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ORIGINAL ARTICLE

# Development of doubled haploids from an elite *indica* rice hybrid (BS6444G) using anther culture

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**Abstract** A total of 200 doubled haploids (DHs) were generated from an elite rice hybrid, 'BS6444G' for which an androgenic method was developed by manipulating the physical and chemical factors. The spike pretreated at 10 °C for 7–8 days was effective for callusing and green plant regeneration. The maximum callus frequency was achieved when the anthers cultured in N6 medium supplemented with 2.0 mg $L^{-1}$ 2,4-diclorophenoxyacetic acid, 0.5 mg $L^{-1}$ 6-benzylaminopurine and 3% maltose. Calli induced in N6 media also showed significant green shoot regeneration in MS medium supplemented with 0.5 mg $L^{-1}$ 1-napthalene acetic acid, 0.5 mg $L^{-1}$ kinetin, 1.5 mg $L^{-1}$ benzylaminopurine and 3% sucrose producing 210 green plants. Assessment of the ploidy status showed 95.71% fertile diploids and 4.2% polyploids; no haploids were observed. A total of 38 sequence-tagged microsatellite (STMS) markers proved able to discriminate a heterozygote from all the 200 DHs. The DHs grown in the field showed significant variation for their agronomic traits. Comparison of traits with control indicates homogeneity within each DH line and significant variance of traits between DH lines. Nine DH lines produce higher grain yield than the hybrid parent which suggests the possibility of exploiting hybrid vigor in *indica* rice through the development of DH lines of high yielding hybrids.

**Keywords** Doubled haploid · Androgenesis · Rice hybrid · *Indica* rice · Anther culture

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## Introduction

Anther culture (AC) techniques have proved to be an efficient aid to plant breeding providing rapid production of doubled haploids (DHs) (Suriyan et al. 2009). Generation of DHs through AC helps in rapid fixation of homozygous lines in comparison to conventional breeding which requires 6–7 generations of self pollination. Double haploid techniques not only accelerate the breeding cycle but also allow better discrimination between progeny genotypes (Marassi et al. 2006). The fixation of recessive traits in DH populations simplifies conductance of genetic studies. Though a number of varieties and improved parental lines have been developed through androgenic approaches mostly in *japonica* hybrids (Lee et al. 1989), the use of AC as a routine technique for breeding of *indica* rice is extremely limited due to poor induction of androgenic calli and subsequent plant regeneration (Sripichitt et al. 2000). The recalcitrance of *indica* cultivars has been attributed to low callusing ability, poor plant regeneration potential, and frequent occurrence of albino plant regeneration (Dewi et al. 2009) which limits the practical application for production of homozygous lines. Low androgenic potential in anthers has been overcome with application of different media combinations (Bishnoi et al. 2000; Echavarri and Cistue 2016; Uno et al. 2016) which aided in developing an *indica* salt tolerant cultivar (Senadhira et al. 2002).

With rice reaching a yield plateau after the green revolution, obtaining high yields through use of hybrid rice has gained significant interest worldwide. Rice hybrids have been shown to have significant yield advantages over inbreds (Yuan 1994) in terms of both productivity and production per unit area. Although a number of *indica* rice hybrids (RHs) have been released for commercial cultivation in India, high seed costs and poor grain

cooking quality have limited their popularity among farmers. Because production of RH requires a three lines system (A, B and R lines) in India, non-synchronization of male and female lines also constrains hybrid rice seed production. Production of fertile, stable recombinant DH cultivars from RH via androgenesis has been proposed as a viable option, capable of generating homozygous lines in a single generation (Forster and Thomas 2005).

The possibility of converting the yield advantages from heterosis into fixed partial or complete dominance in DH lines through anther culture of hybrid rice has led to the release of several varieties (Han and Huang 1987). Though AC is an attractive approach for DH production, generation of sufficient number of DHs is the prerequisite for using it for practical breeding purposes. However, a number of methods were established by manipulating the physical and chemical factors for DHs production in *indica* rice for which the methods varies from genotype to genotype (Bagheri and Jelodar 2008). With reports to date being meager for production of DHs from elite *indica* RHs (Mishra et al. 2015), the present study describes the optimum conditions for establishing callus culture and green plant regeneration for large scale DHs production in one elite RH, BS6444G through anther culture along with the evaluation of DHs based on agronomic performance. This study also reports on the molecular characterization of DHs using sequence-tagged microsatellite sites (STMS) markers. Besides, this study describes the androgenic method standardized for generation of doubled haploids in a popular indica rice hybrid for minimizing the limitations of the rice hybrids cultivation where the frequency of albinos and haploids were less in the culture; this is the first report of its kind in *indica* rice.

