ORIGINAL RESEARCH ARTICLE OPEN ACCESS

# Production of Doubled Haploids from Rice Hybrid KRH-2 through Anther Culture and their Evaluation for Agro-Morphological Traits

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Received: 10th April 2020; Accepted: 30th May 2020

## Abstract

The present study was aimed to optimize conditions of anther culture and production of doubled haploids of a popular rice hybrid, KRH-2. Immature panicles collected from field grown donor plants were pre-treated at 8°C for eight days and plated on four different basic media for callus induction with different hormonal concentrations and supplements. N6 media with maltose as the carbon source, supplemented with 2 mg $L^{-1}$ of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg $L^{-1}$ of kinetin and 10 mg $L^{-1}$ of silver nitrate (designated as N6-4 medium) was found to give the highest callus induction (22.4%). The compact calli, which were transferred to MS regeneration medium supplemented with 2.5 mg $L^{-1}$ of benzyl amino purine (BAP), 0.5 mg $L^{-1}$ of kinetin and 1.5 mg $L^{-1}$ of naphthalene acetic acid (NAA) recorded a good regeneration (25.4%). A total of 125 stable, doubled haploid lines (DHLs) were produced through spontaneous doubling of KRH-2 microspores. The lines showed significant variability with respect to key agro-morphological traits.

**Keywords**-Rice androgenesis; Callus induction; Green plant regeneration; KRH-2; morpho-agronomic evaluation

## Introduction

Among the various alternatives available for increasing rice productivity, adoption of hybrid rice technology is one of the most feasible options (Yuan 1994). Since early 1990s hybrid rice has been cultivated in many Asian countries including India. Presently, China has the maximum area under hybrid rice and the technology is one of the key components of food security in China. Despite, the tremendous potential of hybrid rice technology in improving rice production and productivity, the adoption of hybrid rice technology has not been satisfactory in India. Presently, it is estimated that out of 44.5 Mha of cultivated rice, only 3 Mha is under hybrid rice. Higher seed cost, poor seed-set, low to moderate levels of heterosis and poor grain and cooking quality of rice are the major reasons for limited adoption of the technology(Mishra *et al*. 2013). Among these, in particular, the moderate levels of heterosis is one of the main reasons for its lower adoption in the intensive rice cultivation areas of India and a need has been felt to enhance the genetic diversity among the parental lines to increase the heterosis levels.

In recent years, rapidly changing climatic conditions coupled with increased incidence of biotic and abiotic stresses have posed severe challenges for increasing rice production which has already witnessed a scenario of yield stagnation. One of the feasible options available to enhance the rice production in the short and medium term is through adoption of hybrid rice, which has been proved to have considerable yield advantage over the inbred lines (Yuan 1994). Though many commercially released varieties of Indian hybrid rice have shown higher productivity

and production per unit area, adoption of the latter by Indian farmers has not been very encouraging, primarily due to higher seed costs and poor cooking quality (Mishra *et al*. 2013).

Among the various methods available for breeding and improving the parental lines used for hybrid development, conventional breeding approach is the most commonly deployed one. However, it involves several cycles of breeding-selection and is cost, resource intensive. This necessitates development of other viable options that could surpass these breeding limitations. Through conventional breeding, it takes a minimum of 6-8 years for development of desirable lines and there is a need to shorten the process of development of homozygous lines (Snape 1989; Raina & Zapata 1997; Baenziger *et al*. 2006). One such viable and feasible option for developing homozygous lines in a single generation is through adoption of doubled haploid (DH) breeding strategy (Hu. 1985; Baenziger *et al*. 1989; Wu *et al*. 2012).

Different fixed (i.e. non-segregating) populations namely the recombinant inbred lines (RIL), doubled haploid lines (DHLs), near isogenic lines (NILs) etc., are considered as valuable tools for use in tagging and mapping of novel traits/genes and also as a population improvement strategy, besides being useful in functional genomics studies (Paterson 1996; Collard *et al*. 2005; Forster & Thomas 2005). Among these populations, DHLs are unique in the sense that their development and fixation takes the shortest possible time (i.e. within one-two breeding seasons). DHLs are considered as valuable tools for identifying gene blocks associated with important quantitative traits such as yield, biotic and abiotic stress tolerance and heterosis (Tinker *et al*. 1996; Wang *et al*. 2001; Senadhira *et al*. 2002; Suriyan *et al*. 2009). Even though there are several reports of development and utilization of DHLs for varietal development/ improvement (Han and Huang 1987), their utilization in molecular mapping of heterotic loci/QTL is limited.

Even though protocols for anther culture and doubled haploid production have proved to be quite simple in japonica rice, success rate in indica rice has been limited (Zapata and Arias, 2003; Bagheri and Jelodar, 2008). Though many attempts were made to produce the fertile DH plants by manipulating the factors that govern androgenesis in indica rice, the success of optimized anther culture conditions have been fairly unsatisfactory (Ratheika and Silva, 2007). The reasons identified for the limited success of indica rice is principally due to the recalcitrant nature as a result of early anther necrosis, inherently diminished competence to form callus, higher frequency of albino plant regeneration and lower frequency of green plant regeneration (Balachandran *et al*. 1999; Grewal *et al*. 2009). As limited reports are available on the production of DH lines of indica rice (Mishra *et al*. 2015; Naik *et al*. 2016) this study outlines the optimized culture conditions for an elite Indian rice hybrid, KRH-2 and evaluation of the DH lines.

## Materials and Methods

**Plant material:** A medium duration rice hybrid, Karnataka Rice Hybrid-2 (KRH-2 derived from the cross IR58025A × KMR-3R), with long and bold grain type, developed by Regional Agricultural Research Station (RARS), Mandya, Karnataka was used as the experimental material, along with the parents, IR58025B (recurrent parent) and KMR-3R (donor parent). The parental seed material along with the seeds of varietal checks was obtained from the Hybrid Rice Section, ICAR-Indian Institute of Rice Research, Rajendranagar, Hyderabad, India. For evaluation of the developed DH population, Akshayadhan (AKD) and Varadhan (VRD) were used as varietal checks.

**Heterozygosity assessment of KRH-2 hybrid:** Purity assessment of parents and $F_1$ seeds of the hybrid KRH-2 was done before every batch of KRH-2 hybrid anthers were plated on callus induction medium. This was conducted with a simple sequence repeat (SSR) marker RM19660 located on chromosome 6 that exhibited amplification of unique alleles in KRH-2 as per the procedure described in Yashitola *et al*. (2002) and Sundaram *et al*. (2008).

