calli	12	16	16	8	12	6	3	5	5	9
Number of tubes green pigmentation	4	7	8	0	3	1	0	0	1	2
Plant regeneration percentage	33	43	50	0	26	16	0	0	20	20

Table 3 continued

RCPL 1-1C

	17	10	16	9	8	12	6	15	9	10
Total no. of tubes with calli	17	10	16	9	8	12	6	15	9	10
Number of tubes with green pigmentation	4	2	7	1	(-)	2	(-)	2	(-)	-
Plant regeneration percentage	23	20	43	11	(-)	16	(-)	13	(-)	(-)

- Not observed

RCPL 1-2C

	MSI	MSA	MSB	MSBT	MSC	MSD	MSBS	RGA	RG2	N6-H
Total no. of tubes with calli	17	16	16	8	5	9	12	6	12	9
Number of tubes with green pigmentation	5	4	7	-	-	2	1	-	-	2
Plant regeneration percentage	29	25	43	-	-	22	8	-	-	22

RCPL 1-3C

Total no. of tubes with calli	15	12	12	10	4	12	6	6	7	10
Number of tubes with green pigmentation	2	2	4	2	1	1	1	-	-	1
Plant regeneration percentage	13	16	33	20	25	8	16	-	-	10

DR 92

Total no. of tubes with calli	6	5	9	4	5	6	6	6	-	-
Number of tubes with green pigmentation	1	2	2	-	1	-	1	1	-	-
Plant regeneration percentage	16	40	22	-	20	-	16	16	-	-

- Not observed

Table 3 continued

Pusa 33

	MSI	MSA	MSB	MSBT	MSC	MSD	MSBS	RGA	RG2	N6-H
Total no. of tubes with calli	10	12	9	5	6	5	6	-	-	-
Number of tubes with green pigmentation	1	1	2	1	1	-	1	-	-	-
Plant regeneration percentage	10	8	22	20	16	-	16	-	-	-

- Not observed ~~at~~ Do you mean not tested? If so, why give values such as this

It was observed that light intensity of 2500 to 3000 lux is suitable for plant regeneration. A continuous light for 5 days after transfer to regeneration media followed by 16 hr/8 hr light/dark was found to be the best for regeneration.

In Khonorullo, highest regeneration percentage (50%) was observed in MSB medium (Figs 22 & 23) followed by 43 and 33% in MSA and MSC. In regeneration media MSBT, MSBS and RGA there was no regeneration response.

Almost all cultivars responded well in MSB medium except DR 92 where 40% regeneration was recorded in MSA medium compared to 22% in MSB medium.

Forty three percent regeneration was recorded in RCPL 1-1C anthers in MSB regeneration medium followed by 23, 20, 16, 13 and 11% in MSI, MSA, MSD, RGA and MSBT. There was no regeneration in MSC, RG2, MSBS and N6 media.

Forty three percent regeneration was observed in RCPL 1-2C anthers on MSB medium followed by 29, 25, 22, 22 and 8% in MSI, MSA, MSD, N6 and MSBS, respectively. MSBT, MSC, RGA and RG2 media did not support regeneration. In cases of RCPL 1-3C and Pusa 33 maximum regeneration of 33 and 22% respectively was observed in MSB.

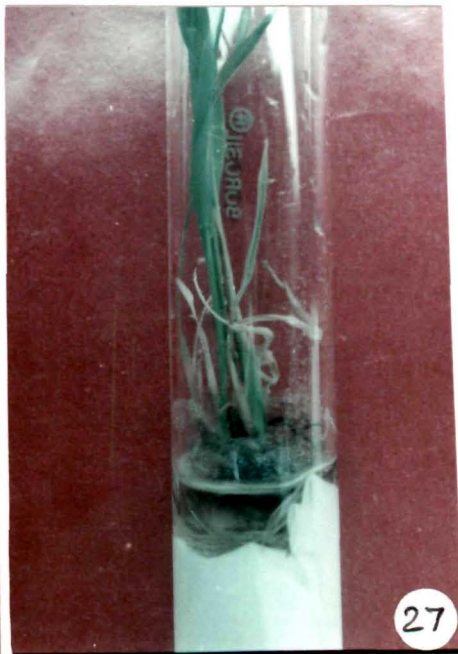
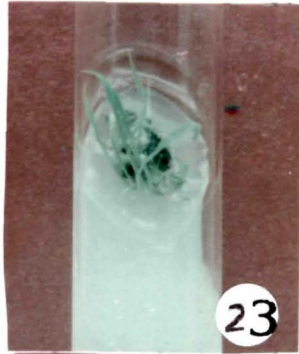
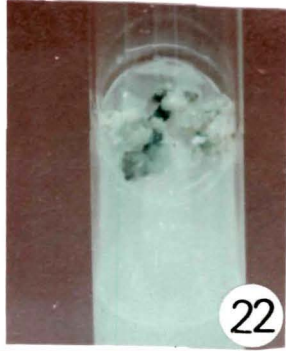
It was observed that MSB, MSI and MSA supported better regeneration than MSC, MSBS, MSBT, RGA, RG2 and N6, MSB being the best for almost all local cultivars.

After the plantlets attained 6 to 7 inches height, they were transferred to Yoshida's liquid culture medium and were covered with tubes to check evaporation (Figs 27-29). After about 15 to 20 days, they were transferred to pots for further growth and maturity.

Ploidy status:

Root tip cytology showed that most (70 to 75%) of the regenerated plants showed diploid (Figs 30 and 31) chromosome numbers, while 15 to 20% were haploids or mixoploids.

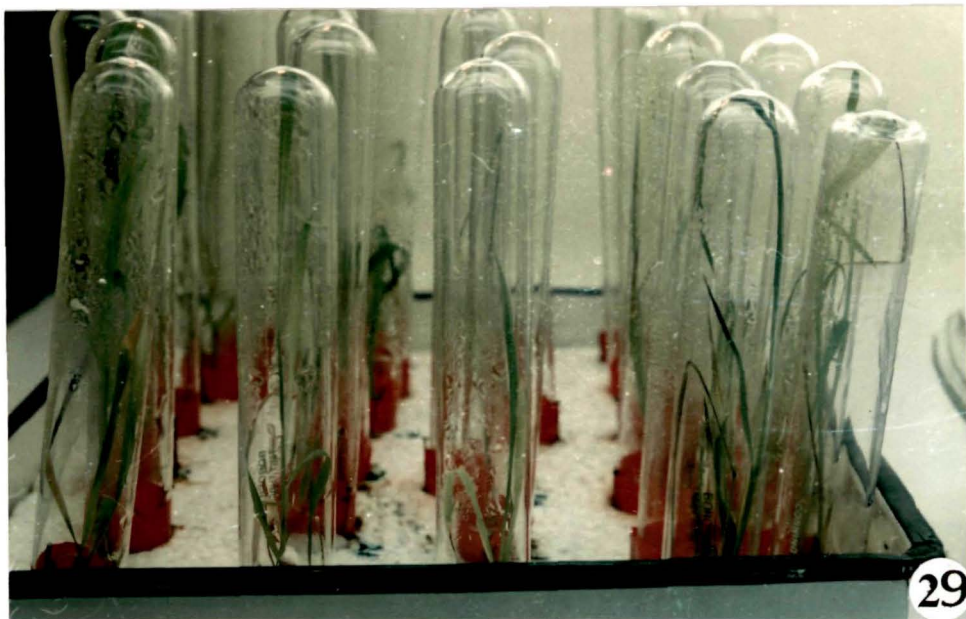
- Figs. 22-27** : Various stages of plant regeneration from anther derived callus of local varieties
- 22** : Initiation of green pigmentation in Khonorullo
 - 23** : Shoot formation in Khonorullo
 - 24** : Leaf initiation in RCPL 1-1C
 - 25** : Plantlet of RCPL 1-1C
 - 26** : Plantlet in advanced stage in RCPL 1-1C and
 - 27** : Plantlet of Khonorullo on paperboat transferred to Yosida's liquid medium



Figs. 28-29 : Plantlets in Yoshida's liquid medium covered
with tubes

28 : Plantlets of RCPL 1-1C

29 : Plantlets of Khonorullo



Figs. 30-31 : Squash preparation from root tip of regenerated plants showing diploid ($2n=24$) chromosomes
(X 3000)



3.2 Production of double haploids

3.2.1 Selection of parents and hybridization

In order to transfer cold tolerance to high yielding genetic background, IR 70, an elite breeding line from IRRI, and Khonorullo, a local cold tolerant cultivar, ~~was~~ selected for hybridization. IR 70 was used as female whereas Khonorullo was used as male. In all, 9 hybrid seeds were obtained.

3.2.2 Raising of F1 and their comparison with parents

The nine hybrid seeds were grown separately in the field. Data were recorded to compare the agrobotanic characters of the F1 plants with their parents. F1 plants showed hybrid vigour and performed better than both the parents in yield and yield contributing characters (Table 4).

Days to 50% flowering (98 days) remained within the range of both the parents which was 109 and 91 days in IR 70 and Khonorullo, respectively. Leaf length (29.0 cm) was reduced as compared to both the parents (34.0 and 36.5 in IR - 70 and Khonorullo, respectively). Plant height remained within the range of both the parents which was 87 cm as compared to 60 and 131 cm in IR 70 and Khonorullo, respectively. Number of ear bearing tiller increased remarkably to 7.1 as compared to 5.3 and 4.2 in IR 70 and Khonorullo, respectively. Likewise, panicle length

Table - 4 : Comparison of some ^Aagrobotanic characters in IR 70 and ^BKhonorullo with their F1 hybrid

	Days to 50 % flowering	Plant Height (cm)	Leaf length (cm)	EBT	Panicle length (cm)	Panicle wt. (g)	Grain wt/ panicles (g)	100 seed wt. (g)	Yield/ plant (g)	Total grain/ panicle	% Sound grain	Grain shape	Endosperm Colour
P1 IR 70	109	60.0	34.0	5.3	21.0	0.90	0.31	1.70	0.6	125	9.1	S	W <i>white</i>
F1 IR 70 X Khonorullo	98	87.0	29.0	7.1	23.9	4.01	2.81	2.85	9.1	167	63.2	M	P1 <i>light</i>
P2 Khonorullo	91	131.0	36.5	4.2	23.5	3.50	2.96	2.89	6.2	149	60.8	B	P3 <i>deep red</i>

(23.9 cm) and panicle weight (4.01 g) also increased from the range of both the parents. Grain weight per panicle (2.81 g) and 100 seed weight (2.85 g) remained within the range of both the parents. Yield per plant (9.1 g) increased remarkably and exceeded both the parents (0.6 g in case of IR 70 and 6.2 g in case of Khonorullo). Total grains per plant (167) also increased and was higher than the parents. Per cent sound grain (63.2%) also increased as compared to both the parents which was 9.1% in IR 70 and 60.8% in Khonorullo. Medium grains were observed in case of F1 as compared to slender grains in IR 70 and bold in Khonorullo. Light red coloured endosperm was observed which was a mixed character of both the parents; IR 70 was white where as Khonorullo, possessed deep red endosperm colour.

3.2.3 Culturing of anthers derived from F1

3.2.3.1 Callusing

Anthers from F1 plants of the cross IR 70 X Khonorullo were cultured as described previously (time of collection of panicle and preparation of staining solutions and procedure) in G5, E24, L8 and MP2 medium. Percentage of callusing ranged from 11.7 (in G5 semi solid medium) to 33.6% (in E24 semisolid medium). Highest percentage of callusing was observed

in one tube containing LB medium (81.6%). In MP2 medium, the frequency of callusing was generally good (31.1%). Frequency of callus induction in different media is presented in Table 5.