## Materials and methods

### Plant material

This study was conducted at the National Rice Research Institute (Formerly CRRI), Cuttack, Odisha, India. The $F_1$ seeds of the popular RH, BS6444G, which is suitable to irrigated and shallow lowland area were obtained from M/S Bayer seed Pvt. Ltd., Hyderabad, Telangana, India and 30-day-old F1 seedlings, raised in dry seed beds under ideal conditions. Seeds were sown in dry bed (1.2 m × 5 m) nursery which was well maintained and equipped with irrigation and drainage facilities. After sowing, nursery was moistened and maintained wet till 20–25 days along with taking all prophylactic measures. To ensure healthy seedlings growth, the beds were applied with 25 kg FYM along with 90 g nitrogen, 45 g phosphorus and 45 g potassic fertilizer. The seedlings were transplanted in a well puddled field with of 20 × 15 cm spacing between and within rows. Recommended doses of N:P:K (100:50:50) were applied in three split doses and need based crop protection measures were undertaken. The experiment was conducted during the dry season of 2013 and wet season of 2014 (The growing condition temperature in dry season was 26.3–35.0 °C along with 12.01 h $day^{-1}$ photoperiod whereas in wet season, temperature and photoperiod were observed as 25–31.6 °C and 11.8 h $day^{-1}$ respectively in PI stage).

### Collection and cold pretreatment of panicles in boot

Healthy panicles at the booting stage, ca. 14–16 cm in length and positioned between the subtending leaf and the flag leaf were collected during 7:00–8:00 am from primary and secondary tillers of the field grown plants. Anthers collected at this early flowering stage with microspores at the mid-late uninucleate stage can lead to callusing or direct embryogenesis (Afza et al. 2000). The appropriate stage for androgenesis was selected using acetocaramine method conducting on some boots till the proper appearance was learned. Then the boots were wiped clean 2–3 times, with a clean muslin cloth moistened with 70% alcohol, wrapped in wet non-absorbent cotton and enclosed in polythene bags in order to prevent desiccation and maintain pollen viability. The wrapped boots were placed in an incubator that was maintained at different temperatures (4, 8, 10, 12 °C) for 2, 4, 6, 8, 10, 12 days in darkness for cold pretreatment. Control spikes were not subjected to cold treatment. The materials for cold pretreatment were prepared by keeping boots intact with their penultimate leaf sheath and node, and trimming off flag leaf and extra basal nodes. Most of the boots then harvested when the growth of anthers reached around one-half to one-third of spikelet length. The boots were surface sterilized with 70% ethanol for 4 min followed by 4% sodium hypochlorite (MERCK, India) for 2 min and rinsed three times with sterile de-ionized water. Before culturing of anthers from a hybrid, the anthers with mid to late uninucleate stages were first determined of microspore which were later traced back with the anthers' position on spikelets. The anthers were removed by cutting off the base of the florets attached to the panicle by a scissors and tapped the florets on top of tubes for uniform distribution in the media. Approx. 40–50 anthers were uniformly dusted in a tube over the surface of the media. A total of 30 boots were used per each treatment taken for study.

### Culture media and condition

Two experiments were conducted for optimization of callus induction. Firstly, N6 (Chu et al. 1975) was prepared containing different concentrations and combinations of growth regulators: (0.1–0.5 mg $L^{-1}$) 6-benzylaminopurine

(BAP), (0.1–0.5 mg $L^{-1}$) kinetin (Kin), (1–2 mg $L^{-1}$) 2,4-dichlorophenoxyacetic acid (2,4-D), (0.5 mg $L^{-1}$), thidiazuron (TDZ) and (5 mg $L^{-1}$) silver nitrate ($AgNO_3$) along with 3% (w/v) maltose for which seven treatments were tested. Secondly, similar combinations of growth adjuvants were tried in N6 media supplemented with 3% different carbohydrate sources: maltose, glucose, fructose, and sucrose.

Callus regeneration media were all MS medium (Murashige and Skoog 1962) containing 3% sucrose along with different concentrations and combinations of growth regulators: (1–2 mg $L^{-1}$) BAP, (0.1–1.0 mg $L^{-1}$) Kin, and (0.01–0.5 mg $L^{-1}$) NAA. A single rooting medium was used throughout the study, MS media supplemented with 2 mg $L^{-1}$ NAA, 0.5 mg $L^{-1}$ Kin and 5% sucrose. The pH of all the media was adjusted to 5.8 using 0.1 N NaOH/0.1 N HCl and solidified with 0.8% (w/v) agar–agar (Himedia, India) before autoclaving. Routinely, 25 mL of molten media was dispensed into culture tubes (25 mm × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheesecloth and sterilized at 121 °C (15 pounds of pressure per square inch) for 15 min. Then the media cooled to about 60–80 °C were dispensed to 25 mL tubes and slanted to get an area of 25 mm × 70 mm. The cultured anthers were incubated in dark at 25 ± 2 °C for callus induction while all other cultures were incubated at 25 ± 2 °C under a light intensity of 42 µmol $m^{-2}/s^{-1}$ from cool, white fluorescent lamps (Philips, India) under 16-h photoperiod.

### Acclimatization and net house transfer

The resulting green regenerants with well-formed roots were removed from agar and kept in normal tap water in shade for 3–4 days at 27–29 °C along with RH of 50–70% for acclimatization. Thereafter, the plants were transferred to 12″ pots containing field soil and kept in net house and necessary culture management was undertaken for good growth and development of the plants.

### Ploidy evaluation

The ploidy status of the green plants was determined based on morphological evaluation such as plant height, spikelet fertility when the plants attained the full maturity stage. Plants with normal morphological appearance and fertility were considered as diploids. Morphologically, tetraploids or polyploids show tall, gigantic growth along with thick dark green leaves with less than 1% of spikelet fertility.

