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# Effect of Cold Pretreatment on Doubled Haploid Production from Mutants of Aromatic Rice (*Oryza sativa* L.) Landraces

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## ABSTRACT

In the present study effect of cold temperature pretreatment duration (4 and 8 days) was examined for generating doubled haploids using anther culture on mutants obtained through *in vitro* mutagenesis of four *indica* aromatic rice landraces. Anthers were cultured on N6 medium supplemented with 2,4-D (2.0 mg/l), BAP (0.5mg/l) and maltose (30 g/l), incubated in dark at 25 ± 2 °C for callus induction. Calli of 2 mm diameter were transferred to half MS medium supplemented with BAP (2.0 mg/l), NAA (0.25 mg/l), Kinetin (0.5 mg/l) and sucrose (30 g/l ) for shoot regeneration with a photoperiod of 12 h and 2000 lux light intensity at 25 ± 2 °C. Cold temperature pretreatment of panicles at 10°C for 4 days produced green shoots. Out of 8 mutants evaluated, Gangabali mutants were found to be responsive to green plant regeneration. Fertile diploids (57.14%) and sterile haploids (42.86%) were recognized on the basis of morpho-agronomic characters

**Keywords** Doubled haploids, Anther culture, Mutants, Callus induction, Kinetin.

## INTRODUCTION

DH technology is the method to obtain genetically pure inbred lines in one step within 1to 2 years, depending on the crop (Rudolf-Pilih *et al.* 2019). The best examples of successful use of DH technology in breeding are reported from anther or microspore culture (Tripathy *et al.* 2018). DH produced through androgenesis can shorten breeding cycle and fix agronomic characters in homozygous state at an early generation compared to conventional breeding which requires at least 6-7 generations of selfing to achieve homozygosity (Mishra and Rao 2016). *In vitro* anther culture for obtaining DH plants has been established as an important biotechnological tool in plant breeding as this technology can be used for simplifying

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systems for mutagenesis, facilitating introgression and supporting basic research in biochemical, physiological, genomic and phenomic studies (Samantaray *et al*. 2021). In this technology the pollen grains are forced to switch from their normal pollen development pathway at middle to late uninucleate stage towards an embryogenic route producing DH plants by reprogramming the gametophytic pathway into sporophytic development (Mayakaduwa and Silva 2017). The change of developmental pathway is achieved by applying stress, mostly cold treatment (Kaushal *et al.* 2014).

DH method for crop improvement is extensively used in *japonica* rice but in *indica* rice its application is limited due to their recalcitrant nature causing early anther necrosis, poor callus induction, proliferation, extremely low green plant regeneration and the most limiting factor being formation of albino plants during regeneration (Silva 2010). Albino plants are produced due to large scale deletions and rearrangements in the plastid genome resulting in development of incomplete membrane structures and different blockages in them. Albino plants contain deleted forms of plastid genome, devoid of 23S and 16S r RNA.

For androgenesis in rice N6 medium which was formulated to increase the frequency of callus induction and maltose as carbon source is preferred over MS medium and sucrose respectively (Rout *et al.* 2016). Auxins NAA and 2,4-D ( 2-3 mg/l) is suitable for callus induction whereas MS (Murashige and Skoog 1962) medium with cytokinin BAP ( 1-3 mg/l), NAA (0.5-1mg/l), Kinetin (0.5-1mg/l) is used for shoot regeneration (Naik *et al*. 2017). Pre treatment method is effective for rice anther culture in which panicles are subjected to cold shock prior to *in vitro* culture where temperature ranges from 4-12 °C for duration of 5-30 days depending upon the genotype (Sakina *et al.* 2020). Pretreatment at 10 °C for 7-9 days was considered optimum for the *indica* cultivars (Mishra *et al*. 2013 and Naik *et al*. 2017).