Table 5: Frequency of callusing of F1 derived anthers of IR 70 X Khonorullo in different media

	Media			
	G5	E24	LB	MP2
Number of anthers inoculated	980	110	1585	170
Total calli obtained	115	37	454	53
% callusing	11.7	33.6	28.6	31.1

3.2.3.2 Regeneration

Calli obtained from the above media were transferred to MSB regeneration medium within 5 to 10 days of their appearance and kept in 2500 to 3000 lux light intensity at 25 q 20C temperature. Green pigmentation was observed after 5 to 10 days of transfer to the regeneration medium. The regeneration percentage obtained from the calli induced in different media is collated in Table 6.

Table 6: Regeneration percentage in the calli obtained from F1 - derived anthers of IR 70 X Khonorullo in MSB medium

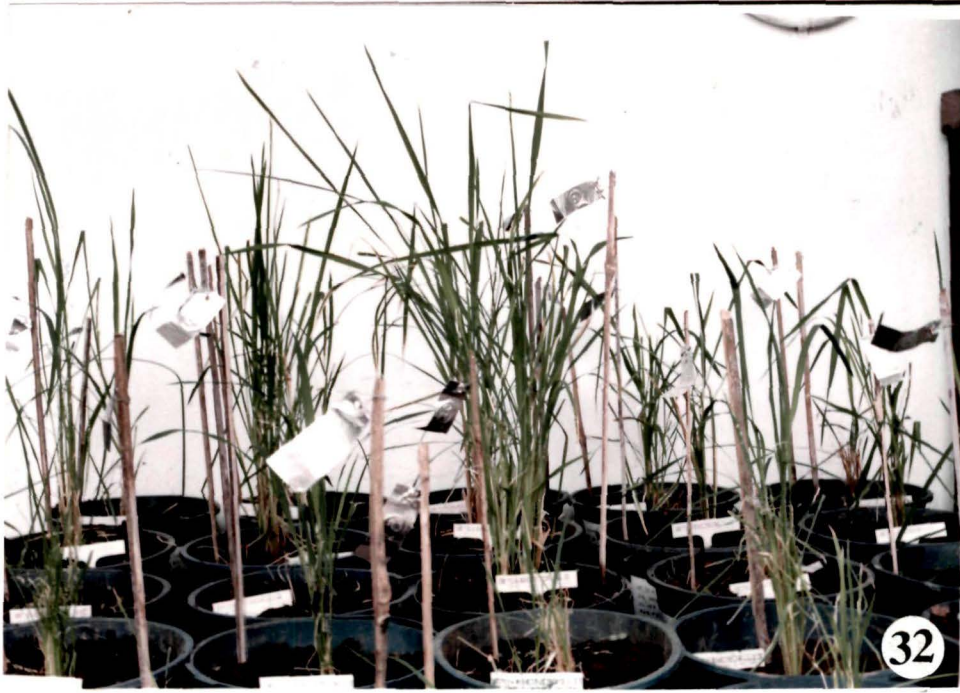
	Media			
	G5	E24	LB	MP2
Total no. of tubes with calli	92	15	174	30
Number of tubes with green pigmentation	—	—	32	—
Plant regeneration Percentage	—	—	18	—

— not observed.

30%
of not

It was observed that green pigmentation and plant regeneration was observed only in those calli which were obtained from LB medium (Table 6). Plantlets after attaining 6 to 8 cm height were transferred to flasks containing semisolid 1/2 MS medium and kept until they attained 18 to 20 cm height. They were, then, transferred to the Yosida's liquid medium and ultimately to the pots after attaining 30 to 35 cm height. Double haploids numbering 21 thus obtained, were grown to maturity in the pots (Figs 32 and 33).

Figs. 32-33 : Double haploid plants growing in pots



3.2.3.3 Evaluation of double haploid (DH) lines

Out of 21 plants regenerated, nine showed promising characters and they were selected. Seeds from these lines were collected and found to be different from each other. Agrobotanic characters of the parents and the double haploid lines are presented in ~~and~~ figures 34 to 67.

Panicle type of IR 70 was compact (Fig. 34) whereas that of Khonorullo was open (Fig. 36). Among double haploid lines, panicle type varied from compact as in line nos. 1,6,15,19 and 21 (Figs. 38,40,45,47 and 48) to open as in line no. 7 13 and 16 (Figs. 42,44 and 48). Line no 10 (Fig. 43) , exhibited intermediate type of panicle. Secondary branching was observed in line nos. 1,6,15,19 and 21 (Figs. 38,40,45,47 and 48) whereas it was absent in the parents. Straight panicle was observed in line nos. 6,13,15,16 and 21 (Figs. 40,44,45,46 and 48) whereas line nos. 1,7,10 and 19 showed droopy panicle (Figs. 38,41,42 and 47).

Shape and size of grains of each line varied greatly. It ranged from bold (Khonorullo) to slender, (IR 70). Line no. 1 and 21 showed slender grains with thin and short hair on upper portion of lemma and palea. (Figs. 51 & 67). Line no. 6 showed bolder grains with glabrous lemma and palea (pubescence) (Fig. 53). Line no. 7 and 10 possessed slender grains with

glabrous lemma and palea pubescence (Figs. 55 & 57). In contrast, line no 13 and 19 showed round grains with glabrous lemma and palea pubescence (Figs. 59 & 65). Line no 15 and 21 showed slender grains with thin and short hair on upper portion of lemma and palea (Fig. 61 and 67). In line no. 16, grains were round with short hair (Fig. 63). A typical character, where lemma and palea remained ~~open~~ at the tip was observed in most of the grains of line no. 13 (Fig. 59). This character was not found in any of the parents.

The endosperm colour, ranged from dark red (Khonorullo colour) to white (IR 70 colour) in the parents, whereas, it was intermediate in the progenies (Fig 70). Line no. 10 and 21 showed white ~~kernel~~ coloured grains. In most of the lines, (line nos. 1,6,7,13 and 15) light red coloured kernel was observed, whereas in line no 19 the kernel colour was ~~more~~ darker. In line no. 16, the kernel colour was dark red like Khonorullo.

Individual anther derived double haploid lines of the cross IR 70 X Khonorullo showed uniformity ^{for} in many agronomic characters. The range of variability among the plants of anther culture derived lines were within the limits of variability of their respective parents.

- Fig. 34** : Panicle of Parent 1, IR 70
- Fig. 35** : Husked and dehusked grains of IR 70
- Fig. 36** : Panicle of Parent 2, Khonorullo
- Fig. 37** : Husked and dehusked grains of Khonorullo

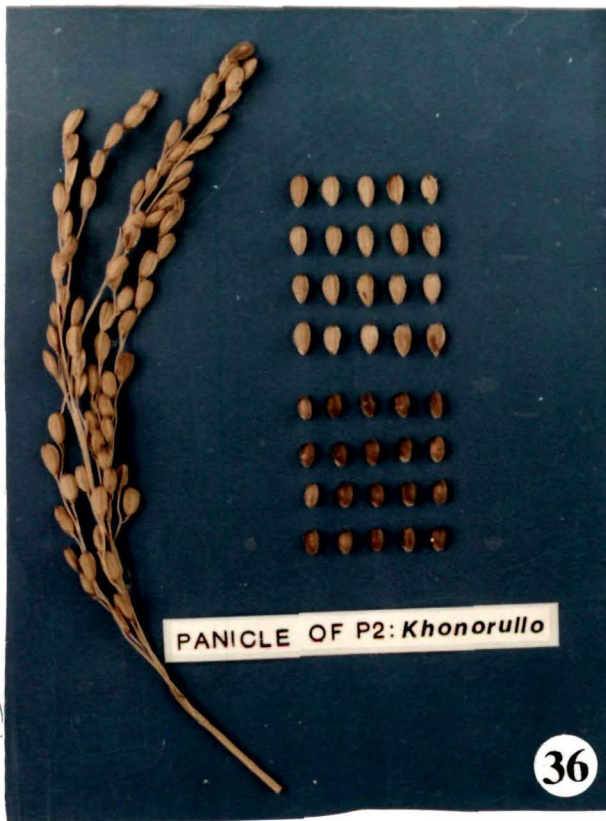
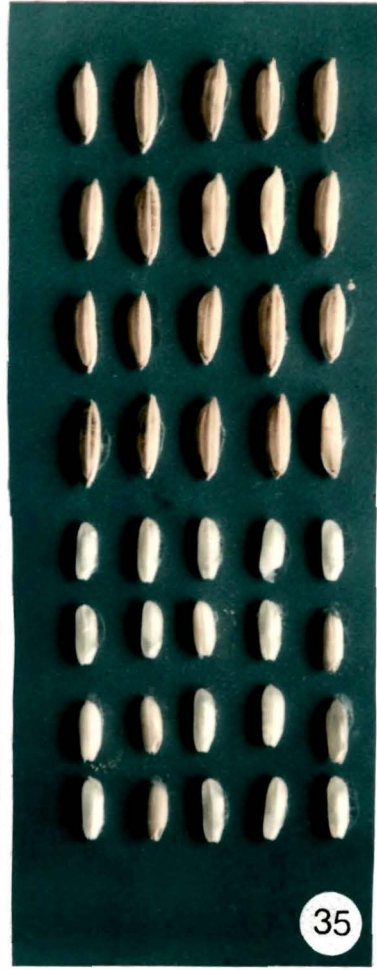
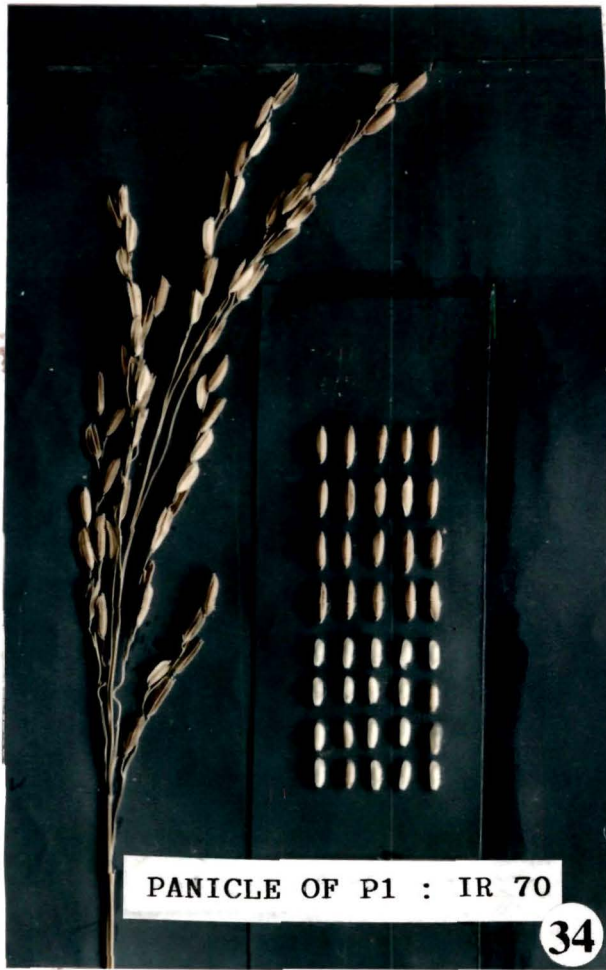