### Analysis of STMS marker

All the 201 plants found to be fertile diploids were differentiated to distinguish the heterozygotes (donor like) and doubled haploids using STMS markers. A total of 650 rice STMS markers were screened out of which 38 pairs showing heterozygosity or parental polymorphism were used. Total genomic DNA was extracted from young leaves by modified cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). Amplification reactions were performed in volumes of 10 µL containing 1× PCR buffer (Biotool B&M Labs, Spain) (75 mM Tris HCl; pH 9.0), 50 mM KCl, 20 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$, 0.25 mM deoxy nucleotide triphosphates (dNTPs) (MBI Ferment Inc., Maryland, USA), 10 pmol of each of the primers, 1.0 unit of *Taq* DNA polymerase (Biotool B&M Labs, Spain) and 25 ng of template DNA. The amplification reaction was carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 36 cycles as follows: 1st cycle of 4 min at 94 °C followed by 35 cycles each of 30 s denaturation at 94 °C, 45 s annealing at 55 °C and 1 min extension at 72 °C. The final step consisted of one cycle of 10 min at 72 °C for complete polymerization. After completion of the polymerase chain reaction (PCR), 2.5 µL of 6× loading dye (MBI Ferment Inc., Maryland, USA) was added to the amplified products and were electrophorized in a 2.5% (m/v) agarose (Bangalore Genei, Bangalore, India) gels with 1× Tris-borate-ethylenediamine tetraacetic acid (TBE buffer), stained with ethidium bromide and documented by a gel documentation system (Syngene, Cambridge, UK). The size of amplification products was estimated by comparing with standard DNA ladder (O'Gene Ruler 50 bp DNA ladder; BR Biochem Life Sciences Pvt. Ltd. India). All the reactions were repeated three times.

### Evaluation of doubled haploid lines for agronomic traits

A set of 200 DH lines generated from an $F_1$ hybrid was evaluated under irrigated conditions using an augmented design with 10 blocks each containing 20 test entry and 3 control plots (Parent; BS6444G, 'Naveen'; mid early duration and 'Tapaswini'; medium duration) during the wet season of 2014 and dry season of 2015 at the NRRI experimental field, Cuttack, Odisha, India. A total of 75 plants of each entry were transplanted in three lines keeping 20 cm spacing between rows and 15 cm between plants. Data were recorded on the following morpho-agronomic traits at maturity stage: plant height (cm), tiller number, flag leaf length and breadth (cm), effective tillers, panicle length (cm) of main culm of the randomly selected plants and days to maturity. Five competitive plants chosen at random from the center row in every three-row plot were used in the data collection. Five panicles selected from five plants chosen randomly were used to determine seed fertility. Seed fertility was computed based on the number of filled spikelets

over the total number of spikelets per panicle. The test weight (g), grain length/breadth ratio, grain yield ($kgm^{-2}$) was recorded for the agronomic characters.

### Observations and statistical analysis

A total of 20 cultures (20 tubes each containing 40–50 anthers) were used per treatment for callus induction and 15 cultures (a tube containing 4–5 calli) for shoot regeneration. Each experiment was repeated at least two times in dry season. The numbers of transferred calli and green and albino plants were recorded. The impact of each treatment was assessed in comparison with the control. The following parameters were used: callus induction (%) = (no. of anthers forming callus/total no of anthers cultured) × 100, plant regeneration (%) = (no. of calli producing green plants/total no. of calli transferred) × 100. Statistical analysis of each parameter related to androgenesis and morpho-agronomic characters was analyzed as completely randomized design (CRD) and augmented design respectively using SAS software (SAS 9.1; SAS Institute 2003). All data were subjected to analysis of variance (ANOVA). The means were separated according to Duncans' multiple range test (DMRT).

## Results

### Callus induction

Anthers containing pollen at the mid uninucleate stage of development were used for culture (Fig. 1a). A preliminary study was undertaken to evaluate the callus induction potential at various temperatures (4, 8, 10 and 12 °C) for a period of 0–12 day from which it was observed that the 10 °C temperature was found to be best irrespective of the combinations and concentrations of growth regulators in N6 basal media. When anthers containing pollen grains of suitable stages were cultured, they turned light brown in color and then swelled in size before calluses were seen bursting the anthers and emerging asynchronously after 3–10 weeks culture (Fig. 1b). The efficiency of callusing for response of the various callus induction media tested was shown in decreasing order: N3 > N1 > N5 > N7 > N2 > N4 > N6 for 7 days incubation at 10 °C (Table 1). The callus frequency was found to be maximum in N6 supplemented with 2 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ BAP along with 3% maltose (N3). Inclusion of kinetin in place of BAP in the media (N6) lowered the frequency of callus induction to almost 1/3. Similarly, addition of 0.5 mg $L^{-1}$ TDZ and 5 mg $L^{-1}$ $AgNO_3$ in the optimum callusing media decreased of callus induction efficiency. On the other hand, anthers incubated in the spikes for 7 days followed by 8 days at 10 °C producing 29.35% and 25.00% respectively (Table 1). The compact pale yellowish calli developed after 3–10 weeks of culture which continued up to 36 weeks. However, anthers incubated for 2 days at 10 °C produced pale whitish watery calli when cultured on the same media composition. With the increase in incubation period from 8 to 10 days, the rate of callus induction decreased to 7.17% significantly. However, surprisingly, anthers incubated for 12 days showed rates of callusing (17.08%) that were increased over 10 day incubation. Development of multiple calli from a single anther was also rarely observed. The effect of different carbohydrates was then tested using N3 hormonal composition and either sucrose, maltose, glucose or fructose at the concentration of 3%. Maltose was found to be superior followed by sucrose in callus induction (Fig. 2).