**Anther culture and double haploid production in KRH-2:** Genetically pure seeds of parents and the elite rice hybrid KRH-2 (derived by crossing IR58025A with KMR-3R) obtained from the Hybrid Rice Section, ICAR-Indian Rice Research Institute, Rajendranagar, Hyderabad, India, were grown during *Kharif* 2015 and the recommended package of practices were adopted for raising the crop. Immature panicles of KRH-2 were collected from the field grown plants at booting stage in the morning between 8-9 AM as described by Balachandran *et al*. (1999). The panicles were packed in wet cloth wrapped in aluminum foil and subjectedto a cold shock pre-treatment at 8°C for a period of eight days in a cold chamber. After, cold shock treatment, spikelets which were in late uni-nucleate to early bi-nucleate stage (determined through microscopy, described by Jahne and Lorz, 1995) were chosen for plating of the anthers. For checking the pollen fertility, excised spikelets were fixed in formaldehyde, acetic acid and 70% alcohol solution (1:1:18). They were then gently macerated on a slide with an autoclaved surgical blade and stained with 1% potassium iodide solution (1% IKI) for observation under bright field microscope for pollen fertility estimation (Mishra *et al*. 2015). Fertile anthers were then aseptically plated on four callus induction medium viz., N6 (Chu *et al*. 1975), B5 (Gamborg *et al*. 1968), MS (Murashige and Skoog 1962) and He2 (Huang *et al*. 1978) following the protocol standardized by Balachandran *et al*. (1999) in 25 batches but with slight modifications. The plated anthers were incubated in dark for 45 days at 25°±1°C. The cultures were examined periodically for contaminations and the development of callus formation was closely monitored.

Compact, pale yellow colored calli measuring 2-3 mm in diameter were transferred to MS regeneration medium (Cho and Zapata, 1988; Balachandran *et al*. 1999) and incubated under illuminated conditions with 16/8 hours' light and dark regime. The regenerated green shoots were transferred to hormone free ½ MS medium (rooting medium) and were maintained under illumination. Well-developed plants with profuse roots were transferred to Yoshida's solution for hardening for two weeks, later they were transferred to green house and maintained till maturity. The response of the anthers for callus induction and green plant regeneration was recorded 40 days after plating.

Optimization studies for callus induction were carried out with four different media namely, N6, B5, MS and He2 with 3% maltose as the carbon source supplemented with additives 0.5 g $L^{-1}$ of casein hydrolysate, 0.1 g $L^{-1}$ of myo-Inositol, 10 mg $L^{-1}$ of silver nitrate and five different hormonal concentrations viz. 0.5 mg $L^{-1}$, 1.0 mg $L^{-1}$, 1.5 mg $L^{-1}$, 2.0 mg $L^{-1}$, 2.5 mg $L^{-1}$ of 2,4-D (2, 4-Dichloro phenoxy acetic acid) + 0.5 mg $L^{-1}$ of Kinetin) and plated in 0.4 % phytagel. Similarly, for green plant regeneration phase, the optimization studies were carried out using the MS medium 3% sucrose as carbon source supplemented with five different hormonal concentrations viz., 0.5 mg $L^{-1}$ of 6-Benzyl Amino Purine (BAP), 1.0 mg $L^{-1}$, 1.5 mg $L^{-1}$, 2.0 mg $L^{-1}$ and 2.5 mg $L^{-1}$; 0.5 mg $L^{-1}$ of Kinetin and 1.5 mg $L^{-1}$ of Napthalene Acetic Acid (NAA) (Table 1). , The *in-vitro* androgenesis of the hybrid, KRH-2 and its parents was carried out during both *Kharif* 2015 and *Rabi* (2015-2016).

The regenerated plants at the end of the *Kharif* 2015 ($D_0$) were grown under greenhouse conditions in earthen pots and upon acclimatization, were maintained till maturity in net-house conditions. These plants were agro-morphologically assessed for the determination of ploidy levels. Total number of regenerated plants, total number of survived plants, total number of sterile (n), diploid (2n) and polyploid plants were recorded. The seeds ($D_0$ generation) of those plants which were observed to be putative fertile doubled haploids (DHLs) were carefully collected. Only the confirmed and true DHLs were advanced to further generations. Similarly, plants obtained during the *Rabi* (2015-2016), were established and ploidy determination and true-fertile DHL advancement was followed.

Duncan's Multiple Range Test (DMRT) was done using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) to assess the effect of different media compositions on callus induction and green plant regeneration. Microsoft excel package was used for the analysis of standard error (SE), standard deviation (SD), coefficient of variation in percentage (CV%) and mean.

**Identification of true doubled haploids using hyper-variable SSR marker**: At the end of *Kharif* 2015 and *Rabi* (2015-2016), in order to determine the origin of putative fertile doubled haploid (2n) anther culture derived plants (across two seasons) from microspores and to ensure thatthey have not originated from the somatic anther wall tissue, genomic DNA was extracted from fresh and healthy leaves of 10-20 randomly selected putative doubled haploid (2n) plants by following the protocol of Dellaporta *et al*. (1983) and amplified using set of eight highly polymorphic SSR markers namely RM15326 (chr. 3), RM15679 (chr. 3), RM19410 (chr. 6), RM21539 (chr. 7), RM22554 (chr. 8), RM22837 (chr. 8), RM6925 (chr. 8), RM23958 (chr. 9) and as described by Yashitola *et al*. (2002), Sundaram *et al*. (2008) and Jaikishan *et al*. (2009) .

**Agro-morphological evaluation of developed DHLs:** A total of 125 regenerated true DHLs ($D_0$) were advanced from $D_1$ to $D_3$ generation were grown for three consecutive seasons (*Rabi* (2016-2017), $D_1$ generation; *Kharif* 2017, $D_2$ generation; *Rabi* (2017-2018), $D_3$ generation) for analyzing their stability, uniformity and for their agro-morphological performance. Seven most crucial yield related agro-morphological characters were recorded from five plants per each entry at suitable stage of the rice plant as per the standard evaluation system recommended by IRRI (IRRI, 2002) in the main field of IIRR, Rajendranagar, Hyderabad, India. For phenotyping of the DHL population, randomized complete block design (RCBD), in two replications, was used. In each replication, five middle plants of each DHL entry were considered for phenotyping. At appropriate growth stage of experimental material, seven traits namely, days to fifty percent flowering (DFF), total grain yield per plant (YLD), total number of grains per panicle (GP), test (1,000) grain weight (TGW), number of productive tillers (PT) and biomass (BM) were recorded from five healthy plants of middle row of each line (in each replication), as per the standard evaluation system recommended by IRRI (IRRI, 2002) and the collected mean data were considered for further analysis.

## Results

The true $F_1$s obtained after crossing IR58025A with its restorer line, KMR-3R, were identified with the help of a parental polymorphic SSR marker RM19660. PCR analysis showed amplification of 250 bp specific to IR58025A and 400 bp band specific to KMR-3R among most of the $F_1$s (**Figure 1**) and the true $F_1$s were then subjected for anther culture to produce doubled haploids.