Fig. 38: Panicle of line no. 1 with husked and dehusked grains

Fig. 39: Closeup of husked and dehusked grains of line no. 1

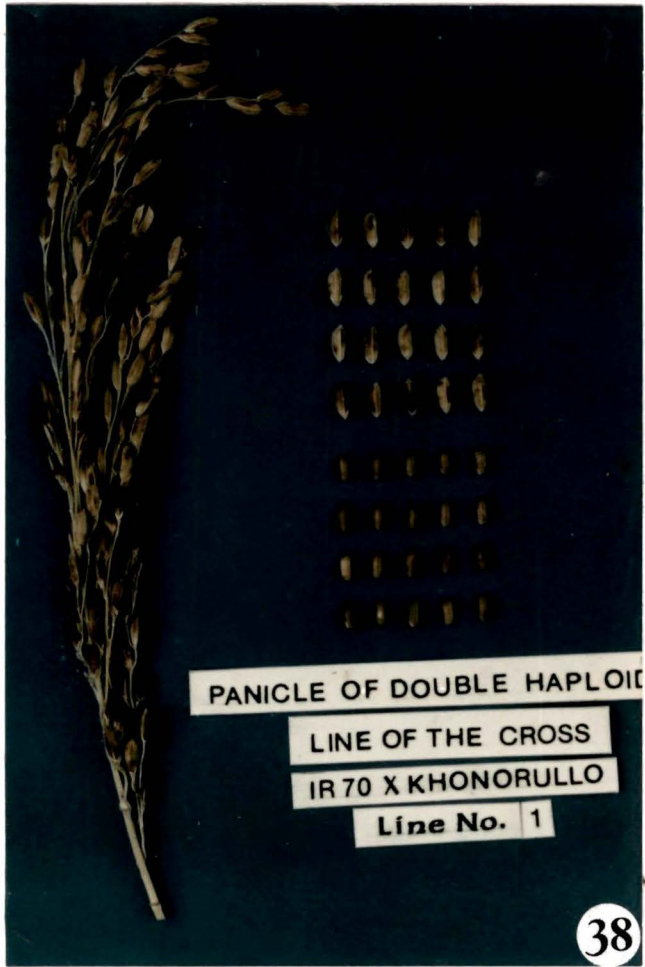


Fig. 40: Panicle of line no. 6 with husked and dehusked grains

Fig. 41: Panicle of line no. 7 with husked and dehusked grains

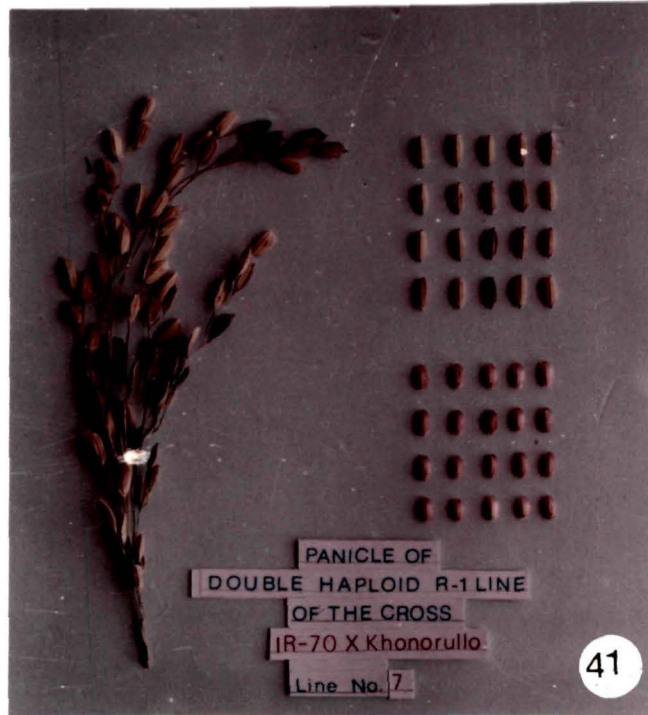
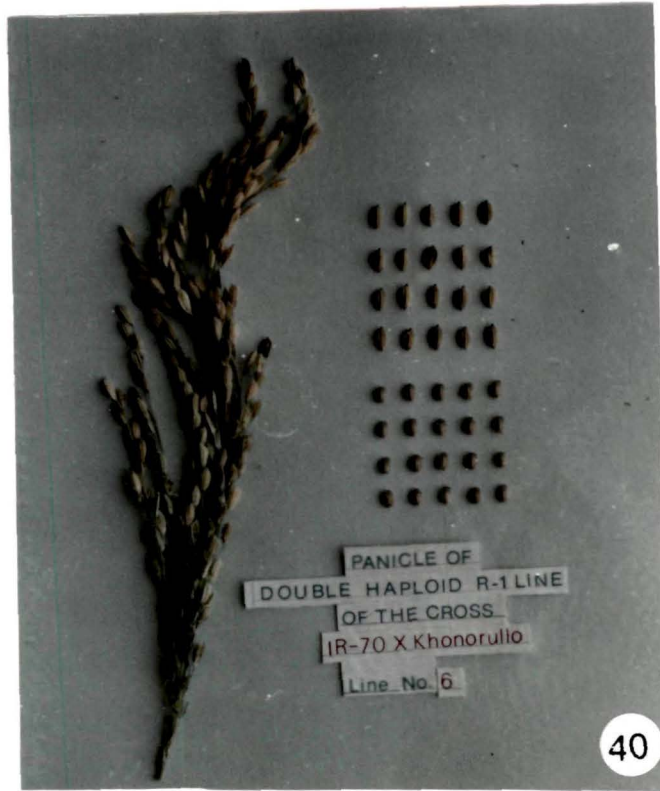


Fig. 42: Panicle of line no. 10 with husked and dehusked grains

Fig. 43: Closeup of husked and dehusked grains of line no. 10

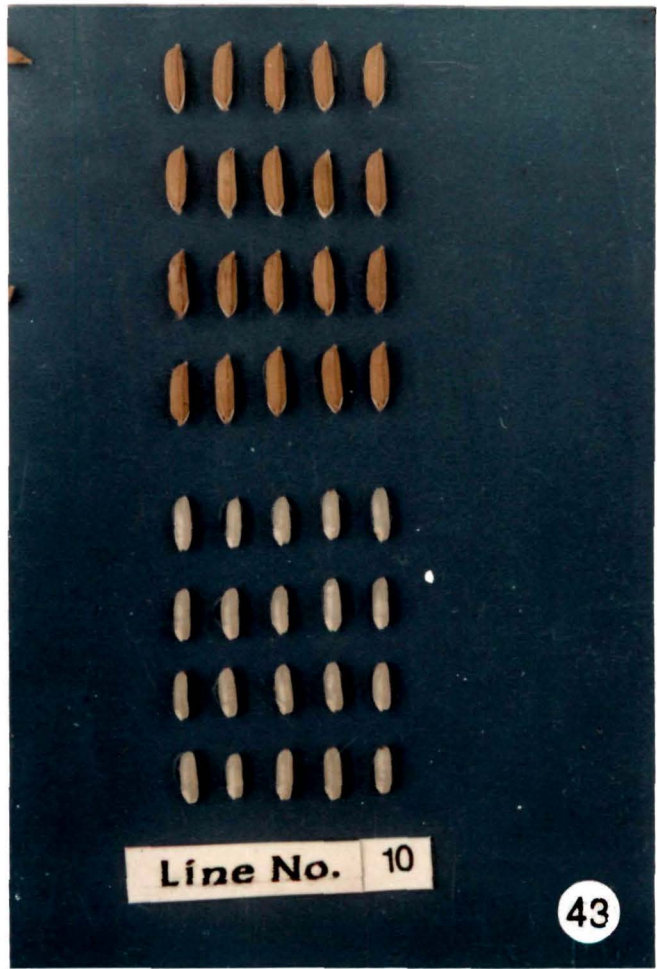
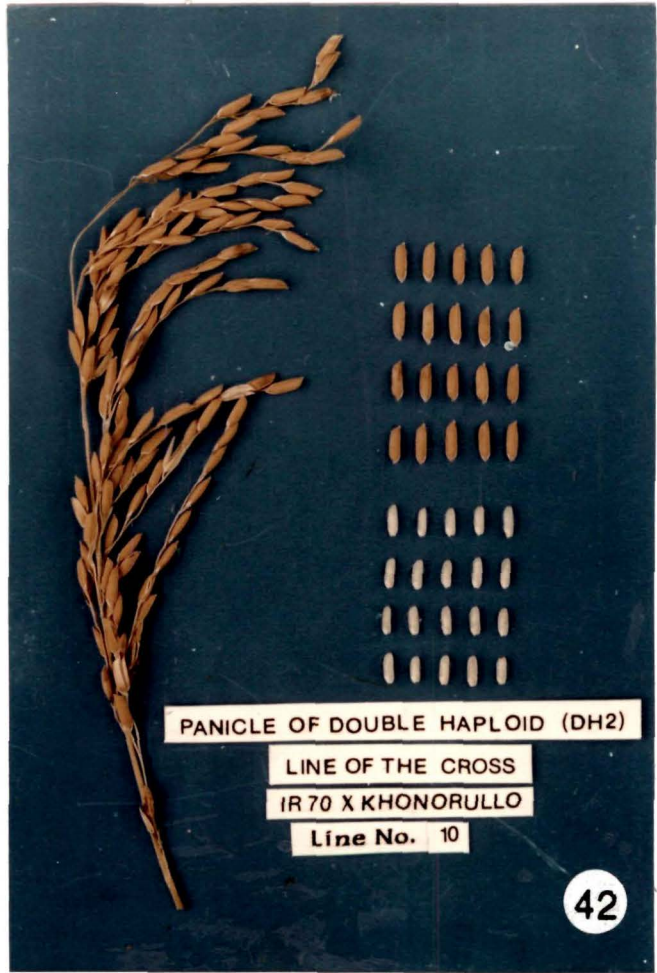