DH system is useful in inducing and fixing mutations by enhancing mutation induction in the plant materials. Selection efficiency to fix and express desirable recessive traits introduced through mutation is possible through DH system (Mishra and Rao 2016). Mutagenic lines subjected to anther culture resulted in stable mutants with higher and earlier flowering than parents. This study was done to fix the agronomic characters by generating homozygous lines through DH technology in the desirable $M_2$ mutants of aromatic rice landraces belonging to *aus* group of indica sub-species, generated through *in vitro* mutagenesis after optimizing plant growth regulators for callus induction and shoot regeneration previously (Das *et al.* 2021).

## MATERIALS AND METHODS

This study was conducted at ICAR- National Rice Research Institute, Cuttack, Odisha, India during November, 2018 to December, 2020. Seeds of 8 $M_2$ mutant lines generated through *in vitro* mutagenesis from four aromatic rice landraces (3 Basumati mutants, 3 Gangabali mutants, 1Kalikati mutant and 1 Karpurajeera mutant) were grown in pots maintained at the net house facility of National Rice Research Institute.

### Collection and cold pretreatment

Boots were harvested from healthy plants at boot emergence stage (4-6 inches distance between flag leaf and penultimate leaf), collected during early morning hours from primary plant tillers. Harvested boots were wiped 2-3 times using muslin cloth moistened with 70% ethanol, wrapped in aluminium foil enclosed in polythene bags to prevent desiccation. Wrapped boots were incubated at 10 °C for 4 and 8 days. The development stage of microspores at early to mid uninucleate was determined before cold pretreatment (Fig. 1).

### Culture media preparation

N6 medium supplemented with 2,4-D (2.0 mg/l), BAP (0.25 mg/l), Kinetin (0.1 mg/l), 3% maltose, 100 mg/l myo-inositol and 0.8% agar poured into each culture tubes (50 ml), autoclaved and used for callus induction. For shoot regeneration-Half strength MS medium containing BAP (2.0 mg/l), NAA (0.25 mg/l) and Kinetin (0.5 mg/l) along with 3% sucrose, 100 mg/l myo-inositol and 0.8% agar was used. Calli of 2 mm diameter were inoculated and incubated at 25 ± 2°C