### Shoot regeneration

A total of 210 green plants resulted from yellow color calli that attained 1.5–2.0 mm size before being transferred to MS basal media supplemented with BAP, Kin and NAA. Calli developed green spots in 7–10 days of transfer (Fig. 1c) and subsequently led to the formation of green shoots after 2 weeks of culture. No calli regenerated from the whiter watery callus that arose from anthers incubated for 2 days at 10 °C though an appreciable increase in size of the calli on MS media was observed. Green shoot regeneration was maximum (68.20%) in MS media supplemented with 1.5 mg $L^{-1}$ BAP, 0.5 mg $L^{-1}$ Kin and 0.5 mg $L^{-1}$ NAA along with 3% sucrose (Table 2; Fig. 1d). Only ten albino shoots were found in whole shoot regeneration system where single albino shoot emerged in the bunch of the green shoots of some culture (Fig. 1e). The calli regeneration potential varied from 45.23 to 68.20% and 49.01–54.04% in 7 day and 8 day incubation respectively (Table 2). The regeneration potential varied in all the treatments used showing decreasing order: M4 > M5 > M2 > M1 > M3 > M6. A different order was found for 10–12 days incubations, with M2 now proving most effective.

### Rooting and acclimatization of in vitro raised plants

A total of 210 micro green shoots formed a high percentage (100%) of roots grown in MS media supplemented with 2.0 mg $L^{-1}$ NAA, 0.5 mg $L^{-1}$ Kin, and 5% sucrose (Fig. 1f). Well developed plants with profuse roots were acclimatized and transferred batch by batch to the net house showing 100% survivability and variability in all the green plants was observed in terms of height, panicle size and grain shape (Fig. 1g).