Fig. 44: Panicle of line no. 13 with husked and dehusked grains

Fig. 45: Panicle of line no. 15 with husked and dehusked grains

Fig. 46: Panicle of line no. 16 with husked and dehusked grains

Fig. 47: Panicle of line no. 19 with husked and dehusked grains

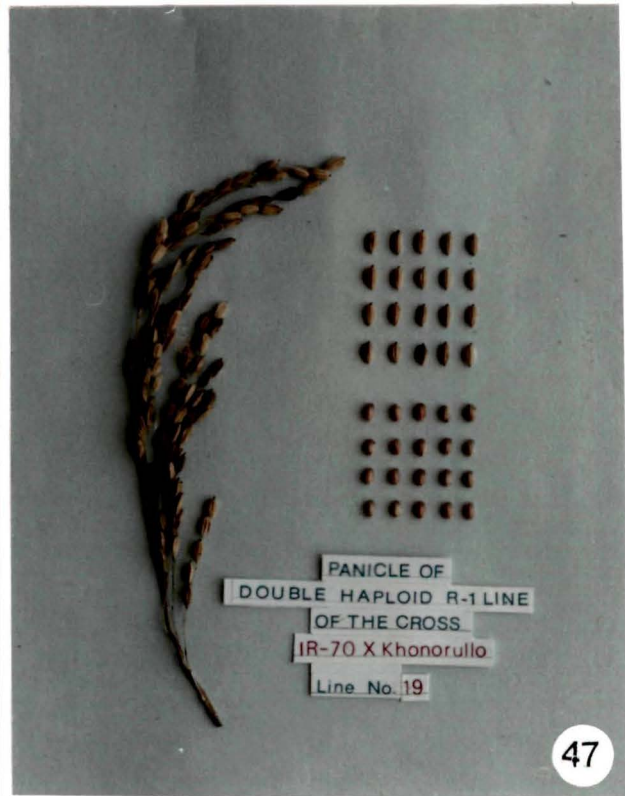
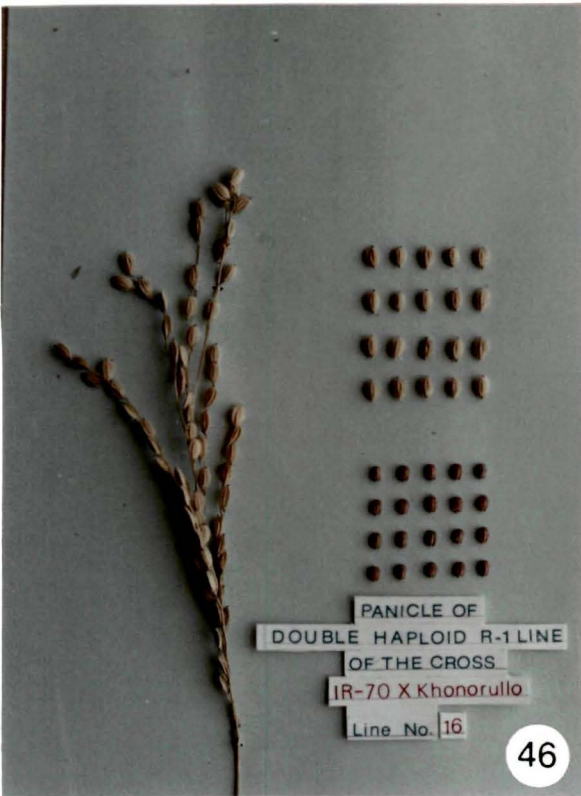
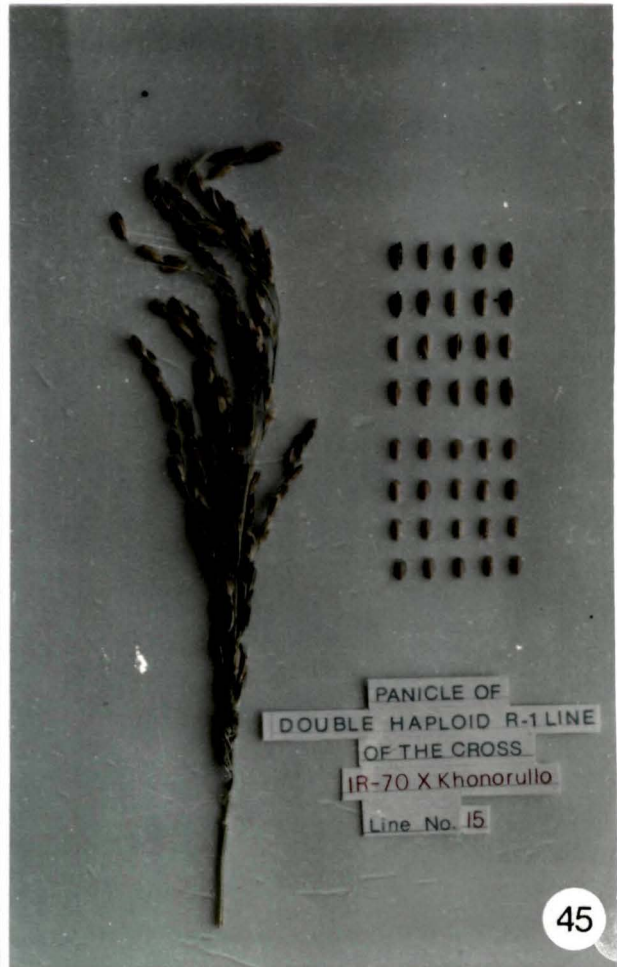
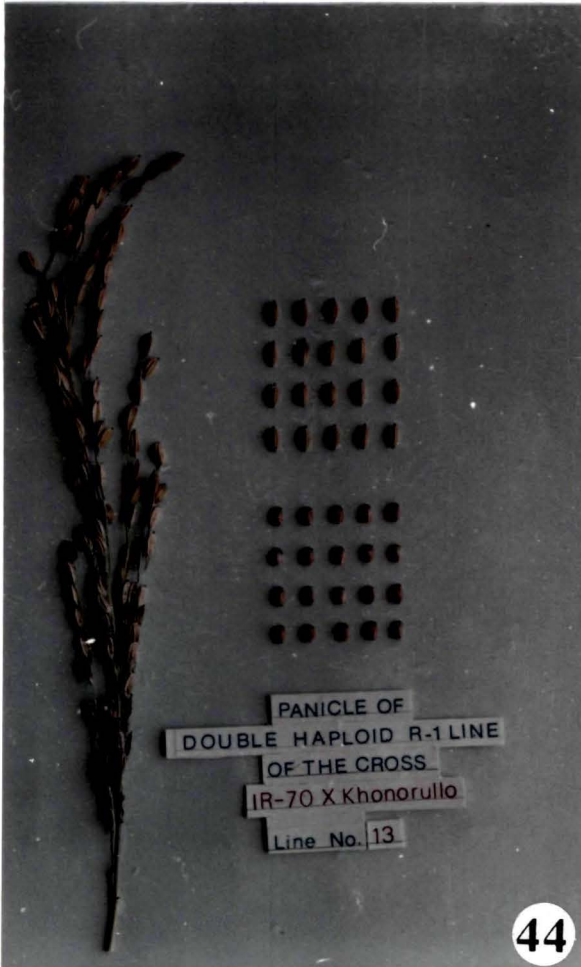


