SUCCESSFUL REGENERATION AND CHARACTERIZATION OF ANTHER DERIVED RICE HYBRID PLANTS FROM *O. SATIVA* L. X *O. RUFIPOGON* GRIFF.

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Abstract: Doubled haploid regenerated through anther culture of wide cross rice hybrids is one of the potential tools to produce various useful genetic and cytogenetic stocks for molecular studies. In this study, an interspecific hybrid derived from *Oryza sativa* and *O. rufipogon* was subjected to anther culture with the aim to regenerate doubled haploid population for their characterization. A total of 21 green and 31 albino plants were obtained from 1904 anthers of *O. sativa* cv. Himali x *O. rufipogon*. Six types of androclonal variants were identified based on morphology and panicle characteristics of regenerated plants. Among 21 plants, only six were found diploid with partially fertility. Despite the sterility and preponderance of wild traits, some of the plants showed useful traits such as increased in panicle and spikelet length, reduced shattering and awning, and large size anther as in *O. rufipogon*. Two of the regenerants were superior for panicle related traits over either parent. The genotyping study using SSR markers also revealed the substantial variation among regenerants. At least three allelic variants were recorded among individual plants based on our preliminary study. The regenerated doubled haploid plants showed large amount of useful variation both at phenotype and molecular level and they can be exploited to enrich the current rice gene pool.

Key words: Anther culturability; Albino; Androclon; Calli; Meiosis; SSR marker.

Abbreviation: 2, 4-D: 2, 4-Dichlorophenoxy acetic acid; $AgNO_3$: Silver nitrate; A I & A II: anaphase I and anaphase II; BAP: 6- benzylaminopurine; cv: cultivar; IRRI: International Rice Research Institute, Manila, Philippines; KI: Kinetin; MI: metaphase I; NAA: á- naphthalene acetic acid; SSR: simple sequence repeat; var: variety.

INTRODUCTION

Tissue culture techniques, such as *in vitro* culture of anthers and embryos of hybrid, when properly integrated in distant hybridization work, provide excellent tools not only to obtain successful hybrids between species, but also to quickly stabilize the recombinants enabling rapid gene transfer across the barriers of genome incompatibility. Wide hybridization has been proved to be a valuable breeding approach for incorporation of alien genetic variation from wild relatives to enrich the cultivated rice (Brar and Khush, 2003). Anther culture, as a tool of biotechnology, is an important technique for immediate fixation of homozygosity there by reducing the breeding cycles and provide opportunity to recessive genes for full expression in hybrid rice itself. Rice cultivars developed using anther culture possessed traits such as earliness, increased grain yield, resistant to biotic and abiotic stresses and superior quality (Zhang, 1992; Miah et al., 1996). Doubled haploid (DH) derived from anther culture of wide cross rice hybrids is one of the potential tools to produce various useful genetic and cytogenetic stocks in molecular studies. Rout and Sarma (1990) noted that it is possible to recover *indica* type recombinant DHs among the anther derived plants with one or two traits introgressed from *O. rufipogon*. Such DH lines permit replicated testing of phenotypes and also facilitate distribution of identical DH population to many different researchers for gene mapping study. Although anther culture has been well integrated into rice breeding programs, especially in China, there still remain problems to be realized it's full potential in *indica* rice and in wide hybridization programme.

High frequency of callus induction and green plant regeneration are prerequisites for the utilization of anther culture technique in rice breeding programs. Most of the tested genotypes however, produced very high proportion of albino plants, which significantly limits the use of this technique (Chen et al., 1991). Beside genotype specificity, many factors such as composition of culture media, pretreatment of panicles, anther condition, growing conditions of the donor plants, and development stage of microspores also significantly effect the efficacy of anther culture of rice (Yamada et al., 1967; Chaleff and Stolarz, 1981; Laxmi and Reddy, 1997; Pande and Bhojwani, 1999; Sharmin and Bari, 2001). Among various factors associated with anther culturability, the most important one is the genotypic

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difference. Many researchers found that different rice species, subspecies, or varieties behaved quite differently in response to anther culture. Therefore, this attempt was carried out to regenerate doubled haploid from interspecific path and to characterize the regenerants at morphology and molecular level to facilitate the establishment of doubled haploid population for mapping and tagging of various useful alien genes in future.