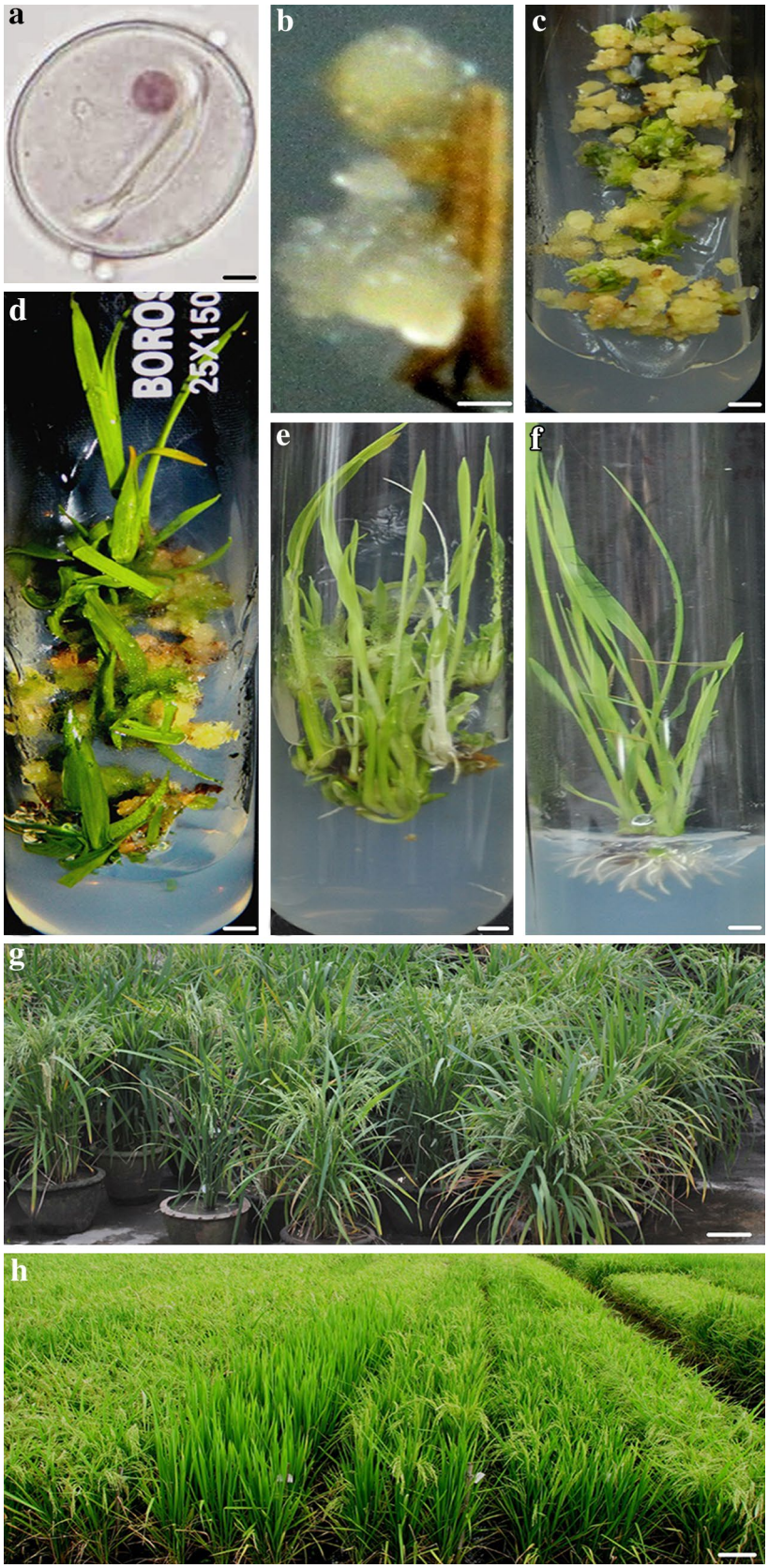


**Fig. 1** Development of plants through androgenesis from BS6444G. **a** Mid-uninucleate stage of microspore (*bar* 0.5 cm). **b** Bursting of anthers showing calli (*bar* 1 cm). **c** Induction of shoot buds from calli (*bar* 5 mm). **d** Micro-shoots elongation (*bar* 5 mm). **e** Albino shoots regenerated from calli (*bar* 5 mm). **f** Rooting in microshoots (*bar* 5 mm). **g** Variability in DH lines grown in net house (*bar* 1 cm). **h** Doubled haploid lines grown in the field for selection of promising lines at A1 generation (*bar* 0.5 cm)

**Table 1** Effect of plant growth regulators in N6 basal media and duration of cold pretreatment on callus induction in anthers of BS6444G

| Media | N6 + PGR (mg $L^{-1}$) | | | | | | % of callus induction (mean ± SE)[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Days of incubation at 10 °C | | | | |
| | 2,4-D | Kn | BAP | NAA | TDZ | $AgNO_3$ | 2 days | 7 days | 8 days | 10 days | 12 days |
| N1 | 1.0 | – | 0.1 | 1.0 | – | – | 5.08 ± 0.10c | 27.86 ± 0.14a | 25.61 ± 0.15b | 3.83 ± 0.05b | 7.96 ± 0.10d |
| N2 | 1.5 | – | 0.1 | 0.5 | – | – | 4.69 ± 0.10c | 24.23 ± 0.24c | 28.27 ± 0.13a | 2.48 ± 0.09c | 3.65 ± 0.06f |
| N3 | 2.0 | – | 0.5 | – | – | – | 10.12 ± 0.15b | 29.35 ± 0.20a | 25.01 ± 0.05b | 7.17 ± 0.03a | 17.08 ± 0.12b |
| N4 | 2.0 | – | 0.5 | – | – | 5 | 5.85 ± 0.12c | 20.66 ± 0.19d | 27.57 ± 0.13a | 3.58 ± 0.08b | 9.14 ± 0.13c |
| N5 | 2.0 | 0.1 | 0.5 | – | – | – | 17.34 ± 0.12a | 26.72 ± 0.12b | 21.36 ± 0.13d | 8.09 ± 0.17a | 24.32 ± 0.24a |
| N6 | 2.0 | 0.5 | – | – | – | – | 5.07 ± 0.08c | 8.57 ± 0.11e | 22.67 ± 0.12cd | 3.52 ± 0.10 bc | 3.83 ± 0.06ef |
| N7 | 2.0 | – | 0.5 | – | 0.5 | – | 3.75 ± 0.11d | 24.31 ± 0.16c | 22.81 ± 0.12c | 1.33 ± 0.11d | 4.36 ± 0.06e |

Means sharing the same letter in a column were not significantly different in Duncan's multiple comparison range test ($p < 0.05$)

[a]20 replicates per treatment; repeated twice

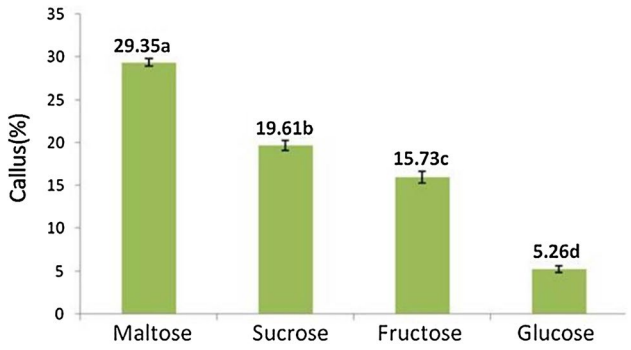


**Fig. 2** Effect of different carbohydrates (3%) supplemented with N6 + 2.0 mg $L^{-1}$ 2,4-D, 0.5 mg $L^{-1}$ BAP on callusing derived from 7 day incubation at 10 °C in BS6444G. *Each bar* is the average of 20 replicates with *error bar* denoting standard error of the mean

## Determination of ploidy status

Out of 210 green plants, 201 showed normal morphological appearance with 65–77% grain fertility. The plants showing gigantic growth along with partial seed setting were identified as tetraploids (4.2%) whereas no differentiation of diploids and doubled haploids based on morphological characters; no haploid plants were observed in all the in vitro raised plants. Since it is difficult to discriminate somatic tissue derived diploids and DHs through morphological characters, molecular markers were used. A total of 38 STMS markers discriminated a single somatic tissue derived heterozygotes out of 200 diploid lines (Fig. 3).