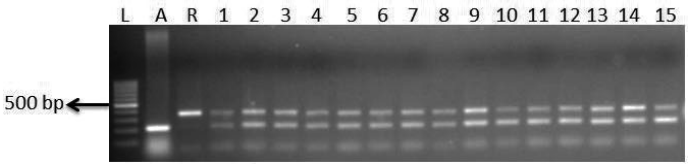


**Figure 1: KRH-2 ($F_1$) purity assessment with RM 19660 (SSR marker); L-100 bp ladder, A-IR58025A (Parent 1), R-KMR-3R (Parent 2), 1-15 KRH-2; $F_1$ samples**

### Anther culture of KRH-2

Only those panicles in which the microspores were in late uni-nucleate (**Figure 2a**) or early bi-nucleate stage (**Figure 2b**) were chosen for anther plating as microspores in these two stages are known to be highly responsive for callus induction. The highest androgenic response was observed in the hybrid when panicles were pre-treated at 8°C for a period of eight days (**Table 5**).

Among the various media used for callus induction, N6 medium was identified to be the best (Table

1). Irrespective of media, among the five different combinations of hormones used for callus induction, a combination of 2 mg $L^{-1}$ 2,4-D and 0.5 mg $L^{-1}$ kinetin elicited the highest callus induction frequency (16.7%-22.4%). Based on the results obtained, N6-4 medium (with 2 mg L-1 of 2,4-D and 0.5 mg L-1 of kinetin) was identified to be the best for callus induction in KRH-2 and its parents, followed by B5-4, MS-4 and He2-4 (Table 1, Figure 3a, Figure 3b). The highest percent of albino plants (25.9%) was observed in parent IR58025B whose calli were induced on He2-4 medium **(Table 1).**

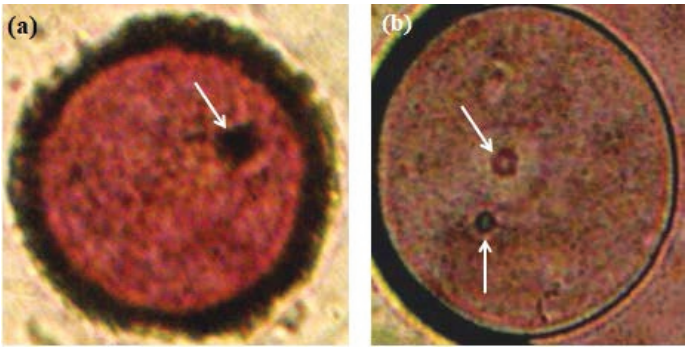


**Figure 2: Anthers in late uni-nucleate/early bi-nucleate stage used for callus induction (nucleus indicated with arrows) (a) Microspore in late uni-nucleate stage after staining with 2% acetocarmine solution (b) Microspore in early bi-nucleate stage showing generative nucleus (small) and vegetative nucleus (big) also after staining with 2% acetocarmine solution**

**Table 1: Response of different media & hormone concentrations on callus induction* and green plant regeneration# in KRH-2 (hybrid) and its parents**

| Culture Medium | Genotype | No. of anthers plated | No. of calli induced | % callus induction ±SE (%)[a] | No. of calli inoculated | No. of calli producing plants | %Green plant regeneration± SE[a] | % Albino plant regeneration±SE[a] |
|---|---|---|---|---|---|---|---|---|
| N6-4 | IR58025B | 7,500 | 1,260 | 16.7±0.56a | 223 | 43 | 19.1± 0.54b | 21.2± 0.68c |
| | KMR-3R | 6,852 | 829 | 12.1±0.58b | 184 | 29 | 15.6± 0.51c | 17.8± 0.81b |
| | KRH-2 | 7,236 | 1,534 | 22.4±0.64c | 280 | 71 | 25.4 ± 0.43a | 15.4± 0.38a |
| B5-4 | IR58025B | 6,500 | 902 | 13.8± 0.11b | 160 | 26 | 16.1± 0.17b | 20.7± 0.47b |
| | KMR-3R | 6,899 | 698 | 10.1±0.66c | 123 | 16 | 13 ± 0.23c | 19.4± 0.68b |
| | KRH-2 | 7,023 | 1,336 | 18.9± 0.82a | 177 | 40 | 22.4 ± 0.62a | 16.4± 0.74a |
| MS-4 | IR58025B | 5,678 | 656 | 11.5± 0.22b | 144 | 18 | 12.4± 0.38b | 24.8± 0.77c |
| | KMR-3R | 6,235 | 575 | 9.2±0.12c | 85 | 9 | 10.5± 0.62c | 20.8± 0.33a |
| | KRH-2 | 7,214 | 1,090 | 15.1±0.85a | 169 | 34 | 20.3 ± 0.67a | 18.7± 0.41b |
| He2-4 | IR58025B | 7,811 | 721 | 9.2 ±0.15b | 121 | 10 | 8.6± 0.14b | 25.9± 0.21c |
| | KMR-3R | 6,778 | 489 | 7.2 ±0.36c | 85 | 8 | 9.2± 0.11b | 24.6± 0.23a |
| | KRH-2 | 5,789 | 644 | 11.1±0.49a | 138 | 22 | 16.2 ± 0.46a | 21.6± 0.54a |

Means with the same letter in a column were not significantly different in Duncan's multiple comparison range test ($p < 0.05$); [a]25 replicates per treatment, repeated thrice

*Callus induction media: N6-1, B5-1, MS-1, He2-1 ( 0.5 mg/L of 2,4-D+ 0.5 mg/L of Kinetin) ; N6-2, B5-2, MS-2, He2-2 ( 1.0 mg/L of 2,4-D+ 0.5 mg/L of Kinetin) ; N6-3, B5-3, MS-3, He2-3 (1.5 mg/L of 2,4-D+ 0.5 mg/L of Kinetin); N6-4, B5-4, MS-4, He2-4 (2.0 mg/L of 2,4-D+ 0.5 mg/L of Kinetin); N6-5, B5-5, MS-5, He2-5 (2.5 mg/L of 2,4-D+ 0.5 mg/L of Kinetin); 0.1 g/l of myo-Inositol , 10 mg/L of silver nitrate; 30 g/L of maltose

#Green plant regeneration medium: MS-1 (0.5 mg/L of BAP+ 0.5 mg/L of kinetin +1.5 mg/L of NAA), MS-2 (1.0 mg/L of BAP+ 0.5 mg/L of kinetin +1.5 mg/L of NAA), MS-3 (1.5 mg/L of BAP+ 0.5 mg/L of kinetin +1.5 mg/L of NAA), MS-4 (2.0 mg/L of BAP+ 0.5 mg/L of kinetin +1.5 mg/L of NAA), MS-5(2.5 mg/L of BAP+ 0.5 mg/L of kinetin +1.5 mg/L of NAA); 30 g/L of sucrose