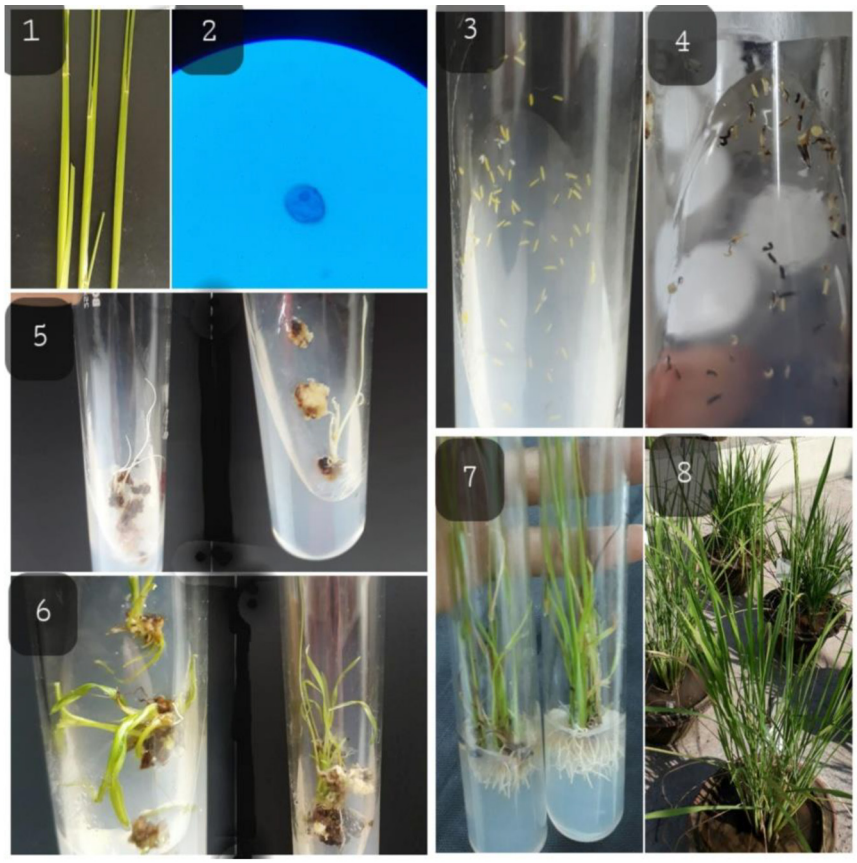


**Fig. 1**. Doubled haploids from mutants of *indica* aromatic rice landraces developed by anther culture. 1. Boots; 2. Uninucleate microspore; 3. Anther inoculation; 4. Callus induction; 5. Albino shoots; 6. Green shoots ; 7. Plantlets; 8. DH plants.

with a photoperiod of 12 h at 2000 lux light intensity. For root initiation, MS medium supplemented with 2mg/l NAA, 0.5mg/l Kn and 5% sucrose was used.

**Surface sterilization and inoculation**

Cold pretreated boots were surface sterilized with 70% ethanol for 4 minutes followed by sodium hypochlorite (4%) solution for 2 minutes and then rinsed 2-3 times with sterile water, 25 to 30 boots were used per treatment. The anthers were removed by cutting off the base of the florets by scissors. These florets were tapped on the top of the culture tubes for anthers (50-60) to fall on the surface of the media. The culture tubes were incubated in dark at 25±2°C. Data on callus induction frequency was taken after 4-6 weeks of inoculation.

**Observations and statistical analysis**

Per treatment 20 culture tubes (40-50 anthers each) were used for callus induction in case of Basumati and Kalikati. 30 and 50 culture tubes (50-60 anthers each) were used for Karpurajeera and Gangabali respectively for each treatment. For shoot regeneration 15 culture tubes (2-3 calli each) for each treatment were used. Each experiment was repeated two times. Observations on number of calli initiated and shoots regenerated (green and albino) were recorded and Callus induction frequency (CIF) was calculated using the following formula:

$$\text{Callus Induction Frequency (\%)} = \frac{\text{Number of seeds production calli}}{\text{Number of seeds inoculated}} \times 100$$

Data was subjected to analysis of variance for completely Randomized Design using excel sheet. The means were separated by Duncan's multiple range test (DMRT), least significant difference ($p<0.05$) using MSTATC.

## RESULTS AND DISCUSSION

### Effect of cold pretreatment on callus induction

The mutant lines showed statistically significant ($p<0.05$) difference in callus induction frequency. Callus induced from anthers of the boots undergone pretreatment of 4 days was found to be more than 8 days pretreatment at 10°C. High callus induction was observed at 2 days of pretreatment in *indica* rice hybrids than at 8 - 10 days duration at same temperature (10°C) (Rout *et al*. 2016 and Naik *et al.* 2017). The extended durations of cold pretreatment was found to be inhibitory for callus induction which is in agreement with earlier studies (Mishra *et al.* 2016). Highest CIF at pretreatment of 4 days was found to be 15.85 % for Kalikati mutant line (KML-3) followed by 14.36 % for Basumati mutant ( BML-1), least CIF (2.17%) was noted in Gangabali mutant (GML-8) (Table 1). CIF from the anthers undergone 8 days pretreatment was markedly low, highest being 3.23% (BML-1) and least was 0.43% (GML-8). These observations are close to the findings of Sen *et al.* (2011) who reported 0-5.06% callus induction frequency in four indica hybrids. As cold pretreatment duration had a significant effect on the CIF %, it can be further optimized for increasing callus induction by decreasing the duration or the temperature of cold pretreatment in these genotypes (Kaushal *et al.* 2014). Callus induction is genotype dependent character and *indica* aromatic rice landraces are known to possess the inherent recalcitrance towards *in vitro* culture, associated with early anther necrosis, poor callus induction and proliferation (Das *et al.* 2021). Another factor responsible for low callus induction is also attributed to the source of anthers. Anthers collected from pot plants placed in net house are considered to be less responsive to callus induction than the anthers collected from the plants grown in the fields. As genotype and nutrient composition both determine callus induction frequency, N6 medium used for callus induction may not be equally effective for callusing in all the genotypes. Liquid N6 medium with modified nitrogen concentrations can be used for increasing callus induction in these genotypes as improved N6 medium with modified concentrations of nitrogen ($NH_4^+$ and $NO_3^-$) was found effective for higher callus induction in the recalcitrant genotypes. Liquid N6 medium increased callus induction in rice hybrids involving aromatic rice whereas, Linsmaier and Skoog (LS) medium is effective in increasing callus induction in indica rice (Medhabati *et al.* 2014 and Premvaranon *et al.* 2011).