MATERIALS AND METHODS

Interspecific hybrid seeds were produced by pollinating *O. sativa* cv. Himali with the pollen of *O. rufipogon* (NPGR Acc. 11057) in 2004 and grown during rice growing season of 2005 in plastic buckets filled with sterilized soil in glasshouse of Biotechnology Unit, Khumaltar, Lalitpur, Nepal. Plants were fertilized with Nitrogen:Phosphorus:Potash at the rate of 90:40:30 kg/ha, respectively. All possible plant protection measures were adopted to maintain the plants as healthy as possible. Boots containing anthers with mid to late uninucleate stages were harvested based on cytological test (Fig. 1) and anther position in spikelet. Lower portion of harvested boots were cleaned with running tap water and partly sterilized by 70% ethanol and cold pretreated at 8±2^{0}C. After seven days of pre-treatment, spikelets from middle portion of boot were collected in a beaker containing 100 ml distilled water. The sterilization was performed by immersing boots in 70% ethanol for one minute and 0.1% mercuric chloride solution for 20 minutes providing occasional shaking. The mercuric chloride solution was drained out and the materials were thoroughly washed three times with sterile distilled water. The sterile spikelets were transferred on several layers of sterile muslin cloth in petriplate. Anthers were excised by holding spikelets cluster with a sterile forceps on one hand and cut was provided near the basal end of the florets below anthers with the help of sterile scissors in the other hand. The cut florets were collected in a sterile petridish lined with two to three layers of filter paper. In each petridish, 90-110 anthers were inoculated uniformly over the surface of the callus induction medium in petridishes by grasping cut floret as open end down and tapping it on petridish rim using sterile forceps (Fig. 2 & 3). After inoculation, the petridishes were sealed with parafilm and kept in dark chamber at 26±1^{0}C with relative humidity around 60 per cent.

Callus induction medium was prepared supplying N6 mineral salts (Chu, 1978) + N6 vitamins 2 mg/l each + myoinositol 100 mg/l + 2,4-D 2 mg/l + KI 0.5 mg/l + $AgNO_3$ 10 mg/l + maltose 50 gm/l. After two to three subcultures in callusing medium followed by regeneration was performed in ½ strength MS medium supplemented with sucrose 20 gm/l, NAA 1 mg/l, BAP 2 mg/l, KI 0.5 mg/l. The pH of the medium was adjusted to 5.8 and solidified with 0.7 per cent agar. Embryogenic calli of 2-3 mm in size were transferred to culture tube containing regeneration medium (Fig. 4 & 5) which were maintained at 26±1^{0}C under 16/8 hours light/dark period. Well developed plants with profuse roots were assigned numerical numbers as 1, 2, 3 and so on and grown in glasshouse. Green plants obtained from Himali/*O. rufipogon* were characterized at phenotype and molecular level. The ploidy status of regenerated plants was confirmed by analysis of meiotic behaviour in pollen mother cells (PMCs) at M I, A I and A II and was also further verified using various morphological attributes. Pollen and spikelet fertility were also determined as method described by Virmani et al., 1997. Nine microsatellite markers viz. RM 5, RM 48, RM 164, RM 222, RM 223, RM 226, RM 234, RM 247, and RM 257 were selected to screen these regenerants. Total DNA was extracted from young leaves of 35 days old seedlings using CTAB method (Murray and