Based on the results obtained, N6-4 medium (with 2 mg $L^{-1}$ of 2,4-D and 0.5 mg $L^{-1}$ of kinetin) was identified to be the best for callus induction in KRH-2 and its parents, followed by B5-4, MS-4 and He2-4 (**Table 1, Figure 3a, Figure 3b**). Similar to callus induction, the highest percentage of green plant regeneration (25.4%) on optimized MS medium was observed in those calli which were induced from anthers pre-treated at 8°C for 8 days (**Table 5**). Highest frequency of green plant regeneration (15.6-25.4%) was observed in those calli that were induced on N6-4 medium (**Figure 3c-3d**) followed by B5-4, MS-4 and He2-4 media. MS-5 medium (concentrations of BAP, kinetin and NAA being 2.5 mg $L^{-1}$, 0.5 mg $L^{-1}$ and 1.5 mg $L^{-1}$, respectively) was identifed to be the best medium for green plant regeneration (**Table 1, Figure 3c-Figure 3d**). Besides, green plants, a substantial percentage of albino plants in the range of 15.4% to 25.9% was observed. The least percent of albino plants was observed in the hybrid KRH-2 whose calli were induced on N6-4 medium. The highest percent of albino plants (25.9%) was observed in parent IR58025B whose calli were induced on He2-4 medium (**Table 1**).

Regenerated shoots with primary roots were transferred to hormone-free ½ MS medium for the development of more roots (**Figure 3e-3f**). The plants were then grown in Yoshida's solution for hardening (**Figure 3g-3h**). Later, the hardened plants were transferred to earthen pots maintained in biosafety screen house (**Figure 3i**) and were maintained till maturity in field (**Figure 3j-3l**).

The details of number of regenerated plants from anther culture of the three genotypes viz., KRH-2, IR58025B and KMR-3R across two seasons are presented in **Table 2**. Regenerated plants constituted fertile doubled haploids, sterile haploid and polyploid plants [**Figure 3k i-iv**]. In *Kharif* 2015, 270 out of 381 regenerated plants of the KRH-2 hybrid were observed to survive. Among them, 58 were fertile double haploids (2n), 140 were sterile haploids (n) and 72 were sterile polyploid plants. Similarly, in *Rabi* (2015-2016), among the 193 plants survived, 67 plants were observed to be fertile diploids (2n), 56 plants were sterile haploids (n) and 70 were sterile polyploids. Similar trend was noticed in case of the other two genotypes, IR58025B and KMR-3R as well.

**Table 2: Details on the number of regenerated plants along with their ploidy status across two seasons of androgenesis in KRH-2 and in its parents**

| Genotype | No. of regenerated Plants | | No. of survived Plants | | No. of fertile plants (2n) | | No. of sterile plants (n) | | No. of sterile polyploid plants | |
|---|---|---|---|---|---|---|---|---|---|---|
| Season | *Kharif* 2015 | *Rabi* 2015-16 | *Kharif* 2015 | *Rabi* 2015-16 | *Kharif* 2015 | *Rabi* 2015-16 | *Kharif* 2015 | *Rabi* 2015-16 | *Kharif* 2015 | *Rabi* 2015-16 |
| KRH-2 | 381 | 247 | 270 | 193 | 58 (21.5) | 67 (34.7) | 140 (51.9) | 56 (29.0) | 72 (26.6) | 70 (36.3) |
| IR58025B | 266 | 138 | 242 | 114 | 79 (32.7) | 23 (20.2) | 116 (47.9) | 67 (58.8) | 47 (19.4) | 24 (21.0) |
| KMR-3R | 333 | 154 | 266 | 138 | 77 (28.9) | 48 (34.8) | 96 (36.1) | 51 (37.0) | 93 (35.0) | 39 (28.2) |

The percentage value of fertile doubled haploid, sterile haploid and sterile polyploid plants is indicated within the parenthesis.

