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

A Thesis Submitted for the Degree of

DOCTOR OF PHILOSOPHY

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We certify that the thesis entitled " Androgenic doubled haploids in rice : *in vitro* induction, plant regeneration and evaluation" submitted by Mr. Rabindra Nath Bhuyan for the degree of Doctor of Philosophy in Botany of the North - Eastern Hill University, Shillong embodies the record of original investigation carried out by him under our supervision. He has been duly registered and the thesis presented is worthy for being considered for the award of Ph.D. degree. The work has not been submitted for any degree of any other university.

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(RABINDRA NATH BHUYAN)

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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 tones. 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 tones 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 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; Nikata 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 a gamete 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 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; Qo and Chen, 1983a).

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 (Zhau, 1983). Qu 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 lead 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 graminae, a high sucrose concentration has been found to be beneficial for plant production through anther culture (Clapham,1973; Ouyang et al., 1973; Oono 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, 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 Oono, 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 Oono,(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 Oono, 1968; Harn, 1969; Woo and Tung, 1972; Guha - Mukharjee, 1973) to continuous illumination (Nishi and Mitsuoka, 1969; Niizeki and Oono, 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 red kerneled, 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 double haploid lines were evaluated for agronomic characters under medium altitude (950 m. msl) vallyland condition and promising ones with high yield and white kernel colour were identified for further evaluation.