**Table 1.** Callus induction frequency from anthers of mutant lines studied. In Duncan's multiple range comparison test ($p<0.05$) means sharing same letter in a column are not significantly different. *20- 50 replicates as required per treatment; repeated twice.

| Mutant lines | Callus Induction Frequency (%) (mean ± SE)* Pretreatment at 10 °C | |
|---|---|---|
| | 4 Days | 8 Days |
| BML-1 | 14.36±0.15b | 3.23±0.13a |
| BML-9 | 9.72±0.11c | 1.77 ± 0.12b |
| BML-10 | 9.32±0.10c | 1.86±0.09b |
| GML-1 | 2.48±0.09d | 0.49±0.10d |
| GML-6 | 2.91±0.11d | 0.57±0.10d |
| GML-8 | 2.17±0.10d | 0.43±0.08d |
| KML-3 | 15.85±0.19a | 3.10±0.20a |
| PML-1 | 3.07±0.13d | 0.88±0.11c |
| LSD ($p<0.05$) | 1.06 | 0.72 |

### Effect of cold pretreatment on shoot regeneration

Both green shoot and albino was observed in the calli induced from the anthers undergone pretreatment for 4 days while no green shoots were observed in case of 8 days pretreatment (Table 2). The frequency of albino plants increased with increase in the pretreatment duration, (Herath *et al.* 2009). Green shoots (7) were observed only in case of Gangabali mutant lines whereas other mutant lines generated albino plantlets. Several factors including pre-treatment, culture medium and stage of the pollen affect the frequency of albinos (Mishra *et al.* 2013). Pre treatment of the anthers is the most important factor deciding the frequency of albino plants during regeneration. Cold pretreatment shock blocks gametophytic development and ensures continuous division of microspores into forming a sporophytic callus which causes instability in microspores development and results in loss of chlorophyll in regenerated plants (albino) during anther culture

**Table 2.** Response of shoot regeneration to cold pretreatment by the mutant lines studied.

| Mutant line | Days of cold pretreatment | No. of anthers plated | No. of anthers forming | No. of green shoots | No. of albino shoots |
|---|---|---|---|---|---|
| BML-1 | 4 | 906 | 88 | - | 2 |
| | 8 | 900 | 16 | - | - |
| BML-9 | 4 | 897 | 84 | - | - |
| | 8 | 909 | 17 | - | - |
| BML-10 | 4 | 1002 | 140 | - | 8 |
| | 8 | 906 | 29 | - | - |
| GML-1 | 4 | 3021 | 73 | 1 | 2 |
| | 8 | 3009 | 15 | - | 1 |
| GML-6 | 4 | 3024 | 88 | 5 | 6 |
| | 8 | 3006 | 17 | - | - |
| GML-8 | 4 | 2996 | 63 | 1 | 3 |
| | 8 | 3003 | 13 | - | - |
| KML-3 | 4 | 909 | 140 | - | 13 |
| | 8 | 903 | 28 | - | 5 |
| PML-1 | 4 | 1512 | 45 | - | 4 |
| | 8 | 1503 | 13 | - | - |