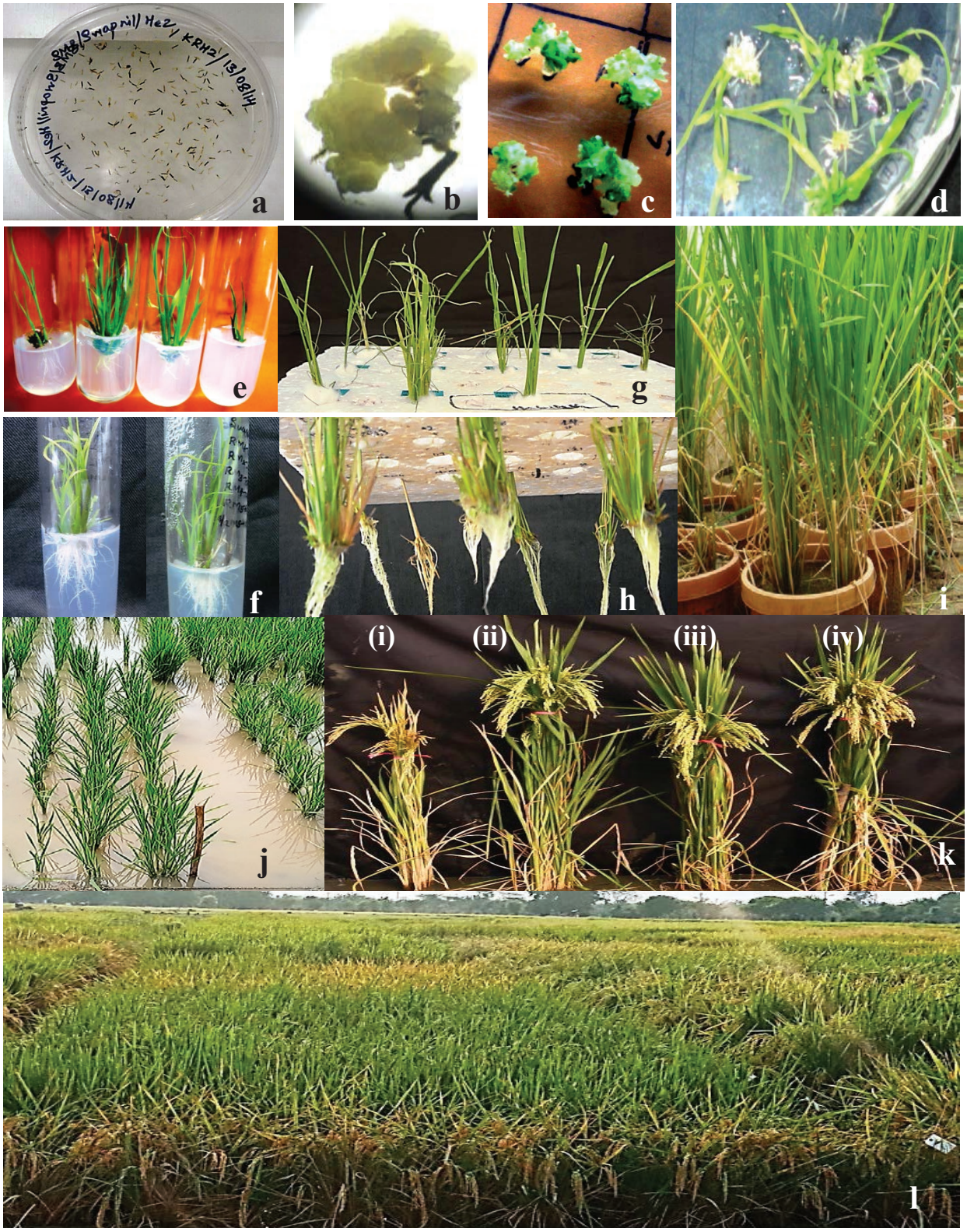


**Figure 3: Various stages of androgenesis in KRH-2**

(a) Plated KRH-2 anthers on callus induction medium (b) Compact calli emerging from anthers of the hybrid 40 days after anther plating. (c-d) Emergence of green and albino shoots from compact calli on MS regeneration medium (RM) after 10 days of transfer of calli on MS RM (e) Regeneration of roots from regenerated shoots in ½ MS medium (hormone free medium) after 10 days of its transfer (f) Root development in half (½) MS medium; (g-h) Plants hardening in Yoshida's nutrient solution (i) Acclimatization of hardened plants in green house (k) Morphological evaluation of anther culture derived plants for ploidy determination at maturity (i) putative haploid sterile plant (ii) sterile polyploid plant (iii)-(iv) fertile doubled haploid plants (j,l) Field establishment of true KRH-2 derived doubled haploid (DH) population (D4 generation) (a) Seedling stage (b) Matured DHLs

**Molecular analysis of hybrids and doubled haploids**

The PCR analysis of regenerated fertile doubled haploid plantlets with parental polymorphic hyper-variable SSR markers namely RM15326, RM15679, RM19410, RM21539, RM22554, RM22837, RM6925 and RM23958 (**Table 3, Figure 4a-4b**) showed amplification identical to either of two parents IR58025A or KMR-3R. Also, 12.67% of induced calli were somatic in origin whereas 68.25% were elicited from microspores. Most of the true and fertile DHLs showed amplification identical to parent KMR-3R.

**Table 3: Details of SSR markers used in the study**

| S. No | SSR/EST-marker name | Chr No. | Physical position (Mb) | Forward Primer's Sequence | Reverse Primer's Sequence |
|---|---|---|---|---|---|
| 1 | RM15326 | 3 | 20.6 | TGAATCTACCGCTCTACTTGTGG | AAACAGTG-CATCCTTCTTGTGG |
| 2 | JGT03-26.8 | 3 | 26.80 | GAGCGTTTGTAGTAAGTTTCATGGAC | GGCCCAACCCAAACA-CAAAT |
| 3 | RM15679 | 3 | 26.87 | TAGATGTATGAGTCGGAATGGAGTCG | CAGACGCAGTGTGTG-TATGAAGTTCC |
| 4 | RM21539 | 7 | 16.44 | GCCCAACTACTTCGACAGCTTCC | CAATGACCTGAGTAG-CATCCAAGG |
| 5 | RM6925 | 8 | 0.64 | GAATGAGAGGACGCTTGAAGAGG | GCATTCAGTC-CCAGCTTGTATCG |
| 6 | RM22554 | 8 | 5.58 | TTGTCAAGATCATCCTCGTAGC | GTCATTCTGCAACCT-GAGATCC |
| 7 | RM22837 | 8 | 12.36 | ACCTGGGTCAGATGTCTGTTTGG | GGTAGAGCTCCATC-CATCTTAGTGC |
| 8 | RM23958 | 9 | 7.94 | CTACCACTGTTTCATTGTGTCTCG | GAATTGAAGGAGAAG-CAGGAAGC |

**Morpho-agronomic performance evaluation in doubled haploids**

Doubled haploids produced in different seasons were evaluated for the following yield and yield related traits: i) days to 50% flowering (DFF), ii) total grain yield per plant (YLD), iii) total number of grains per panicle (GP), iv) test (1,000) grain weight (TGW), v) number of productive tillers (PT) and vi) biomass (BM). Varieties, Akshayadhan (AKD), Varadhan (VRD) and the hybrid KRH-2 were used as checks for comparing the performance of DH population (Table 4).

Flowering duration ranged between 91 days (SMB-7) and 129 days (Akshayadhan, AKD). The mean value of DFF for parents, varietal-hybrid checks along with the DHLs was observed to be 101 days. All better performing DHLs were observed to be early flowering compared with varietal checks and KMR-3R. The total number of grains (GP) varied between 211 (SMB-10) and 364 (SMB-1 (RP6301-189-17-2) with mean value of 310 **(Figure 5)**. Only one DHL, namely, SMB-1, was observed to have more number of grains than the varietal-hybrid checks. The range of TGW trait value was observed to be between 14.6 g