Fig. 48: Panicle off line no. 21 with husked and dehusked grains

Fig. 49: Closeup of husked and dehusked grains of line no. 21

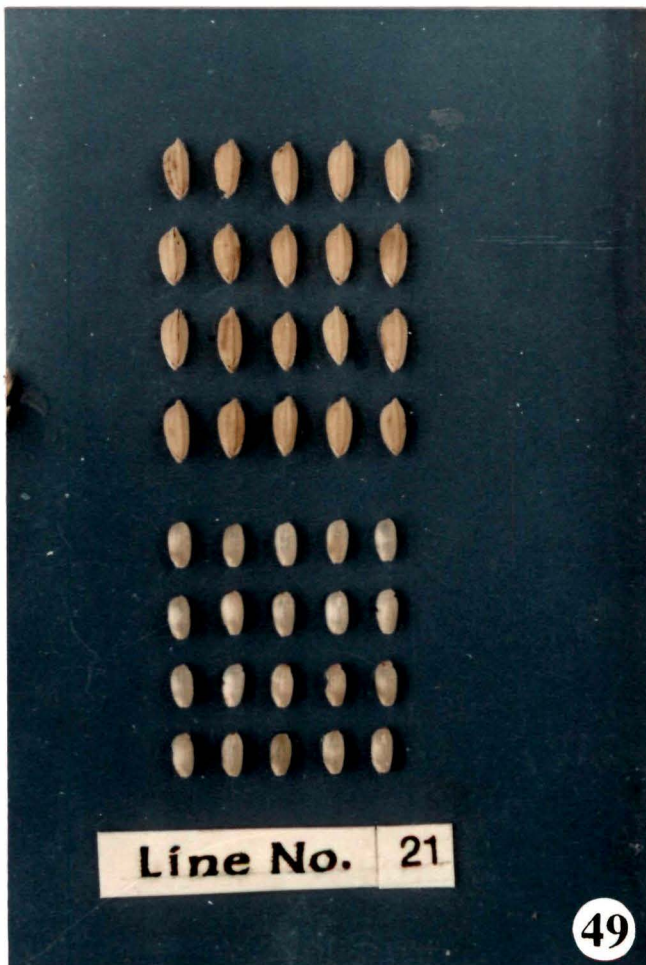


Fig. 50: Grains of double haploid (line no. 1) plant

Fig. 51: A closeup of grains of line no. 1

Fig. 52: Grains of double haploid (line no. 6) plant

Fig. 53: A closeup of grains of line no. 6

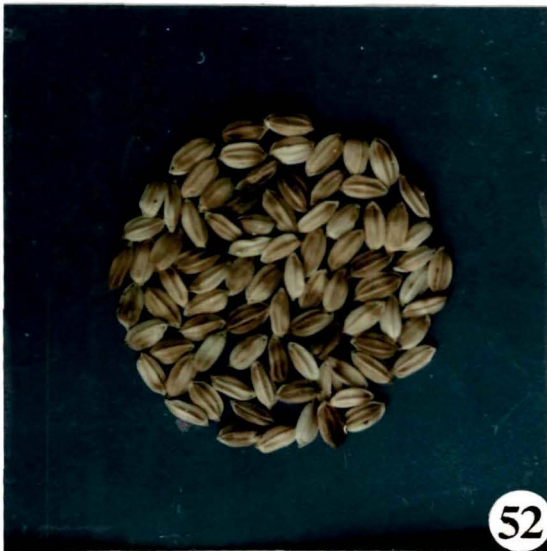


50

Line 1- IR 70 X KHONORULLO



51



52

Line 6- IR 70 X KHONORULLO



5

Fig. 54: Grains of double haploid (line no. 7) plant

Fig. 55: A closeup of grains of line no. 7

Fig. 56: Grains of double haploid (line no. 10) plant

Fig. 57: A closeup of grains of line no. 10



54

Line 7- IR 70 X KHONORULLO



55



56

Line 10- IR 70 X KHONORULLO



57

Fig. 58: Grains of double haploid (line no. 13) plants

Fig. 59: A closeup of grains of line no. 13

Fig. 60: Grains of double haploid (line no.15) plants

Fig. 61: A closeup of grains of line no. 15



58

Line 13- IR 70 X KHONORULLO



59



60

Line 15- IR 70 X KHONORULLO



61

Fig. 62: Grain of double haploid (line no. 16) plants

Fig. 63: A closeup of grains of line no. 16

Fig. 64: Grains of double haploid (line no. 19) plants

Fig. 65: A closeup of grains of line no. 19



Line 16- IR 70 X KHONORULLO



Line 19- IR 70 X KHONORULLO



Fig. 66: Grains of double haploid (line no. 21) plants

Fig. 67: A closeup of grains of line no. 21



Line 21- IR 70 X KHONORULLO



Figs. 68-69: Double haplod (DH) lines growing in pots

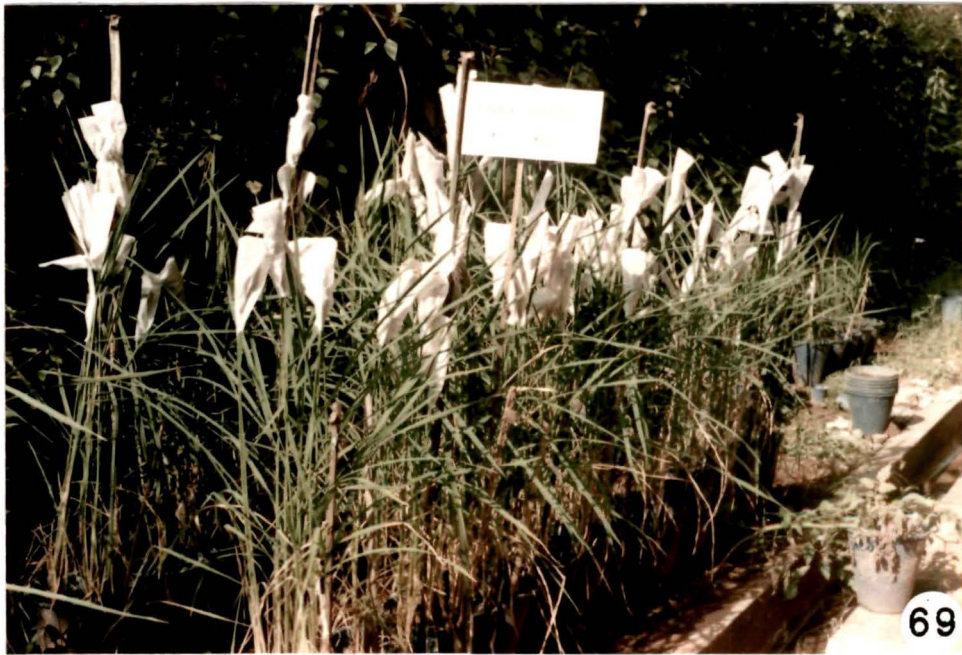
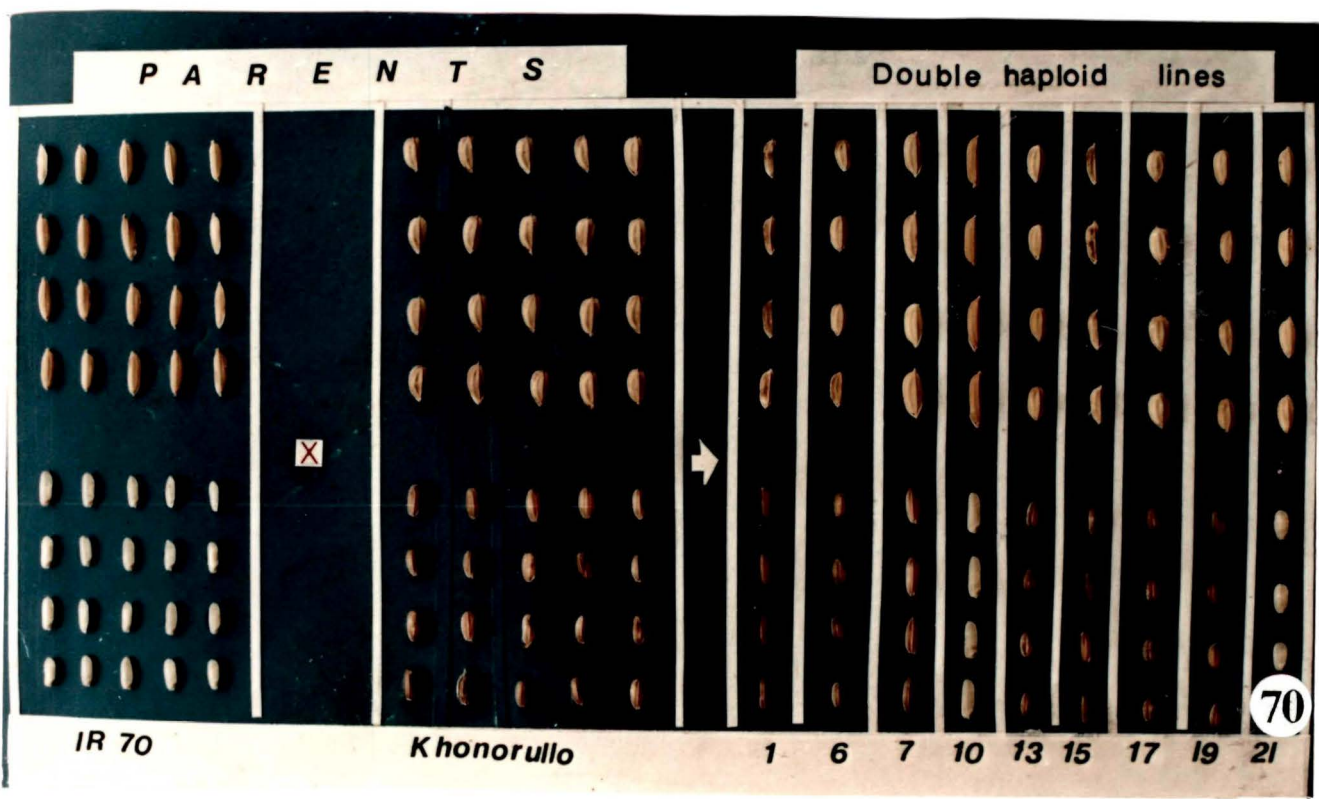


Fig. 70: Comparison of grain shape and color of different double haploid (DH) lines with their parents i.e., IR 70 and Khonorullo



P A R E N T S

Double haploid lines

IR 70

Khonorullo

1 6 7 10 13 15 17 19 21

70

3.3 Field evaluation and performance of doubled haploid (DH) lines

Seeds obtained from the double haploid plants were grown during next year for field evaluation and in pots^(Fig 68-69) and data were recorded to compare the agrobotanic characters with the parents (Table 8). Means, ranges, genetic variability, analysis of variance, simple correlation coefficient and path coefficient analysis were worked out.

3.3.1 Means and ranges for different characters

The mean values of nine characters, their ranges and SE values from the field experiment conducted in lowland are presented in Table 7, 8 and Fig 71 - 74.

The parent 2 , Khonorullo attained maximum height followed by line no. 21 whereas least plant height was observed in parent 1 (IR 70). Maximum panicle length was observed in parent 2 . Maximum and minimum grain weight per panicle were observed in DH 1 and parent 2, respectively.. Similarly maximum panicle weight was observed in parent 2 and minimum weight was observed in parent 1. Maximum seed fertility was observed in line no.10 whereas parent 1 showed minimum seed fertility. Maximum ear bearing tiller was observed in parent 2 and the least was in line no.6. 1000 grain weight was highest in line no.7 and lowest in line no 6. Maximum spikelet per

Table 7. Means and ranges for different characters

Character	Mean	Range	SE
Plant height	84.87	57.20 - 129.20	2.25
EBT	3.75	2.80 - 5.00	0.37
Penicle length	20.24	17.04 - 23.00	0.60
Spikelet/Penicle	140.64	77.00 - 203.00	7.18
Penicle weight	2.43	0.78 - 3.52	0.66
Spikelet/fertility	73.26	8.10 - 94.80	0.73
Grain weight/penicle	2.12	0.22 - 2.64	0.07
1000 grain weight	24.54	19.60 - 30.78	0.65
Grain yield	3.06	0.70 - 3.94	0.08

Table 8. Comparison of grain yield and components in DH lines and parents under field condition of medium altitude (1000m msl)

Line	Plant Height (cm)	EBT (no.)	Panicle length (cm)	Spikelet/ panicle (no.)	Panicle weight (gm)	Spikelet fertility (%)	Grain weight/ panicle (%)	1000 grain weight	Grain yield (t/ha)
DH 1	87.00	3.80	22.00	203.00	3.1	77.30	2.64	22.00	3.94
DH 6	66.80	2.80	17.04	160.00	2.74	87.50	2.60	19.60	3.27
DH 7	80.80	3.20	20.02	132.00	2.20	91.40	1.90	30.78	2.97
DH 10	78.40	3.20	21.06	77.00	2.20	94.80	2.06	30.20	3.12
DH 13	68.80	3.60	19.18	119.00	2.38	68.20	2.30	22.80	3.23
DH 15	80.00	4.00	20.16	178.00	2.32	72.42	2.20	21.64	3.15
DH 16	89.90	3.40	20.04	118.00	2.42	81.82	2.28	26.80	3.45
DH 19	95.90	4.00	20.16	133.00	2.44	82.04	2.36	22.00	3.66
DH 21	100.60	4.20	20.02	159.00	2.64	81.54	2.48	24.30	3.55
IR 70 (P ₁ - C)	57.20	5.00	20.00	123.00	0.78	8.10	0.22	25.60	0.70
Khonorullo (p ₂ - C)	129.20	4.00	23.00	145.00	3.52	60.70	2.30	24.30	2.60
General mean	84.87	3.75	20.24	140.64	2.43	7.26	2.12	24.55	3.06
SE	2.25	0.37	0.60	7.18	0.06	0.73	0.07	0.65	0.08
CD 5 %	6.43	1.05	1.72	20.53	0.17	2.10	0.19	0.86	0.23
CV (%)	5.93	21.96	6.64	11.42	5.54	2.24	6.88	5.93	5.93

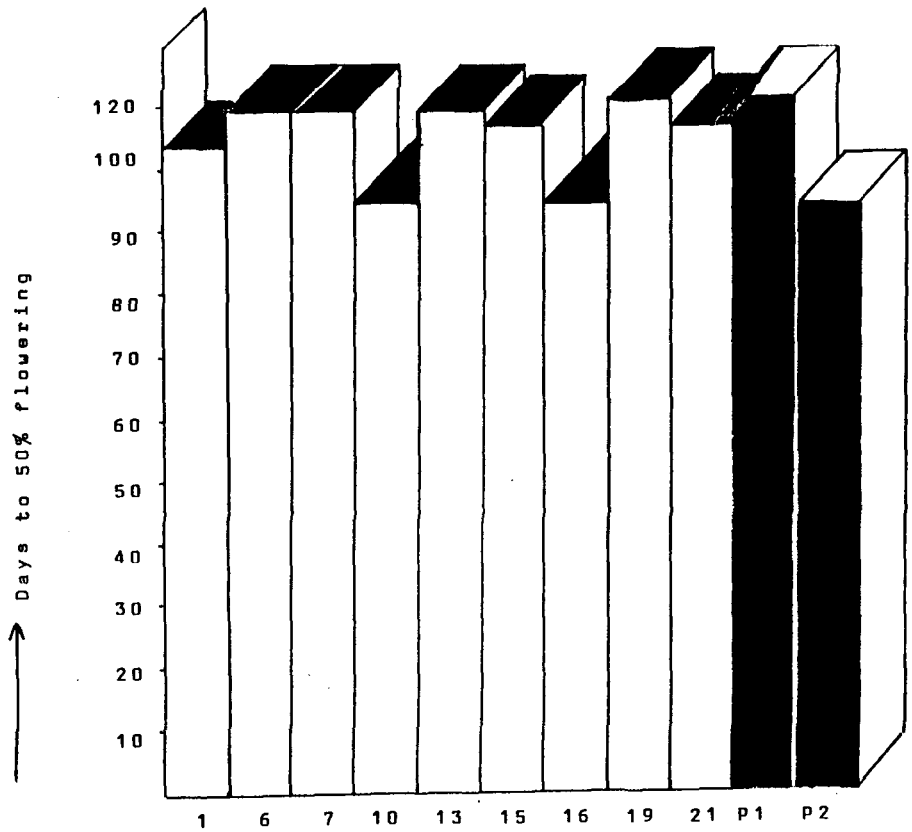


Fig. 71 → DH lines

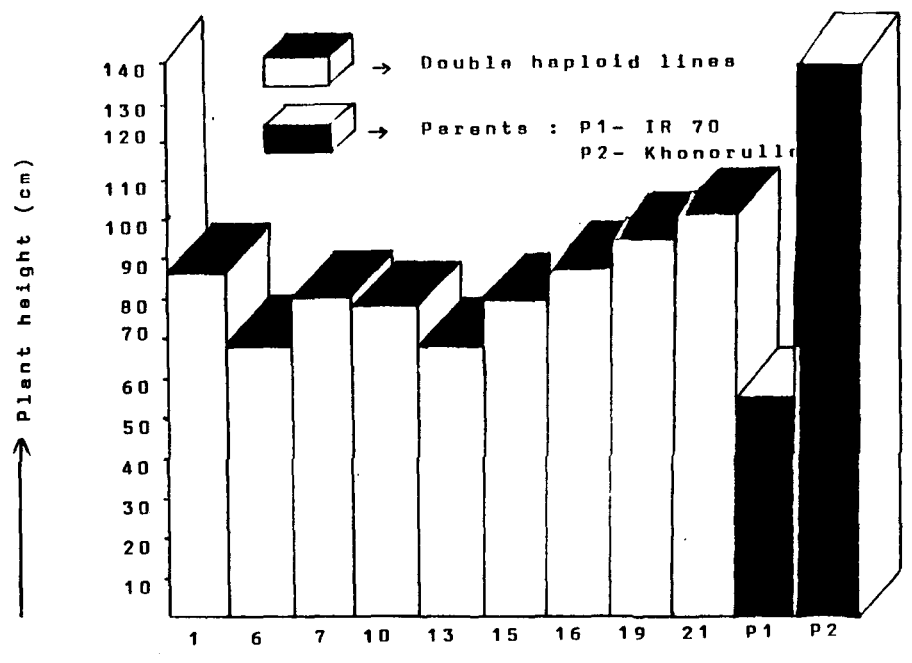


Fig. 72 → DH lines

Fig. 71 : Variation in days to 50% flowering of different lines with respect to their parents
 Fig. 72 : Variation in Plant height of different lines with respect to their parents