(Khatun *et al.* 2012). Albinos are produced mainly due to large scale deletions and rearrangements in the plastid genome resulting in development of incomplete membrane structures and different blockages in them (Kumari *et al*. 2009).Variation in the frequency of albino plants obtained through anther culture of *indica* rice is attributed to its extreme variety or genotype specificity (Medhabati *et al.* 2014). Albinism in *indica* aromatic rice has been confirmed to be the serious bottleneck in anther culture for producing DH plants where most or all regenerated plants have been found to be useless albinos (Silva 2010). Low shoot regeneration and high albinos in the aromatic rice landraces may be due to their recalcitrant nature to *in vitro* culture (Das *et al.* 2021).

### Different ploidy levels in regenerated plants

Callus developed from anthers sometimes gets contaminated with the callus formed from the anther wall tissues and the resulting plantlets may be haploids, diploids, triploids and tetraploids (Dunwell 2010). The diploids were identified by their appearance. Out of the 7 plants generated through androgenesis, 4 (57.14%) were found to be diploid plants formed due to natural chromosome doubling during callus formation and 3 (42.86%) were haploids. The findings were similar to the observations of Ambarwati *et al.* (2009) who reported that diploids regenerated in *indica* rice cultivars varied from 50% to 65 % with normal morphological features, usually the same phenotypic characters as that of the parent plants with around 70-80 % grain fertility (Segui-Simarro and Nuez 2008). The haploids were found to be short statured (30%-35%) reduced plant height, panicle length and leaf length with double the number of the tillers than the parent plant and infertile flowers (Ambarwati *et al.* 2009). Duration and temperature of cold pretreatment is established factor for enhancing the blockage of the gametophytic development of microspores diverting them into sporophytic development during anther culture (Samantaray *et al.* 2021). Naik *et al*. (2017) reported 90-99% doubled haploid regenerations from the calli of the anthers undergone pretreatment of 7-8 days at 10°C. The frequency of spontaneous genome doubling in the present study may have reduced due to the reduced duration of cold pretreatment which may have resulted in less number of sporophytic calli in the culture. The frequency of doubled haploids can be increased by optimizing the temperature and duration of cold pretreatment for these genotypes.

## CONCLUSION

Successful DH plant generation depends on number of factors important being the genotypic factor. Aromatic rice landraces belonging to i*ndica* sub-species showed poor callus induction, shoot regeneration and produced albinos at a higher frequency. Duration of cold pretreatment of the anthers showed negative relation with the green shoot regeneration. High frequency of albinos was the major drawback in androgenesis of *indica* aromatic rice landraces. The DH plants generated from the desirable Gangabali mutant lines to be evaluated in the field conditions for their agro- morphological characters and yield potential.

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**REFERENCES**


Ambarwati AD, Somantri IH, Utami DW, Apriana A, Moeljopawiro S (2009) Rice anther culture to develop doubled haploid population and blast resistant lines. *J Agro Biogen* 5(2): 71-77.

Das S, Pradhan B, Sahu S, Samal KC, Parida S, Samantaray S (2021) Genotype-media interaction in aromatic rice landraces of India, relation with hypoxic germination. *Biol Forum* 13 (13a): 817-823.

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. *Pl Biotechnol J* 8: 377-424.

Herath HMI, Bandara DC, Samarajeewa PK, Wijesundara DSA (2009) Effect of low temperature pre- treatment on anther culture in selected *indica,* japonica rice varieties and their inter sub-species hybrids. *Cey J Sci* 38: 11-16.

Kaushal L, Sharma R, Balachandran SM, Ulaganathan K, Shenoy V (2014) Effect of cold pretreatment on improving anther culture response of rice (*Oryza sativa* L.). *J Exp Biol Agric Sci* 2: 233-242.

Khatun R, Shahinul ISM, Ara I, Tuteja T, Bari MA (2012) Effect of cold treatment and different media in improving anther culture response in rice (*Oryza sativa* L.) in Bangladesh. *Ind J Biotechnol* 11: 458-463.