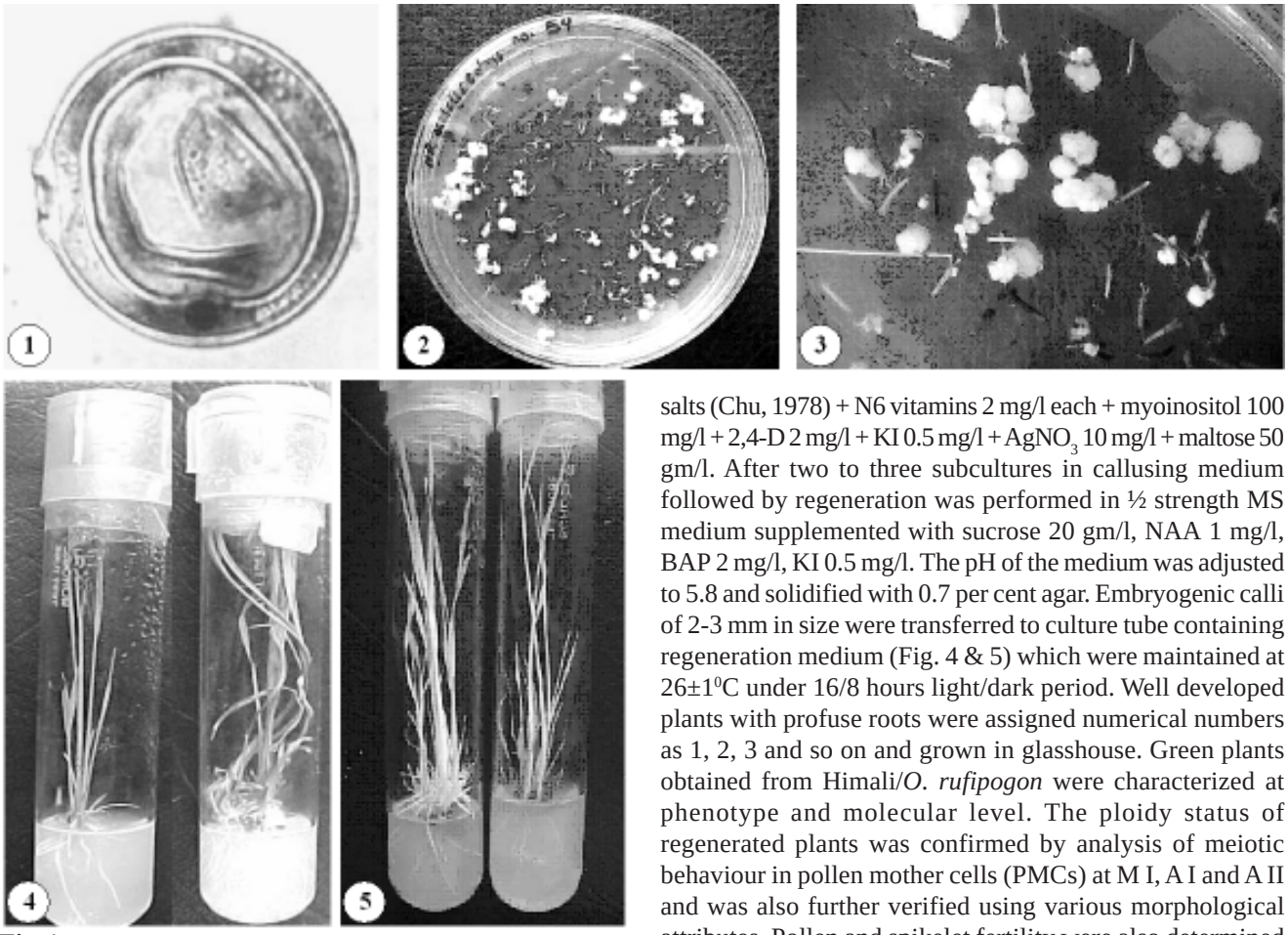

Fig. 1: Mid-uninucleate microspore- a large vacuole is formed, nucleus located at one end of the microspore. Figure 2-5. Androgenesis in interspecific (*O. sativa* var. Himali/*O. rufipogon*) rice anther culture; **Fig. 2:** Calli induced from anther culture; **Fig. 3:** Proliferating embryogenic calli; **Fig. 4:** Regenerated green plants with well developed shoot and root; **Fig. 5:** Albino plants.

Thompson, 1980). PCR was performed in 10 µl reaction mixture following IRRI based Gene Array and Molecular Marker Applications Laboratory's protocol. Each reaction mixture consist of following reagents: PCR amplification was done in a Programmable Thermal Controller (MJ Research, INC, Waltham, MA, USA) with the following settings: 94^{0}C for 5 minutes followed by 35 cycles of 94^{0}C for 1 minute, 55^{0}C for 1 minute and 72^{0}C for 2 minutes and finally 72^{0}C for 7 minutes then hold at 4-^{0}C for storage. PCR products were resolved in 3 per cent agarose gel. Gels were stained with ethidium bromide, and photographed on UV-transilluminator. Photographs were used to score and compare the bands of respective regenerants for further characterization.

Observation on response to callus induction was carried out during 60-80 days after plating considering that each callus piece was originated from a single anther. The frequencies of callus induction and regeneration were estimated as follows: Callus induction frequency (%) = (number of anther producing calli/number of anthers plated) x 100; green or albino plant frequency (%) = (number of green or albino plants recovered/ number of calli cultured) x 100.

RESULTS AND DISCUSSION

A total of 1904 anthers from rice interspecific hybrid between *O. sativa* var. Himali and *O. rufipogon* were cultured (Table 1.). The response of hybrid anther to callus induction was 8.3 per cent. One hundred and fifty calli were transferred for regeneration. A total of 21 green plants (14%) and 31 albino plants (20.67%) were obtained. The present anther culture results were similar to the report of Rout and Sarma (1990) for callus induction and regeneration.