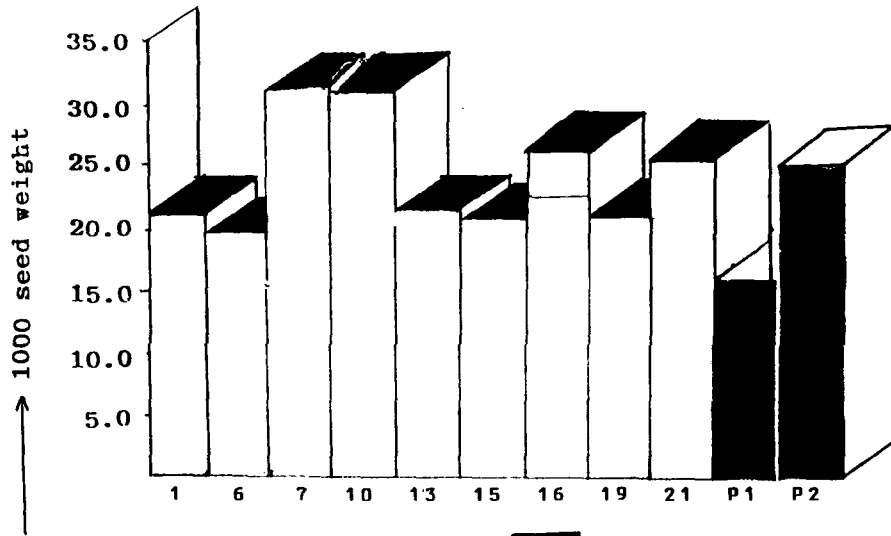


Fig. 73

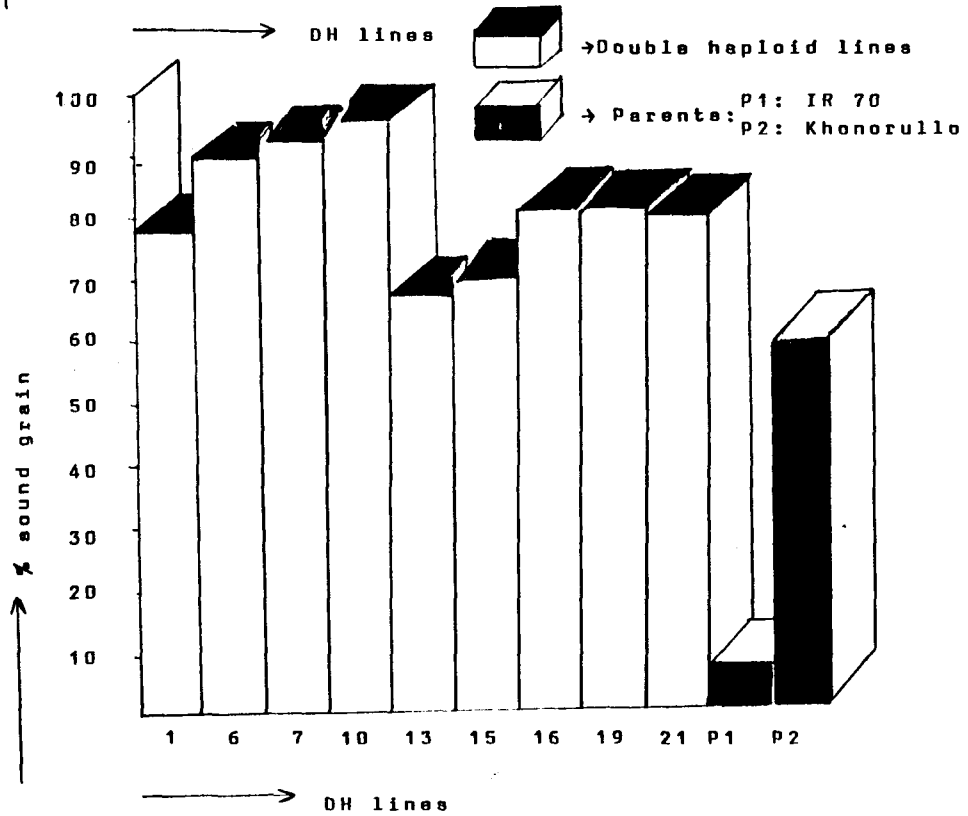


Fig. 74

Fig. 73 : Variation in 100seed weight of different lines with respect to their parents

Fig. 74 : Variation in % sound grain of different lines with respect to their parents

panicle was observed in line no.1 whereas it was minimum in line no.10. Maximum grain yield was observed in line no.1 while parent 2 recorded the lowest grain yield.

3.3.2 Genetic variability

Phenotypic and genotypic coefficient of variance (PCV & GCV) heritability estimates (h^2) (in broad sense), genetic advance (GA) and genetic advance as percentage of mean (GA%) for different characters ~~recorded~~ have been presented in Table 10

Genotypic coefficient of variation (GCV) were , in general, lower than that of phenotypic coefficient of variation (PCV) in all characters studied. Both GCV and PCV were highest for the spikelet fertility (32.54 and 32.46 % respectively) followed by grain weight /panicle (32.03 and 31.29 % respectively) and the lowest values were observed in panicle length (9.56 and 6.87 % respectively). Heritability (in broad sense) was also highest in spikelet fertility (99.53 %) followed by panicle weight (96.24 %), grain yield (95.71 %) grain weight /panicle (95.45 %) and plant height (93.65 %) whereas it was minimum in EBT (25.01 %). Spikelet /panicle exhibited maximum GA (72.15 %) while it was lowest in EBT (0.57) followed by grain weight /panicle (1.57) and panicle weight (1.61) . When the genetic advance was calculated as percentage of mean, grain weight/panicle exhibited the highest

Table 9 Genetic variability of nine characters among DH lines and parents

Character	Phenotypic Coefficient of variation (%)	Genotypic Coefficient of variation (%)	Heritability (in broad sense) (%)	Genetic Advance	Genetic Advance as percentage of mean. (%)
Plant height	23.52	22.76	93.65	45.23	53.29
EBT	25.35	12.68	25.01	0.57	15.20
Penicle length	9.56	6.87	51.67	2.42	11.96
Spikelet/penicle	26.18	23.56	80.97	72.15	51.23
Penicle weight	28.46	27.92	96.24	1.61	66.26
Spikelet/fertility	32.54	32.46	99.53	2.24	3.06
Grain weight/penicle	32.03	31.29	95.45	1.57	74.06
1000 grain weight	15.41	14.22	85.20	7.80	31.77
Grain yield	28.63	28.01	95.71	2.03	66.34

GA % (74.06 %) followed by grain yield (66.34 %) and panicle weight (66.26 %) . On the other hand, spikelet fertility showed the least GA % (3.06 %) followed by panicle length (11.96 %) .

3.3.3 Analysis of variance

Highly significant differences ($P \leq 0.01$) among the genotypes were observed for yield and all other agronomic components except in case of number of ear bearing tillers which was significant at 5 % level (Table 10).

3.3.4 Simple correlation coefficient^{ts} among some major yield contributing characters

Nine characters, namely, plant height, panicle length, grain weight/panicle, panicle weight, spikelet fertility (%), ear bearing tillers/plant, 100 seed weight, spikelet/panicle and yield/plant were subjected to analysis for simple (phenotypic) correlation to find out the extent of association among the characters. The results are presented in Table 11.

Grain yield/plant showed positive significant relationship with panicle weight ($r=0.70$) only. However, relationship of grain yield with plant height, panicle length, grain weight/panicle and spikelet/plant were positive but non-significant. The association of grain yield with spikelet fertility and 100 seed weight was negative. Significant positive

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Table 10 Analysis of variance of yield and components

Source of variation	df	Plant height	EBT	Panicle length	Spikelet/panicle	Panicle weight	Spikelet fertility	Grain weight/panicle	1000 seed weight	Grain yield
Replication	4	3.209 ^a	0.336	1.034	81.909	0.024	9.538†	0.017	5.887†	0.0009
Line	10	1890.531††	1.804†	11.479††	5745.273 [?]	2.324††	2830.470††	2.223††	63.032††	3.700††
Error	40	25.299	0.676	1.809	257.909	0.018	2.691	0.021	2.118	0.033

† Significant at 5% †† Significant at 1%

df - Degree of freedom

^a - MSS - Mean sum of squares

Table 11. Simple correlation coefficients of nine characters among DH lines under field condition

	PL	GW/P	PWT	^F SFTL	EBT	SW	SPL/P	GY/P
PH	0.35	0.15	0.16	-0.04	0.31	0.09	0.07	0.48
PL		- 0.08	0.22	0.01	0.09	0.26	0.09	0.31
GW/P			0.79**	- 0.22	0.17	- 0.64*	0.51	0.59
PWT				- 0.22	0.20	- 0.55	0.66*	0.70*
SFTL					-0.32	0.64*	- 0.37	- 0.23
EBT						- 0.37	0.16	0.27
SW							- 0.61	- 0.41
SPL/P								0.41

PH : Plant height, PL : Panicle length , GW/P : Grain weight/panicle, PWT : Panicle weight, SFTL : Spikelet fertility, EBT : Number of ear bearing tillers/plant, SW : Seed weight, SPL/P : Spikelet/panicle

$$GY/P = \text{Grain yield / plant?}$$

panicle weight ($r=0.79$), between spikelet/plant and panicle weight ($r=0.66$) and also between 100 seed weight and spikelet fertility. A negative significant correlation was observed between 100 seed weight and grain weight/plant. All other correlations were either of low magnitude or negative.

3.3.5 Direct and indirect effects of different characters on grain yield

Path analysis was carried out to know the extent of direct and indirect effects of component characters on seed yield by partitioning the correlation coefficients. The results are presented in Table 12.

In low land condition, panicle weight exerted the highest positive influence on yield followed by plant height and panicle length. The direct effects of seed weight, spikelet/panicle and ear bearing tillers on yield was in negative direction.

Table 12 DIRECT AND INDIRECT EFFECTS OF EIGHT CHARACTERS ON GRAIN YIELD UNDER LOW LAND CONDITION

	PH	PL	GW/P	PWT	FTL	EBT	SW	SLP/P	Correlation with yield
PH	<u>0.400</u>	0.053	0.003	0.088	-0.001	-0.020	-0.026	-0.013	0.48
PL	0.140	<u>0.151</u>	-0.002	0.122	0.000	-0.006	-0.080	-0.017	0.31
GW/P	0.061	-0.031	<u>0.020</u>	0.432	-0.003	-0.011	0.197	-0.090	0.59
PWT	0.064	0.034	0.016	<u>0.548</u>	-0.003	-0.013	0.170	-0.118	0.70*
FTL	-0.016	0.001	-0.004	-0.120	<u>0.015</u>	0.021	-0.196	0.065	-0.23
EBT	0.126	0.013	0.003	0.109	-0.005	<u>-0.065</u>	0.112	-0.028	0.27
SW	0.035	0.039	-0.013	-0.303	0.009	0.024	<u>-0.327</u>	0.108	0.41
SPL/P	0.030	0.014	0.010	0.363	-0.005	-0.010	0.186	<u>-0.178</u>	0.41

-0.41

Residual effect=0.42

PH : Plant height ; PL : Panicle length ; GW/p : Grain weight per panicle ; PWT : Panicle weight ;
PTL : Seed fertility ; EBT : Ear bearing tiller ; SW : 100 seed weight ; SLP/p : Spikelet per panicle

DISCUSSION

The agricultural productivity needs to be increased for securing food as a counter-measure of population growth. Breeding should contribute to the expansion of yield potential and to the improvement of the quality of products. The efficiency of breeding depends on the efficient process of broadening the genetic variability, selection, fixation and rapid propagation.

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Conventional breeding in which each process is

performed at population and individual plant level has yielded excellent results. However, recent progress in biological sciences reveals that some stages of breeding procedures can be performed more efficiently by adopting tissue, cell culture and molecular manipulation.

The present investigation on androgenesis was conducted with a view to bringing together the desirable characters of both indica and japonica rice varieties. The experimental results of the present investigation have been discussed in the following pages.

4.1. Standardization of conditions for callusing of microspores in local cultivars :

Environmental factors such as temperature, humidity, sunshine hour etc. ^{exert} ~~play~~ significant ^{influence on the physiology of donor plants which is affected in} ~~role in~~ androgenesis.