Kumari M, Clarke HJ, Small I, Siddique KHM (2009) Albinism in plants: A major bottleneck in wide hybridization, androgenesis and doubled haploid culture. *Crit Rev Pl Sci* 28(6): 393-409.

Mayakaduwa DMRG, Silva TD (2017) A cytological indicator allows rapid assessment of microspore maturity, leading to improved *in vitro* anther response in *indica* rice (*Oryza sativa* L.). *in vitro* cellular & developmental biology. *Plant* 53(6): 591-597.

Medhabati K, Das KR, Henary C, Singh T D, Sunitibala H (2014) Androgenetic callus induction of the indica rice hybrid of Chakhao Amubi and Basmati 370. *Int Res J Boil Sci* 3(4): 73-79.

Mishra R, Rao GJN, Rao RN (2013) Effect of cold pretreatment and phytohormones on anther culture efficiency of two *indica* rice (*Oryza sativa* L.) hybrids: Ajay and Rajalaxmi. *J Exp Boil Agric Sci* 1(2): 69-76.

Mishra R, Rao GJN (2016) *In vitro* androgenesis in rice : advan tages , constraints and future prospects. *Rice Sci* 23: 57-68.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Pl* 15: 473-497.

Naik N, Rout P, Umakanta N, Verma RL, Katara JL, Sahoo KK, Singh ON, Samantaray S (2017) Development of doubled haploids from an elite indica rice hybrid (BS6444G) using anther culture. *Pl Cell Tissue Organ Cult* 128 (3): 679-689.

Premvaranon P, Vearasilp S, Thanapornpoonpong S, Karladee D, Gorinstein S (2011) *In vitro* studies to produce double haploid in *indica* hybrid rice. *Biologia* 66: 1074-1081.

Rout P, Naik N, Ngangkham U, Verma RL, Katara JL, Singh ON, Samantaray S (2016) Doubled haploids generated through anther culture from an elite long duration rice hybrid, CRHR32: Method optimization and molecular characterization. *Pl Biotechnology* 33: 177- 186.

Rudolf-Pilih K,Petkovsek M, Jakse J, Stajner N, Murovec J, Bohanec B (2019) Proposal of new hybrid breeding method based on genotyping, inter-pollination, phenotyping and paternity testing of selected elite $F_1$ hybrids.*Front Pl Sci* 10: 111.

Sakina A, Mir S, Najeeb S, Zargar SM, Nehvi FA, Rather ZA (2020) Improved protocol for efficacious *in vitro* androgenesis and development of doubled haploids in temperate japonica rice. *PLoS ONE* 15(11) In prees.

Samantaray S, Ali J, Nicolas KLC, Katara JL, Verma RL, Parameswaran C, Devanna BN, Awadhesh K, Dash B, Bhuyan SS (2021)Doubled haploids in rice improvement: Approaches, applications and future prospects. Rice Improvement, Ali J,Wani SH (eds) pp 425-447.

Segui-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: Changes associated with embryogenesis induction and microspore-derived embryogenesis. *Physiol Pl* 134: 1-12.

Sen C,Singh RP, Singh MK, Singh HB (2011) Effect of cold pretreatment on anther culture of boro rice hybrids. *The Int J Pl Repro Biol* 3: 69-73.

Silva TD (2010) *Indica* rice anther culture: Can the impasse be surpassed?. *Pl Cell Tiss Organ Cult* 100(1): 1-11.

Talebi R, Rahemi MR, Arefi H, Nourozi M, Bagheri N (2007) *In vitro* plant regeneration through anther culture of some Ira nian local rice (*Oryza sativa* L.) cultivars. *Pak J Biol Sci* 10(12): 2056-2060.

Tripathy SK, Swain D, Mishra D, Prusty AM, Behera SK, Tripathy P, Chakma B (2018) Elucidation of the genetic basis of anther culture response and its breeding perspective in rice. *Eur J Biotech Biosci* 6 (2): 26-30.