Six types of androclonal variants were observed based on morphology and panicle characteristics. Among 21 green plants, only six were found partially fertile and diploid (Fig. 6). Fifteen plants were found to be haploid (Fig. 7) in which meiotic behaviour were largely abnormal particularly bridges and laggards at A I and A II (Table 2; Fig. 9, 10 & 11). Morphologically, haploid plants were small in size without auricle and ligules and were completely sterile (Fig. 7). Despite the sterility and preponderance of wild traits, some of the plants showed useful traits such as increased in panicle and spikelet length, reduced shattering and awning, and large size of anther as in *O. rufipogon* (Table 2 and Fig. 8). Two of the regenerants were superior for panicle related traits over either parent. Such high sterility was also reported in anther culture derived doubled haploid lines from *O. sativa*/*O. glaberrima* and *O. sativa*/*O.rufipogon* (Rout and Sarma, 1990; Brar and Khush, 2003). The genotyping study using SSR markers also revealed substantial variations among regenerants (Fig. 12). This study also confirmed the results

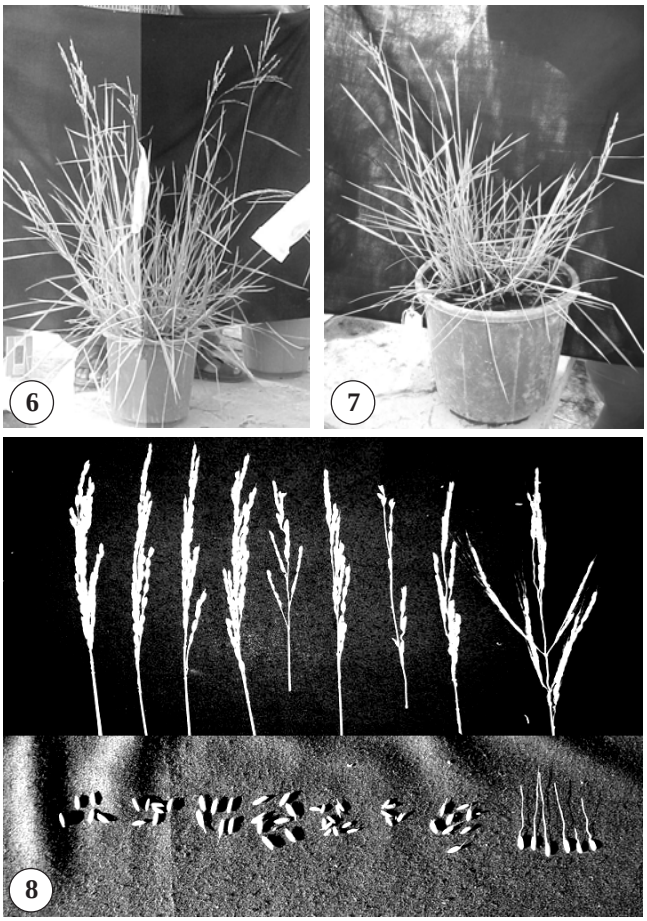

Fig. 6-8: Regenerants derived from anthers of interspecific hybrid between *O. sativa* var. Himali and *O. rufipogon*: **Fig. 6:** Doubled haploid plant with partial fertility; **Fig. 7:** Haploid plants with completely sterility; **Fig. 8:** Different types of androclonal variants for panicle and spikelet related traits.

of others that the anther culture is an effective method not only to develop rice inbred varieties but also equally effective to create the novel genes. For example, in this study regenerants number (#) 2, 4, 5, 7, 8, 9, 10 and 11 showed new alleles that are absent in either parents for RM 223 (Fig. 12). At least three allelic variants were recorded among individual plants based on our preliminary study. However, to resolve the actual polymorphism and relationship among regenerants more number of primer pairs need to be tested.

SUMMARY

The regenerated doubled haploid plants showed large amount of useful variations both at phenotype and molecular level and they can be exploited as genetic and cytogenetic stock for enriching the rice gene pool. Sterile plants can be utilized in the hybrid rice breeding programme.

Table 1: Frequency of regeneration from anther culture of rice interspecific hybrid between *O. sativa* var. Himali and *O. rufipogon.*

Frequency	Anther		Calli transferred for regeneration	Regeneration			
	plated	response		Albino	Green	Haploid	Doubled haploid
Number	1904	158	150	31	21	15	6
%	-	8.30	-	20.67	14.00	71.43	28.57

Table 2: Morphological and cytological characterization of anther culture derived regenerants of Himali/*O. rufipogon*.