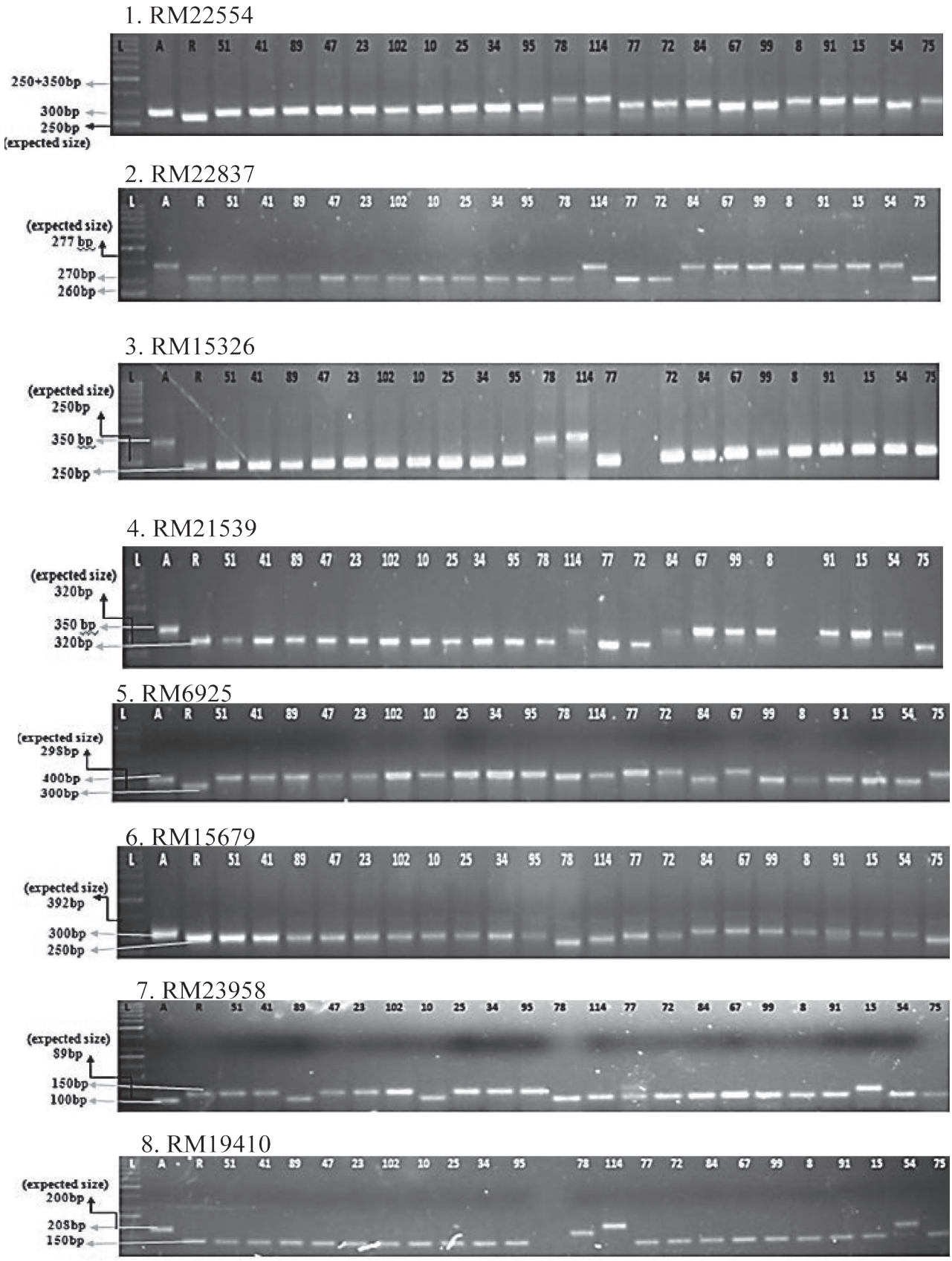


**Figure 4a-4b: Determination of true doubled haploid lines (DHLs) with hyper variable SSR markers; L-100 bp ladder, A-IR58025A (Parent 1), R-KMR-3R (Parent 2), Sample no. 51 to Sample no. 75 are the randomly selected DHLs chosen for confirmation.**

(IR58025B) and 23.68 g (SMB-1) with a mean value of 18.79 g. Number of productive tillers ranged from 8 (SMB-8) to 14 (KMR-3R) with a mean value of 10. No DHL was observed to have more number of productive tillers than KRH-2. For the trait biomass (BM), the range varied from 47.98 g (IR58025B) to 59.98 g (SMB-1) with a mean value of 55.07 g. Among all the DHLs, SMB-1 showed the highest biomass. For grain yield (YLD), the range of values were observed between 18.79 g (IR58025B) and 33.42 g (SMB-1) with a mean value of 24.45 g. Three DHLs namely, SMB-1, SMB-2 (RP6300-188-23-5) and SMB-3 (RP6301-189-27-3-2) were observed to be higher yielding than KRH-2.

**Table 4: Performance of doubled haploid (DH) lines derived from the hybrid IR58025A × KMR-3R**

| | DFF±SE | GP±SE | TGW±SE | PT±SE | BM±SE | YLD±SE |
|---|---|---|---|---|---|---|
| IR58025B | 102±0.23 | 289±0.96 | 14.6±0.98 | 10±0.36 | 47.98±0.13 | 18.79±0.11 |
| KMR-3R | 110±0.15 | 320±0.34 | 18.01±0.77 | 14±0.37 | 52.36±0.11 | 24.23±0.74 |
| KRH-2 | 106±0.36 | 358±0.85 | 22.15±0.32 | 9±0.33 | 58.68±0.59 | 27.94±0.31 |
| AKD | 129±0.12 | 335±0.30 | 20.05±0.15 | 11±0.25 | 51.23±0.45 | 20.08±0.42 |
| VRD | 112±0.15 | 327±0.12 | 22.03±0.11 | 12±0.45 | 53.69±0.23 | 22.03±0.13 |
| ***SMB-1*** | ***98±1.20*** | ***364±0.36*** | ***23.68±0.33*** | ***12±0.67*** | ***59.98±0.71*** | ***33.42±0.26*** |
| ***SMB-2*** | ***92±0.88*** | ***350±0.18*** | ***20.78±0.29*** | ***9±0.88*** | ***58.99±0.69*** | ***30.84±0.43*** |
| ***SMB-3*** | ***104±0.88*** | ***322±0.08*** | ***20.14±0.33*** | ***11±0.58*** | ***57.66±0.62*** | ***29.97±0.33*** |
| SMB-4 | 93±0.33 | 286±0.61 | 19.21±0.29 | 9±0.88 | 56.55±0.50 | 25.77±0.43 |
| SMB-5 | 97±0.88 | 294±0.12 | 19.07±0.58 | 10±1.20 | 50.38±0.58 | 25.38±0.35 |
| SMB-6 | 92±0.58 | 322±0.41 | 18.04±0.58 | 10±0.76 | 55.45±0.62 | 23.44±0.58 |
| SMB-7 | 91±0.88 | 310±0.21 | 16.22±0.67 | 11±0.88 | 56.45±0.51 | 22.78±0.29 |
| SMB-8 | 99±1.20 | 289±0.70 | 17.44±0.58 | 8±0.45 | 58.44±0.28 | 21.48±0.21 |
| SMB-9 | 93±1.20 | 277±0.52 | 15.11±0.33 | 9±0.89 | 54.77±0.39 | 19.44±0.08 |
| SMB-10 | 101±0.67 | 211±0.52 | 15.33±0.58 | 9±0.84 | 53.44±0.32 | 21.22±0.41 |
| Mean | 101 | 310 | 18.79 | 10 | 55.07 | 24.45 |
| SD | 10.14 | 38.48 | 2.75 | 1.58 | 3.52 | 4.41 |
| SE | 2.62 | 9.93 | 0.71 | 0.41 | 0.91 | 1.14 |
| CV(%) | 10.01 | 12.40 | 14.64 | 15.39 | 6.40 | 18.02 |

DFF-Days to 50% percent flowering; GP-Total grains/panicle; TGW-Test (1,000) grain weight (g); PH-Plant height (cm); PT-Number of productive tillers; BM-Biomass (g); YLD-Total grain yield/plant(g); SD-Standard Deviation, SE-Standard Error, CV%-Coefficient of Variation (CV) in percentage; The best performing three DHLs shown in italics and bold, AKD-Akshayadhan, VRD-Varadhan.