## Variation in agronomic performance between and within doubled haploid lines

A set of 200 DH lines derived from parent ($F_1$s of BS6444G) was field evaluated for different agronomic traits: days to maturity, plant height, tiller number, length and breadth of flag leaf, panicle length, seed fertility, 1000-grain weight and grain yield. All traits showed a wide variation among the AC-derived DH lines. Results of the analyses as presented by the *F* values generated from the ANOVA are given in Table 3. Doubled haploid (DH) lines having phenotypic values significantly higher or lower

**Table 2** Effect of plant growth regulators in MS media and days of incubation of spikes at 10 °C derived calli on shoot regeneration in BS6444G

| Media | MS + PGR (mg $L^{-1}$) | | | % of shoot regeneration from calli derived at 10 °C (mean ± S.E)[a] | | | |
|---|---|---|---|---|---|---|---|
| | | | | Days of spike incubation at 10 °C | | | |
| | BAP | Kin | NAA | 7 day | 8 day | 10 day | 12 day |
| M1 | 1.0 | 0.1 | 0.1 | 50.21 ± 0.30c | 51.63 ± 0.39ab | 33.84 ± 0.58bc | 11.15 ± 0.15b |
| M2 | 1.0 | 1.0 | 0.01 | 52.99 ± 0.53bc | 49.02 ± 0.45c | 38.27 ± 0.99a | 14.72 ± 0.26a |
| M3 | 1.5 | 1.0 | 0.1 | 50.95 ± 0.52c | 51.30 ± 0.57bc | 32.84 ± 0.35c | 5.48 ± 0.14d |
| M4 | 1.5 | 0.5 | 0.5 | 68.20 ± 0.51a | 54.05 ± 0.81a | 33.65 ± 0.37bc | 9.76 ± 0.21b |
| M5 | 1.5 | 1.0 | 0.01 | 54.44 ± 0.48b | 50.94 ± 0.24bc | 32.71 ± 0.67c | 7.39 ± 0.13c |
| M6 | 2.0 | 0.5 | 0.2 | 45.24 ± 0.52d | 51.30 ± 0.26bc | 36.24 ± 0.38ab | 1.17 ± 0.00e |

Means sharing the same letter in a column were not significantly different in Duncan's multiple comparison range test ($p < 0.05$)

[a]15 replicate per treatment; repeated twice

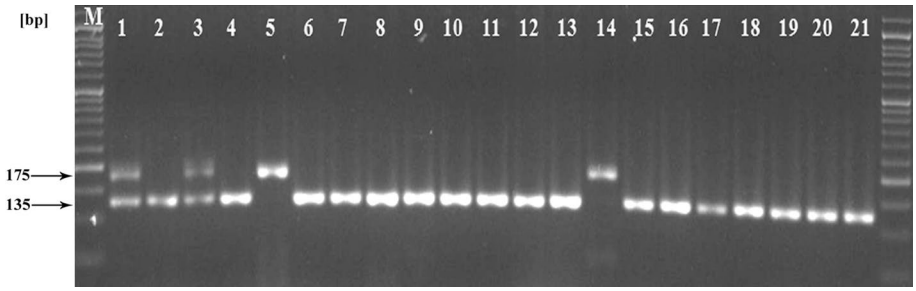


**Fig. 3** Screening of doubled haploids for identification of heterozygotes and subsequent field evaluation. **a** SSR marker (RM206) differentiating somatic tissue derived heterozygote from doubled haploids (*1* BS6444G, *2–13* and *15–21* representatives of doubled haploids, *14* heterozygote, *M* 50 bp DNA ladder)

than those of the parent (transgressive segregants) were observed for all the traits (Table 4). For maturity, two lines exhibited longer duration than the parent. For plant height, 195 lines were shorter than the parent whereas five lines were taller than BS6444G. In terms of percent seed fertility, 146 lines showed superiority over the parental hybrid. Panicle length was found to be higher in 39 DHs as compared to their parent, and nine DHs had higher grain yield. The values of different traits were well distributed among DH lines, e.g. percent of seed fertility varied greatly among the DH plants derived from BS6444G. The mean and variance of the DH lines revealed that the population represents a random sample for the parental gametes which is also confirmed by molecular markers.

Homogeneity within DH lines was examined by comparing the intra-variance of each DH line with the pooled variance of the checks (Table 4). The *F* value of the DH lines versus the pooled variance of the checks revealed significant difference for all the traits tested at the 0.01% level. The results indicated that the plants within each DH line evaluated in the field were homozygous. The homogeneity of plants within DH lines was visible for plant height, grain type, and general plant type (Fig. 1h). The nine DH lines exhibiting higher grain yield over the parent were selected for further advancement.