Panicles collected between 15th to 17th September (mean temperature - minimum 18.2⁰C, maximum 26.3⁰C; humidity 83 to 100 %; average sunshine hours/day - 2.50 to 4.75) showed maximum callusing. Panicles collected beyond these days did not respond so well though the temperature for cold pretreatment and culture condition remained the same. This shows that a temperature between 18.2 to 26.3⁰ C , humidity between 83 to 100 % and sunshine hours between 2.50 to 4.75 hours/day renders anthers in the best metabolic states for the

callusing through androgenesis.

Cold shock to the anthers before inoculation is known to induce better response to callusing and green plant regeneration. This beneficial effect was first reported by Nitsch and Norrel (1973) in *Datura innoxia* and by Chaleff et al. (1975) in *Oryza sativa*. The ~~time needed~~ ^{duration of} for cold pretreatment depends on the temperature used. Wang et al. (1974) demonstrated that shorter the period of treatment ~~lower~~ ^{lower} should be the temperature and vice versa. Cold shock for 10 days at 8° C was found to be the best for callusing as well as for the green plant regeneration. The microspores subjected to this temperature presumably reduce their metabolic activity which results in the accumulation of larger percentage of microspores at the most suitable stage for effecting the developmental shift required for androgenesis. Cold pretreatment prolongs the life span of the anther and weight of the anther also increases during the cold pretreatment period (Zhou and Cheng, 1983).

One of the important factors for haploid production through androgenesis, is the culturing of microspores at the mid-uninucleate stage of development (Chen, 1977). When cultured at this stage, there is a shift from the normal pathway of pollen development and after repeated mitotic divisions of the microspores, calli are formed. Thus, staging of microspores for correct identification of mid-uninucleate stage helps in

obtaining high frequency of callusing.

For staging of the microspores, acetocarmine stain did not work satisfactorily, however, Feulgen reagent stained the nucleus distinctly (Gupta et al., 1987), but it takes 2 to 5 hours and hence this technique had limited application especially when a large number of anthers are to be plated everyday. Acetic acid - iron-alum - haematoxylin stains the microspore nucleus quickly (within 2- 5 min) and the nucleus becomes very distinct (Gupta and Borthakur, 1987). The clarity of nuclear staging using this stain may be due to the use of iron -alum which acts as a mordant and haematoxylin has long been known as a stain for nucleus / DNA.

4.2. Anther Microspore culture:

Induction of callusing in japonica rice has been generally high. However, it has not been so in indica. Consequently, work on haploid production on indica rice has been slow. In addition to hormonal and other growth supplements (Guha et al., 1970; Niizeki and Ono, 1971), the concentration of nitrogen plays an important role in callusing (Chu et al., 1975 ; Song et al., 1978). In the present investigation, G₅, E₂₄ and minimal (semisolid) media were found more suitable than others. The highest frequency of callusing was recorded in E₂₄ (with G₅ salts) which contains 26.72 mM of total nitrogen as against 60

mM in MS (Murashige and Skoog, 1962). Fifty four percent callusing was observed in RCPL 1-3c in E₂₄ medium. This particular variety performed equally well in almost all media. It gave 41% , 12% and 58 % callusing in minimal, N₆ and E_{10G} media, respectively. In the minimal medium where callusing was high, the concentration of nitrogen, phosphorous, potassium and calcium was lowered to 1/3rd and micronutrients like zinc, boron, iodine, copper, molybdenum and cobalt were eliminated as suggested by Song et al.(1978). It is thus clear that low concentration of nitrogen is favourable for callus induction. This is in agreement with the results of Chu et al. (1975). They added only 3.5 mM (NH₄)₂SO₄ to the basic medium that resulted in an increased callus production. In addition, they found that NH₄⁺ at low concentration was advantageous to pollen callus formation, while high concentration inhibited the frequency of callus induction. It appears that total amount of N affects the growth of pollen callus, possibly by inhibition of enzymes of the Kreb's cycle.

In G₅ medium, Khonorullo and RCPL1-2C recorded 50 and 45.5% callusing respectively. In liquid medium, RCPL 1-1C showed highest percentage (77%) of callusing on E_{10G} medium. RCPL 1-3C showed 58% callusing in medium E_{10G} which contains 35 mM nitrogen and 47 mg/l glutamine. The addition of amino acids viz. glutamine increases the formation of embryo and green

plantlets. This is in conformity with the results of Trottier et al. (1993) in wheat where 50% embryo formation and green plant regeneration was reported.

It can, therefore, be said that B₅ and N₆ basic media are suitable for callusing of indica rice. However, small variations have been observed in many cases. Genotypic differences in anther response to callusing have already been reported (Guha - Mukherjee, 1973 ; Maheshwari et al., 1982; Thengane et al., 1994). Khonorullo responded well to almost all media (57%, 50%, 32%, and 11.7% in N₆, G₅, E₁₀^G, minimal and E₂₄, respectively) whereas in Pusa 33, anthers did not respond well in almost all the media and the frequency of callusing in both semisolid and liquid media ranged from 6.1 to 15 % only. This also indicates that genotypic difference plays a vital role in anther response to callusing. Two types of calli, (embryogenic and non-embryogenic) were observed in cultivated rice (Raghava Ram and Nabors, 1985; Thompson et al., 1986, Wang et al., 1974). Embryogenic calli are capable of differentiating into shoots and roots whereas non - embryogenic calli mostly give rise to roots. In this study, the embryogenic calli, on transfer to N₆ medium without growth regulators, proliferated and formed embryoids which developed coleoptile, scutellum and roots. These structures, in turn, gave rise to plantlets with shoots and roots.

During regeneration, it was observed that proliferating calli tend to lose the capability of regeneration as compared to the primary calli. This is evident from the fact that higher response of regeneration from the primary calli which ranged from 22 % to 50% as compared to 10% in proliferating calli. Plant growth regulators like auxins and cytokinins play crucial role in the process of androgenesis. This is in agreement with the increase in production of regenerated plant, by a factor of 10 by an early transfer of callus in maize (Barloy and Beckert, 1993). Earlier, 2,4-D was thought to be the only hormone essential for androgenesis and it was invariably added to the culture medium with or without cytokinin and/or other auxins (Niizeki and Dono, 1968; Harn, 1969; Nishi and Mitsuoka, 1969; Guha et al., 1970; Iyer and Raina, 1972; Woo and Tung, 1972; Guha-Mukharjee, 1973; Wang et al., 1974). The inhibitory effect of 2,4-D and that it can be replaced by NAA was first reported by Niizeki and Dono (1971). Afterwards it was also found out that cytokinin is essential for obtaining callus of high morphogenic potential (Chen, 1977; Chaleff and Stolarz, 1981; Lee and Chen, 1982). The concentration of hormones required may not be uniform for all the cultivars as genotypic differences have been reported (Liang, 1978; Cornejo -Martin and Primo - Millo, 1981).

Other factors that influence anther response, are

composition of the gas mixture in the culture vessel (Johansson et al.,1982) and the density of anther inoculated in the culture medium (Bajaj et al., 1977; Sundarland et al.,1981).

In the present study, it was observed that MSB was the best regenerating medium as compared to other media. MSB medium contained 0.5 mg/l NAA and 3.0 mg/l kinitin. The regeneration percentage of different varieties in MSB medium was 50 % in Khonorullo, 43 % in RCPL 1-1c and RCPL 1-2c, 33 % in RCPL 1-3c and 22 % in DR 92 and Pusa 33.

Light intensity of 2500 to 3000 lux and a continuous light for 5 days after transfer to regeneration media followed by 16 hr/8hr light/dark period was the best for regeneration.

The light conditions used for rice anther culture range from complete darkness to continuous illumination. For callus induction, inoculated anthers are typically placed in the dark (Niizeki and Dono 1968; Harn 1969; Woo and Tung 1972; Y. Chen et al., 1974). However, Nishi and Mitsuoke (1969) and Niizeki and Dono (1971) also reported callus induction under continuous light. A typical current practice is to use dark incubation for callus induction followed by 16 hour light per day at 2000-3000 lux for plant regeneration (Chu et al., 1984; Croughan and Chu, 1991).

4.3 Ploidy status of plants derived through anther culture

The ploidy of plants derived from anther or microspore culture is highly variable and in most instances diploidy predominates. In the present investigation also around 70 to 75% diploid plants were observed, 10 to 20% were haploids and rest mixaploids. Haploid to pentaploid plants are reported in *O. sativa* by Nishi and Mitsuoka (1969) and Niizeki and Ono (1971). The range of diploids in regenerated plants was 50 to 60% against 30 to 40% haploids (Ono, 1975; Mok and Woo 1976; Chen and Lin, 1976). The ~~high frequency~~ of regeneration of diploids are known to be favoured over haploids through a selection mechanism (Ono , 1978) and this is desirable for its application to breeding. The higher the frequency of diploids, the greater the efficiency. / int

The wide range of ploidy levels seen in androgenesis has been attributed to endomitosis and/or the fusion of various nuclei (Narayanaswamy and Chandy, 1971; Engvild et al., 1972; Raquin and Pilet, 1978; Engvild 1973 ; Sunderland and Dunwell, 1974). In general endomitosis would cause diploidy and tetraploidy, whereas nuclear fusion would give rise to triploids and pentaploids. Chromosomal variation and atypical mitosis leads to polyploidy and aneuploidy which are common in plant tissue culture (D'Amato, 1965; Torrey, 1967;

Murashige, 1974; Matthews and Vasil, 1976). Variation in the ploidy of plants observed in anther culture appears to be a function of the developmental stage of the anthers at the time of culture, with higher ploidy levels more prevalent in anther culture following microspore mitosis. This is expected in view of the specific timing of DNA synthesis with generative and vegetative nuclei following microspore mitosis and the fusion of nuclei at the 1c or 2c levels resulting in various ploidy levels.

4.4 Production of promising double haploid lines and their evaluation

The usefulness of *in vitro* selection during anther culture was reviewed by Nabors and Dykes (1985). The selected tolerance is likely to be useful because it is inherited and stable. Wenzel (1985) and Wenzel et al., (1987) also stressed that cereals offer an ideal system for isolated microspore culture. *In vitro* selection against limiting factors such as water stress, cold tolerance etc. is a very effective way of obtaining modified variants of already highly adapted varieties. Many researchers have confirmed that pollen-derived lines are homozygous and about 90 % of the plants produced are uniform and stable in all major traits (Oono, 1975; Kumaravadivel and Sree Rangaswamy, 1994). The rapidity with which homozygosity ^{in plants} can be

produced ^{through} in anther culture is one of the most important ^{features} attributes of this technique. In view of the totipotency of plant cells, a single cell can be considered as an intact hybrid plant, with full genetic diversity. In ^{anther} pollen culture of hybrid plants, ~~with different genotypes~~, 1 ml of medium, may be sufficient for the cultivation of thousands of plantlets. Haploid tissues are quite susceptible to change in ploidy level during cell proliferation and growth *in vitro* (Melchers and Bergman 1958). In the present investigation, homozygosity was observed in the double haploid lines as evidenced from lack of segregation within the lines. This was further confirmed by insignificant difference (CD) ^{among} within the lines. However, significant differences between the double haploid lines indicate to their origin from different recombinants ^{ions}.

With a view to transferring cold tolerance to high yielding background, IR70 , an elite IRRI line and Khonorullo, a local cold tolerant cultivar were crossed. The F₁ hybrid showed hybrid vigour and performed better than the parents. F₁ anthers of the cross IR70 x Khonorullo were cultured in four semisolid (G₅ , E₂₄ , L8 and MP2) media. Percentage of callusing ranged from 11.7 (in G₅ medium) to 33.6 % (in E₂₄ medium)

The results of this investigation show that higher frequency of callus induction and subsequent green plant

regeneration from anthers of the hybrid between two varieties could be achieved by proper combination of growth regulators in the induction medium. By and large LB and MP2 media were found to support callusing in IR lines. Green plant regeneration was obtained from calli inoculated mostly in LB medium (with 1mg/l 2,4-D and 3mg/l NAA). Highest frequency of callusing i.e., 33.6 % was observed in crossed anthers of IR70 x Khonorullo on E₂₄ medium which contained 1 mg/l 2,4-D and 0.5 mg/l IAA.