**Table 5: Two seasons mean data of cold shock pre-treatment regimes on callus induction on N6 medium and on green plant regeneration percentage on MS medium of parents IR58025B, KMR-3R and hybrid KRH-2 in *Kharif* 2015 and *Rabi* (2015-2016)**

| Temperature-4°C | | | | |
|---|---|---|---|---|
| **S.No** | **Genotype** | **Duration of cold shock (days)** | **Callus induction (%) on N6 medium (Mean±SE)** | **Green plant regeneration (%) (Mean±SE)** |
| 1 | IR58025B | 2 | 2.17±0.40 | 4.73±0.94 |
| | | 4 | 4.96±0.57 | 6.04±0.59 |
| | | 6 | 7.13±0.71 | 7.12±0.13 |
| | | 8 | 8.56±0.54 | 9.97±0.64 |
| | | 10 | 8.15±0.59 | 9.92±0.84 |
| 2 | KMR-3R | 2 | 1.96±0.43 | 2.7±0.52 |
| | | 4 | 4.21±0.78 | 3.8±0.81 |
| | | 6 | 5.96±0.43 | 4.53±0.32 |
| | | 8 | 7.86±0.61 | 5.78±0.50 |
| | | 10 | 5.69±0.59 | 8.74±0.26 |
| 3 | KRH-2 | 2 | 2.91±0.55 | 12.26±0.46 |
| | | 4 | 5.09±0.73 | 13.76±0.69 |
| | | 6 | 7.62±0.85 | 19.41±0.06 |
| | | 8 | 10.51±1.01 | 25.67±0.63 |
| | | 10 | 5.89±0.76 | 15.96±0.13 |
| **Temperature-8°C** | | | | |
| 1 | IR58025B | 2 | 3.79±0.68 | 5.73±0.23 |
| | | 4 | 5.80±0.59 | 7.30±0.07 |
| | | 6 | 6.45±0.47 | 8.72±0.47 |
| | | 8 | 8.60±0.86 | 10.05±0.80 |
| | | 10 | 6.99±0.84 | 14.53±0.37 |
| 2 | KMR-3R | 2 | 2.87±0.70 | 4.18±0.32 |
| | | 4 | 4.87±0.55 | 6.72±0.93 |
| | | 6 | 6.16±0.68 | 7.43±0.60 |
| | | 8 | 6.88±0.79 | 8.63±0.66 |
| | | 10 | 5.15±0.69 | 7.60±0.53 |
| 3 | KRH-2 | 2 | 12.77±0.18 | 12.35±0.21 |
| | | 4 | 14.17±0.25 | 14.81±0.37 |
| | | 6 | 14.82±0.22 | 18.72±0.22 |
| | | **8** | **17.59±0.23** | **26.37±0.02** |
| | | 10 | 12.25±0.02 | 16.61±0.27 |

| Table 5 (continued) | | | | |
|---|---|---|---|---|
| **S.No** | **Genotype** | **Duration of cold shock (days)** | **Callus induction (%) on N6 medium (Mean±SE)** | **Green plant regeneration (%) (Mean±SE)** |
| **Temperature-12°C** | | | | |
| 1 | IR58025B | 2 | 4.27±0.87 | 5.90±0.89 |
| | | 4 | 5.36±0.45 | 8.10±0.25 |
| | | 6 | 7.89±1.86 | 9.63±0.14 |
| | | 8 | 9.05±0.83 | 11.07±0.35 |
| | | 10 | 7.89±0.70 | 9.35±0.38 |
| 2 | KMR-3R | 2 | 3.26±0.73 | 4.17±0.17 |
| | | 4 | 4.78±0.61 | 9.82±0.27 |
| | | 6 | 5.87±0.79 | 8.56±0.61 |
| | | 8 | 6.17±0.86 | 9.45±0.68 |
| | | 10 | 5.24±0.47 | 6.78±0.17 |
| 3 | KRH-2 | 2 | 8.57±0.94 | 14.05±0.18 |
| | | 4 | 10.10±0.74 | 17.19±0.25 |
| | | 6 | 11.57±1.22 | 14.82±0.22 |
| | | 8 | 14.90±1.02 | 17.59±0.23 |
| | | 10 | 10.88±0.85 | 12.25±0.02 |

The highest response for both the stages is indicated in bold

**Figure 5**: **The best performing DH line (SMB-1) showing larger panicles and more number of productive tillers and of highly desirable medium slender (MS) grain type.**

## Discussion

The purpose of the study was to develop the doubled haploid (DH) lines from an elite rice hybrid through which trait fixation viz., yield/plant (YLD) could be possible. The stable, fixed and homozygous DHLs were utilized for QTL mapping of yield and its allied traits. KRH-2 is one of the elite, stable and widely adaptable rice hybrid across the country with higher yields and with desirable grain type. Long term goal of the study was to develop DHLs from a high yielding rice hybrid, KRH-2 and to utilize the developed DHLs as pre-breeding material for producing positively heterotic novel hybrids than KRH-2. Moreover, the successful development of doubled haploids has been routinely undertaken from some of the elite hybrids in the recent past for improving the yield/plant, grain quality and grain type. A similar kind of study was done by Mishra *et al*. (2015) where promising DHLs were produced through anther culture of two elite rice hybrids.

The KRH-2 hybrid was produced in every season by undertaking crosses between its parents viz., IR58025A and KMR-3R at IIRR. In order to rule out the KRH-2 hybrid's seed production from out-crossing, the heterozygosity assessment (purity assessment) of the KRH-2 hybrid along with its parents was undertaken with a hyper-variable SSR marker, which amplified specific alleles for both the parents. As fresh KRH-2 hybrid seeds were produced in every season and which were employed for DHL production, therefore, commercial hybrid was not used in the study. Checking the pollen fertility of the newly developed test-cross derived hybrids is one of the routinely followed procedures for checking the fertility restoration in novel hybrid. In this study, the pollen of freshly produced KRH-2 hybrid is expected to be completely fertile as KMR-3R (its donor parent) is an elite restorer. To validate the success of crosses made in every season and to validate complete fertility restoration in fresh KRH-2 hybrid, the pollen fertility test was done. We did not have any mechanism to avoid the plating of sterile anthers as most of the anthers were fully fertile.

As opined by Balachandran *et al.* (1999), the androgenic response was genotype specific, so there was a necessity to optimize the anther culture conditions for every genotype. Therefore, as a part of optimization of the anther culture conditions for the hybrid KRH-2 and its parents, many media-hormonal combinations were tried. Out of many anther culture protocols published by this group, the highest androgenic response was observed when protocol enlisted in Balachandran *et al*. (1999) was followed.

Several factors are reported to influence the anther culture response in crops. The process of androgenesis is genotype specific (Kaushal *et al*. 2015). Other factor that significantly influences the successful regeneration of fertile plants through androgenesis is the nutrient medium employed for callus induction and plant regeneration. Our study clearly demonstrated that the anther culture response is significantly dependent on the interaction between the genotype and media and not a single factor is responsible for bringing out the best anther culture response. Similar observations have been made in previous studies (Chen, 1991; Khalequzzaman *et al* 2005; Bagheri and Jelodhar, 2008). Through the present study, we optimized a few components of anther culture media and established that N6 medium (Chu *et al*. 1975) was the most suitable for callus induction from anthers of KRH-2. This is in congruence with few earlier reports (Mishra *et al* 2011; Mishra *et al* 2015; Naik *et al* 2016).