## Discussion

Anther culture is considered as a potential technique for production of DHs. However, it could not be fully exploited in *indica* rice due to this group's typically poor response to tissue culture. This necessitated manipulating the physical and chemical factors for optimization of androgenesis in the elite *indica* RH, BS6444G.

### Callus induction

Our result showed that the 7 day spike incubation at 10 °C resulted in the best callus induction in the $F_1$s of BS6444G at the mid-uninucleate stage followed by 8 day which corroborated the earlier findings in *japonica* cultivar (Genovesi and Magill 1979; Zapata-Arias 2003). Since extended temperature and period was reported to be inhibitory for callus induction (Lenka and Reddy 1994) however, interestingly, the period of incubation for 12 days showed a fairly high callusing in our study.

In addition to incubation temperature and period, the plant growth regulators play very significant role in optimization of callus induction. Our results showed that N6 medium supplemented with 2 mg $L^{-1}$ 2,4-D, 0.5 mg $L^{-1}$ BAP and 3% maltose turned out the highest frequency of callus induction. Though a fairly high concentration of 2,4-D (2 mg $L^{-1}$) alone produced relatively high rate of callus induction in some genotypes of *indica–indica* and *indica–japonica* crosses (Raina and Zapata 1997), however, combination of low concentration (0.5 mg $L^{-1}$) of BAP with 2,4-D enhanced the rate of calli response considerably in our study, as it did for Bohorova et al. (1995). It might be the synergistic effect of these two plant growth regulators (PGRs) used for callus induction. Though application of silver nitrate enhanced the callus induction frequency for *indica* and *japonica* cultivars (Niroula and Bimb 2009), our study shows an opposite effect for callusing. Since maltose in the AC medium is hydrolyzed more slowly than sucrose (Lentini et al. 1995) and yields only glucose upon hydrolysis, the beneficial effects of maltose in improving calli induction have been proven in this study (Roberts-Oehlschlager et al. 1990).

### Shoot regeneration

The present study showed that cold stress at 10 °C could be effective for the improvement of *indica* rice anther culture.

**Table 3** Variations among doubled haploid (DH) lines, between DH lines and parent ($F_1$ hybrid of BS 6444G) along with two checks for different agronomic traits under replicated trials using *F* statistics

| Source of variation | Mean squares ± SE | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Plant height (cm) | Days to 50% flowering | No. of effective tillers | Flag leaf length (cm) | Flag leaf width (cm) | Panicle length (cm) | Spikelets fertility % | Test weight (g) | Grain length width ratio | Grain yield (kg $m^{-2}$) |
| Control | 1.55** | 1.311* | 1.02** | 0.46 | 0.72 | 1.10** | 0.59 | 0.60 | 0.83* | 0.65 |
| Treatment (DH lines) ± SE | 511.28** ± 0.61 | 62.72** ± 0.21 | 4.58** ± 0.09 | 13.92* ± 0.30 | 0.03** ± 0.01 | 11.47** ± 1.15 | 38.35* ± 0.43 | 6.79* ± 0.14 | 3.43** ± 0.04 | 0.15** ± 0.01 |
| CV % | 7.52 | 2.84 | 12.90 | 13.81 | 10.31 | 7.31 | 6.40 | 10.46 | 12.65 | 27.60 |
| CD at 5% | 5.97 | 2.52 | 1.11 | 3.21 | 0.10 | 1.61 | 4.66 | 1.70 | 0.46 | 0.20 |
| CD at 1% | 7.84 | 3.31 | 1.46 | 4.22 | 0.13 | 2.12 | 6.13 | 2.24 | 0.61 | 0.27 |

*Control* Tapaswini, Naveen and BS6444G; *SE* standard error

*, **Significant at 5% and 1% level of significance respectively

**Table 4** Mean values for parent (BS 6444G) and DH lines (200 DHs derived from rice hybrid, BS 6444G) with checks (Naveen and Tapaswini) for ten agronomic traits using *F* statistics

| Traits | Mean ± SE | | No. of DH lines[a] | | *F* value |
|---|---|---|---|---|---|
| | | | BS 6444G | | |
| | BS 6444G | DH lines | Higher | Lower | |
| Plant height (cm) | 105.60 ± 0.70 | 87.90 ± 0.61 | 5 | 195 | 0.51** |
| Days to 50% flowering | 110.00 ± 0.73 | 101.06 ± 0.21 | 2 | 198 | 0.15** |
| Flag leaf length (cm) | 41.20 ± 0.02 | 26.60 ± 0.29 | 1 | 187 | 0.22** |
| Flag leaf width (cm) | 1.30 ± 0.06 | 1.12 ± 0.01 | 4 | 187 | 0.20** |
| No. of effective tillers | 11.20 ± 0.24 | 9.70 ± 0.09 | 10 | 161 | 0.38** |
| Panicle length (cm) | 27.40 ± 0.60 | 26.40 ± 1.16 | 39 | 157 | 0.13** |
| Spikelets fertility % | 81.80 ± 0.33 | 82.60 ± 0.41 | 146 | 53 | 0.28** |
| Test weight (g) | 20.20 ± 0.07 | 18.34 ± 0.14 | 28 | 169 | 0.39** |
| Grain length width ratio | 2.90 ± 0.02 | 4.45 ± 0.04 | 199 | 0 | 0.44** |
| Grain yield (kg $m^{-2}$) | 1.33 ± 0.01 | 0.89 ± 0.01 | 09 | 190 | 0.46** |