In anther culture, selection can start even in the 1st. (DH1) generation itself, because it uncovers both the genotypic and phenotypic values even of additive genes in this generation. Using single seed descent (SSD) method of breeding with 200 recombinant genotypes, one homozygous line will be found, with a probability of 68% for characters coded by 48 independent genes (Farouqhi-Wehr and Wenzel, 1990). The SSD method is comparable with haploid method, but in SSD, selection starts in F₆, whereas in DH progeny, one can successfully select at DH1. Repeated rounds of recombination in F₂ and in F₃, results in too much recombination between desired linked genes. This is the disadvantage of classical backcrossing where there is possibility of important characters to be lost, because the difference in the progeny are too small for effective selection. Haploids can also increase the efficiency for most complex breeding problems that is the transfer of quantitatively

genotypes to cultivars. In the present investigation nine out of 21 lines regenerated, showed promising characters. Seeds of these lines showed differences in agronomic characters from each other indicating their ~~the~~ origin from different recombinants.

Panicles of different lines showed mixed characters i.e., from compact (in IR70) to open (in Khonorullo). Same was the case with shape and size of the grains. It ranged from bold (like Khonorullo) to slender (like IR70). The endosperm colour ranged from dark red (Khonorullo) to white (IR70) in the parents. Among double haploid lines, DH no. 10 and 21 showed white kernel grains whereas all the other lines showed light red coloured endosperm.

In the present study, IR70 and Khonorullo and their F1 hybrids showed partial spikelet fertility (8.1, 60.7 and 62.2%, respectively) because of suboptimal temperature prevailing throughout the life cycle of the plants. In contrast, the androgenic double haploid lines recorded a significant increase in spikelet fertility which ranged from 77.3% (line 1) to 94.8% (line 10) . This was because of selection for desirable DH lines. Maximum number of grains per panicle numbering 203 was recorded in DH line 1 as compared to 123 and 145 in IR70 and Khonorullo respectively. Increase in 1000 seed weight was also observed in three DH lines (line no. 7, 10 and 16) as compared to the parents. Maximum 1000 seed weight was

recorded in line No.7 (30.8 gm.) and it ranged from 19.6 to 30.8 gm in DH lines as compared to 25.6 and 24.3 gm in the case of IR70 and Khonorullo, respectively. These results suggest that sterility barrier (for realising genetic recombinants and fixation of fertile homozygous lines) in an indica-japonica hybridization programme could be overcome through F1 anther culture technique.

4.5 Field evaluation and performance of double haploid (DH) lines

Days to 50% flowering in double haploid lines ranged from 95 to 111 days in lines 10 and 19, respectively, and it remained within the limit of both the parents which was 95 days in case of Khonorullo and 111 days in case of IR 70 (Fig. 71).

Range of plant height also remained well within the parents which was 57 cm in case of IR 70 and 129 in case of Khonorullo. Plant height ranged from 67 to 101 cm in case of double haploid lines(Fig. 72). Leaf length was reduced in most cases but in line no. 19, 37 cm long leaf was observed as compared to 32 cm in IR 70 and 27 cm in Khonorullo.

Higher number of ear bearing tillers (EBT) was observed in line nos. 15, 19 and 21 whereas others showed

reduction in number of EBT than the parents which is 5 and 4 in IR 70 and Khonorullo respectively.

Length of panicle remained within the range of parents except in line no 6 and 13 where it was reduced to 17 and 19 cm respectively . Panicle weight also remained well within the range of the parents in all lines. Yield on the other hand, increased in all the DH lines (Table 8).

There was a significant increase in 1000 seed weight in lines 7 and 10 (30.8 and 30.0 g respectively) than the parents which was 25.6 and 24.3 gm in IR 70 and Khonorullo, respectively (Fig.73).

There was also a sharp increase in total grain and percent fertility in many lines. In line no 1, there were 203 grains as compared to 123 and 145 in IR 70 and Khonorullo respectively. A record number of 94.8% fertile grains was recorded in line no 10 followed by 91.4% in line no 7 as compared to 8.1 and 60.7% in IR 70 and Khonorullo respectively. All other lines also showed increase in fertility over their parents (Fig.74).

Slender grains were observed in line no 10 which is a distinct character of IR 70. Medium type of grain was observed in line nos 1,7,15 and 21 whereas bold grains were observed in rest of the lines.

White kernel grains (like IR 70) were observed in line no 10 and 21 . Most of the other lines showed light red colour kernel except in line no 16 and 19 where deep red colour (like khonorullo) kernel was observed. Panicle shape, grain type and kernel colour of different double haploid lines, together with their parents are presented in Figs. 34 - 49.

A wide genetic base with acceptable level of productivity is essential in the crop populations under improvement. Therefore, the study of variability in crops is essential for selecting the desirable genotypes. Knowledge of the nature and magnitude of genetic variation governing the inheritance of quantitative characters like yield and its components is essential for effecting genetic improvement. Since only the genetic portion of the total variability contributes to grain yield under selection, the importance to breeders of information about the parameters of the genotype - environment complex is clear (Allard, 1960). Burton (1952) has suggested that the genetic coefficient of variation together with the heritability estimates would give the best picture of the amount of advance to be expected from selection. Yield being a complex, polygenic character is the ultimate expression of many components.

Highly significant differences were observed among the DH lines and parents for all the characters studied. The

phenotypic coefficient of variation (PCV) was higher than the genotypic coefficient of variation indicating the larger influence of environment on the expression of characters. Bhattacharjee and Mishra (1981) and Gomathinayagam et al. (1990) have made the same observations in rice genotypes. All the characters except number of ear bearing tillers/plant (EBT) showed wide variation. In the present study the level of GCV and PCV were not of high order.

The broad sense heritability estimates ranged from 25.01% in EBT/plant to 99.53% in spikelet fertility. High heritability estimate was noticed with panicle weight, grain yield, plant height, grain weight/panicle, spikelet fertility, 1000 seed weight and spikelet/panicle while panicle length had only moderate heritability (51.67%). According to Burton (1952) a character having high GCV value with high heritability would be more valuable for selection. According to Johanson et al. (1955) high heritability coupled with high genetic advance would be more useful in predicting the results and effects of selection. High heritability and genetic advance was shown only by spikelet/panicle and plant height indicating the preponderance of additive gene action (Fanse, 1957). The range of genetic advance as percentage of mean (GA%) was 3.06% (spikelet fertility) to 74.06% (grain weight/panicle). The relatively lower estimates of heritability and GA for the EBT as observed in the present study

was reported by Bhattacharjee and Mishra (1981). The higher value of heritability exhibited by plant height was in accordance with the findings of SwamyRao and Gaud (1969). High heritability coupled with high genetic gain observed in the present study was also reported by Shukla et al. (1972) and Chaudhury et al. (1973). Since the heritability (H^2) is an estimate of the heritable portion of the variation, a high H^2 value in quantitative characters was useful as they provide the base of selection on the phenotypic performance.

observed between
was shown by
Significant positive association (among DH lines) was shown by yield/plant and panicle weight. There was also positive, significant correlation between panicle weight and grain weight/panicle, between spikelets/panicle and panicle weight and between 100 seed weight and spikelet fertility. The interrelationship between 100 seed weight and grain weight/panicle was negatively significant.

When the correlations were further partitioned, maximum positive direct effect contribution was given by panicle weight followed by plant height and panicle length. Even though spikelet/panicle showed positive correlation, its direct contribution was only on the negative side. Hundred seed weight and EBT had direct negative contribution while the remaining traits were either in negative direction or of low magnitude. Except for panicle weight the indirect effect via grain

Except for panicle weight the indirect effect via grain weight/plant and spikelet/panicle all other indirect effects were also either very low or negative.

From the present study it is evident that the performance of DH lines possessing cold tolerance was quite satisfactory when compared ^{to} ~~with~~ both the parents. The DH lines showed an increase in yield to the tune of 79.23% over parent 1 (IR 70) and 22.85% over parent 2 (Khonorullo), respectively. However, multilocational testing will be needed for recommendation of these lines for cultivation in mid and low altitude areas.

SUMMARY

Anther culture-mediated breeding has now become a routine procedure used for compressing breeding cycle in crop plants in general and rice in particular. The major advantage of androgenesis is the reduction of time needed to obtain pure lines. Doubled haploid lines are completely homozygous and are easier to study the genetic effects because dominance effects are eliminated. The technique is more useful in situations where rapid generation advance is not possible because only one crop can be grown every year.

Rice grown in high altitude areas of North Eastern Hills of India gets exposed to cold at flowering/ripening phase and faces the problem of incomplete panicle exertion, asynchronous flowering and poor seed setting. This results in yield losses ranging from 20 - 50% depending on severity of cold stress. In addition, due to long crop season and severe winter, only one crop of rice can be grown each year in these areas and thus conventional breeding requires long time.

In order to use the technique of anther culture in breeding rice for these areas, we first standardized conditions for anther callusing in local varieties of rice. All together, six varieties and F₁-derived anthers (of IR70 x Khonorullo) were tested for anther callusing on 5 semisolid and 2 liquid media. RCPL1-3c was found the most responsive followed by Khonorullo, RCPL 1-2C and RCPL 1-1C. Apart from favourable climatic conditions characterized by temperature (min 17.2°C, max 26.3°C), humidity (83 - 100%), sunshine hours (3.10/day); cold pretreatment at (8°C for 10 days), inoculation of anthers at mid uninucleate stage and low concentration of nitrogen (26 to 35 mM) were the most important factors required for high frequency callus induction.

Early transfer of primary calli to regeneration media was found essential for production of green plants. In

addition, exposure of calli to continuous light for first five days after transfer to regeneration medium and then to a 16/8 hour light /dark period was found to be the best for maximum green plant regeneration. Majority of the regenerated plants were found to possess diploid chromosome number indicating doubling of chromosomes during culture.

In order to transfer cold tolerance to high yielding genetic background, anthers of F_1 plants obtained from cross, IR70 x Khonorullo; were cultured and the double haploid lines, thus obtained, were screened for desirable traits. Out of 21 DH lines, 9 were found promising and they were field evaluated for yield and yield contributing characters. Among the 9 lines, line no. 10 and 21 were white kernelled, a desirable character, coupled with high spikelet fertility (94.8 and 81.54%, respectively). Line no.1 was although red kernelled yet the highest yielder. Field evaluation at Barapani (1000m msl) showed line no.1, 19 and 21 as high yielders. Altogether, DH lines showed an increase of 79.2 and 22.85% in yield over parent 1(IR70) and parent 2 (Khonorullo), respectively.

Highly significant differences were observed among the DH lines for the agronomic characters studied. The phenotypic coefficient of variation was more than the genotypic coefficient of variation indicating a high influence of environment. High heritability estimates were recorded for several characters like

panicle weight, grain yield, plant height, grain weight/panicle, spikelet fertility, no.of spikelet/panicle and 1000 grain weight. High heritability and genetic advance, observed in plant height and no. of spikelet/panicle, suggested additive gene action. Genetic advance as percentage of mean was highest in grain weight/panicle.

Among the DH lines, yield/plant and panicle weight showed significant positive association. Characters, like panicle weight and grain weight/panicle, no.of spikelet/panicle, panicle weight, 100 seed weight and spikelet fertility showed significant positive correlation. When correlations were partitioned, maximum positive direct effect on yield was shown by panicle weight. Plant height and panicle length were the next two higher contributors. Hundred seed weight and number of ear bearing tillers contributed negatively.

Field evaluation of the DH lines showed that these lines possess cold tolerance and performed better than both the parents. Multilocational testing is being conducted at low and mid altitude areas for identifying the high yielding DH lines coupled with desirable attributes.

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