Androclonal variants	Plant no. belong to specific class	Descriptive characters	Meiosis*	Fertility status
Class 1	4,6,9,10,12 and 21	intermediate growth habit, awnless, large panicle and anther, white stigma, fine and long grain with shattering, straw grain color and compact panicle	abnormal mostly laggards and bridges at A I and A II	completely sterile
Class 2	16, 17 and 19	wild type growth habit, short and partly awned, large panicle and anther, white stigma, long slender grain with straw color, compact panicle, and no shattering	normal (12II) at M I with minor irregularities such as laggards at A I	pollen fertility: 15% spikelet fertility: 25%
Class 3	1, 3 and 5	wild type growth habit, awnless, purple stigma, fine and long slender grain with brown color, small anther and compact panicle, and no shattering	normal (12II) at M I with minor irregularities such as laggards at A I	pollen fertility: 27% spikelet fertility: 30%
Class 4	2, 7, 8, 13 and 15	normal growth habit, short and partly awned, purple stigma, fine and long grain with straw color, large anther, open type of panicle with shattering	abnormal mostly laggards and bridges at A I	completely sterile
Class 5	11 and 18	plant type dwarf, growth habit like female parent, white stigma, small panicle with fine long spikelets, open type panicle, and no shattering	abnormal mostly laggards and bridges at A I	completely sterile
Class 6	14 and 20	tall, vigorous, intermediate in growth habit, awnless, purple stigma with minute anthers, partially exerted panicle with degenerated spikelets	abnormal mostly laggards and bridges at A I	completely sterile

* 50 Pollen Mother Cells (PMCs) were observed in each stages of meiosis.

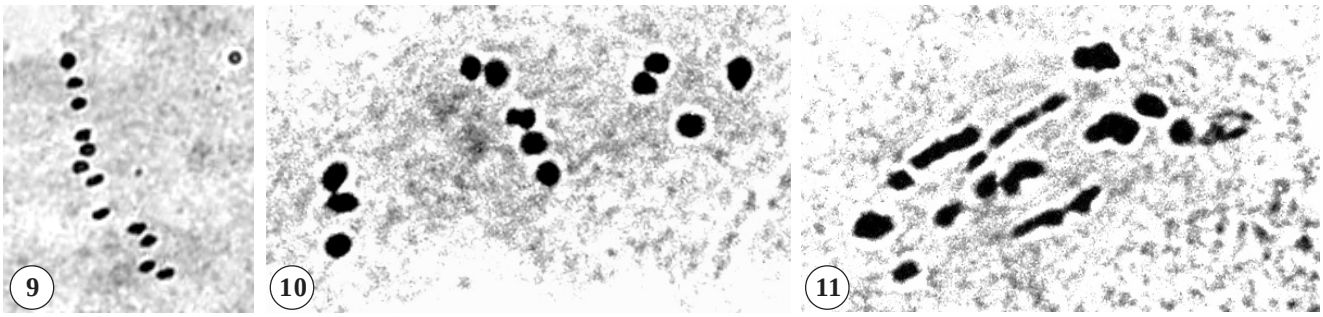

Fig. 9-11: Meiosis in anther derived regenerated plants: **Fig. 9:** Normal meiosis in doubled haploid plant with 12 II at M I; **Fig. 10:** Abnormality in cell division with five laggard chromosomes in the center of the cell plate at A I (cells prepared from regenerant No. 21); **Fig. 11:** Bridges during cell division at A I from regenerant No. 15, (cells with laggards and bridges indicated abnormal meiosis and plant with such abnormality during meiosis is completely sterile *i.e.* problems in cell division in haploid cell).

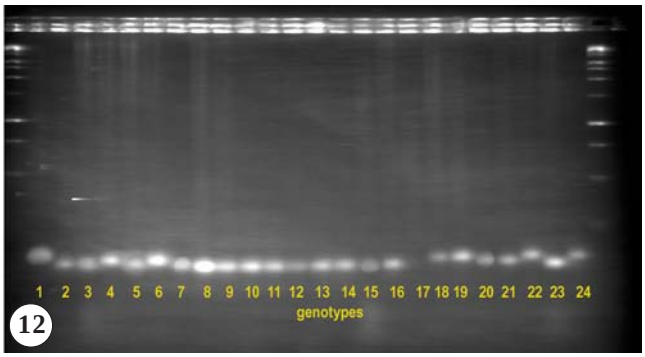

Figure 12: SSR banding pattern in 21 regenerants detected by RM 223 primer pair (from left to right): ladder; 1. *O. sativa* var. Himali (Female parent); 2. *O. rufipogon* (Male parent); 3. *O. rufipogon* (Population); 4-24. Regenerants; and ladder.

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