*SE* standard error ratio within DH lines variance versus pooled variance of the checks

*, **$p < 0.05$ and 0.01%, respectively

[a]Number of DH lines differing from the parents $F_1$s at 5% level of significance using scheffe's test

Different reasons have been suggested for the positive effect of cold pretreatment in androgenesis (Shariatpanahi et al. 2006; Shahvali-Kohshour et al. 2013). Cold pretreatment may protect microspores from toxic compounds released in decaying anthers because it slows down degradation processes in the anther tissues. It may induce starvation of microspores disconnected from the tapetum in cold-treated anthers. In addition, cold increases the total content of free amino acids, which might be conducive to an adaptation of microspores to the metabolic changes for androgenesis. Moreover, the calli texture and color also is associated with androgenesis. The compact calii with yellowish color resulted in better green plant regeneration whereas soft calli with white color proliferated and resulted in callus multiplication which ultimately became necrotic and die. We observed that 7 day spike pretreatment at 10 °C resulted in best shoot regeneration followed by 8 day from the anther derived calli grown in N6 media. Though the 12 day incubated spikes showed appreciable callusing, it did not respond for promising green shoot regeneration.

In the present experiment, the shoot regeneration was found to be best in MS medium supplemented with BAP and Kin in combination with NAA. Though plant growth regulators play an important role in green shoot regeneration, their effects on green plant production has been analyzed to a lesser extent than in the case of callus induction. In this study, a combination of BAP, Kin and NAA adequately supported green plant regeneration from subcultured callus which might balance the auxin /cytokinin in endogenous levels. However, increasing the concentration of BAP from 1.5 to 2 mg $L^{-1}$ reduced the green shoot regeneration frequency by 1.5 times. The donor, BS6444G displayed high callusing followed by best shoot regeneration as compared to other *indica* cultivars studied which is corroborated with earlier finding (Javed et al. 2007).

The occurrence of a large proportion of albinos among AC regenerated plants remains a formidable obstacle in rice anther culture (Khatun et al. 2012) especially in *indica* rice. In this study, 7–8 day spike incubation at 10 °C and the anther derived calli cultured only for 12–14 days reduced the frequency of albinos compared to panicle incubation at more than 10 days which is corroborated with the earlier reports (Asaduzzaman et al. 2003). Cold pretreatment is reported to enhance the blockage of the gametophytic development of microspores and guide the continuous division of the microspores into forming callus/embryo, a sporophyte during culture (Heberle-Bors et al. 1996); this type of shifting from gametophytic to sporophytic stage may cause instability in microspore development resulting in loss of chlorophyll. However, very low amount of albinos in this study might be due to the fixing of the microspore in uninucleate stage at this 10 °C moved towards sprorophytic stage instead of gametophytic stage. Moreover, surprisingly, no haploids were found in this culture for the reason being either the spontaneous doubling or the merging of the same microspores in anthers at low temperature. This study provides the first report in production of low albinos and no haploids in *indica* rice.

Flow cytometry is an attractive approach to assess the ploidy status of in vitro raised plants (Ochatt et al. 2009), but it cannot discriminate DHs from somatic tissue derived diploids. Application of STMS markers successfully provided the fair information on heterozygosity of the allelic loci for all the 200 diploid lines derived through AC (Grewal et al. 2011) which were used for

further studies. Molecular analysis revealed that of the 201 fertile diploid lines, 200 originated from the pollen and one from somatic tissue of the $F_1$s.

### Agronomic performance

The hybrid, BS6444G and the checks taken in this study showed significant variation for all agronomic traits. Interestingly, transgressive segregants for grain yield along with the biochemical grain quality characters have led to the selection of nine lines for on station trial along with a single line showing the grain yield performance at par with the parent. Since the plants within each DH line evaluated in the field were homozygous which were clear from marker data as well as plant height, plant type and grain type, and general appearance of the plants, this again confirms the origin from microspore after ploidy evaluation (Suhartini and Hanarida 2000).

The method established here is different from those published previously in certain key points: (a) 7 day incubation at 10 °C of spike produced a fairly frequency of callusing and green shoot regeneration in *indica* rice; (b) 17 week cultured anthers responded for callusing which produced significant frequency of green shoot regeneration; (c) the culture was totally free from haploids along with low frequency of albinos; (d) promising DH lines showed better grain yield than the RH which proved that fixing of heterotic attributes in the homozygous condition due to chromosome doubling.

## Conclusions

In conclusion, this method for AC was developed and optimized to produce DH lines for an elite RH, BS6444G manipulating the physical and media components. This method will certainly help others to move fast from the in vitro method to large scale field trials, thereby accomplishing the field assessed DHs in a short period of time as compared to traditional breeding. In this study, a total of nine promising lines were further advanced for on station trial which could be released as varieties (Zapata et al. 1991) if shows consistent performance in the grain yield.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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