The androgenesis of the parents was undertaken in order to assess the difference between the parents and the hybrid for androgenic response. Further, the magnitude of difference between the parents was used to gauge KRH-2 hybrid's anther culture response. As opined by Kaushal *et al* (2014), the utility of parents for anther culture along with the hybrid is useful in knowing which of the two parent's gene (s) contribute for hybrid's higher androgenic response. Among the two parents, IR58025B showed higher callus induction percentage (16.7%) than KMR-3R (12.1%), while the callus induction in the hybrid, KRH-2, was observed to be 22.4%. The highest green plant regeneration percentage in the optimized MS medium (MS-5) was observed in IR58025B (19.1%), followed by KMR-3R (15.6%) while the hybrid showed 25.4% (Table 1). The $F_1$ hybrid recorded a higher callus induction and green plant regeneration response than both the parents. Similar results were reported by Herath and Bandara, 2011.

Plant growth regulators have been categorically proved to significantly affect the callus induction and green plant regeneration phases of anther culture (Ball *et al* 1993). We demonstrated that an optimum concentration of 2 mg $L^{-1}$ of 2,4-D and 0.5 mg $L^{-1}$ of kinetin promoted callus induction efficiency as shown earlier by Mishra *et al* (2015) and Naik *et al* (2016). The same concentration of 2,4-D and kinetin was used by Hooghvrost *et al* (2018) for the development of DHLs.

In addition to callus induction, regeneration of green plants is another vital step in anther culture. In this study, the green plant regeneration was carried out in MS medium supplemented with BAP, kinetin and NAA. Optimum concentration of BAP, kinetin and NAA for successful shoot regeneration in MS-5 medium was 2.5, 0.5 and 1.5 mg $L^{-1}$. As observed by Naik *et al* (2016), the increase in the concentration of BAP from 1.5 to 2 mg $L^{-1}$ decreased the rate of shoot regeneration from anther calli by 1.5 times. Similar observation was reported by Kaushal *et al* (2014) where higher concentration of BAP (2.5 mg $L^{-1}$) decreased the frequency of shoot regeneration. In the present study, we observed an increment in rate of shoot regeneration by 23% when BAP concentration was increased from 2 to 2.5 mg $L^{-1}$. Similarly, the rate of primary root formation in regenerated shoots was observed to increase by 17% when the concentration of NAA was increased from 1 to 1.5 mg $L^{-1}$ in our study against the earlier reports (Kaushal *et al* 2014; Mishra *et al* 2015). Thus, based on the results of the present study it is clear that N6 medium is the most suitable for successful callus induction at optimized concentrations of 2,4-D (2 mg $L^{-1}$), kinetin (0.5 mg $L^{-1}$), maltose (30 g $L^{-1}$) and silver nitrate (10 mg $L^{-1}$) and for plant regeneration MS medium with 2.5 mg $L^{-1}$, BAP, 0.5 mg $L^{-1}$ kinetin and 1.5 mg $L^{-1}$ NAA, was confirmed to be optimum. The Duncan's multiple range test (DMRT) showed the significant differences among the genotypes under study which was due to effect of different media-hormonal compositions on callus induction and green plant regeneration phases.

The initial determination of the ploidy of the regenerated plants was determined based on plant morphology and its fertility/sterility status. Later, the doubled haploidy of the fertile plants was confirmed using a set of hyper-variable SSR markers. A thorough analysis of the DHLs for their ploidy status was done by employing hyper-variable SSR markers (co-dominant in nature, therefore suitable for the analysis). Three different categories of the doubled haploids (DH) are reported to be regenerated from microspores. Those diploid plants which are produced as a result of chromosome doubling (normal reduced gametes), the second being the somatic cell, particularly anther wall induced diploids and the third, are produced from unreduced gametes (Chani *et al* 2000). In 12.67% of the calli that were induced, it was observed that they showed amplification identical to both the parents IR58025A and KMR-3R when assessed with a set of hyper-variable SSRs indicating their origin from the somatic (2n) anther cell wall whereas 68.25% of the induced calli showed allelic amplification of either of the two parents (as shown in Figure 4a-4b) indicating their origin from microspores (n). Further, the utility of the SSR markers for the identification of true doubled haploid has been well-documented in rice and in other crop systems (Tang *et al* 2006; Shahid *et al* 2013; Wu *et al* 2015). The analysis of the DHLs with carefully selected hyper-variable SSR markers viz., RM15326 (chr.3), RM15679 (chr.3), RM19410 (chr.6), RM21539 (chr.7), RM22554 (chr.8), RM22837 (chr.8), RM6925 (chr.8), RM23958 (chr.9) (www.gramene.org; Jaikishan *et al*. 2009; Rajendrakumar *et al*. 2009) demonstrated that these markers for multiple loci in different DHLs indicating the regenerants to be true DHLs. Moreover, these markers showed clear amplification and true segregation of alleles of both the parents of the hybrids which was sufficient to show that doubled haploids were indeed true and fertile. Our observations are in accordance with Zhang (1989), Olufowote *et al* (1997) and Tang *et al* (2006).

Pertaining to the polyploid plants, we observed that these plants were unusually tall and more importantly were completely sterile. Such plants were discarded after their morphological assessment at maturity stage. The utility of the SSR markers was done to identify/differentiate those doubled haploids which were produced as a result of spontaneous chromosome doubling. The other two groups of doubled haploids *viz*., those induced from anther cell wall (somatic in origin) and those from unreduced gametes were of least interest to us. Therefore, as can be inferred from Figure 4a-4b, only those doubled haploids which

showed an allelic amplification to either of the two parents were considered to be true DHLs. Therefore, determination of polyploidy using SSR markers was not possible and polyploids were discarded based on their plant morphology and sterility, upon their maturity.

Agro-morphological evaluation of 10 KRH-2 derived DHLs showed a range of variation for prominent agronomic traits such as DFF, YLD, TGW and PT. Negligible variation was observed within the population for successive generations indicating a high level of uniformity/stability. The agro-morphological data of the 10 DHLs along with the parents, hybrid KRH-2 and varietal checks presented in Table 4 is the three seasons' mean for all the yield related traits when ten plants (replicates) were considered. As the standard error (SE) of the mean data of each DHLs for yield related traits was observed to be below 1, we inferred that there was negligible agro-morphological variation within the individuals (replicates) of each DHL across three consecutive seasons demonstrating the stability of these DHLs.

Iso-cytoplasmic restorer lines are developed as a process of selfing the promising hybrids. The process of androgenesis or doubled haploidy is akin to selfing as the haploid (n) genome of microspores of a hybrid undergoes endo-reduplication to produce diploid (2n) plants on proper stimulation. The fertile DH plants possess genomes from either of parents but not from both. Iso-cytoplasmic restorer lines carry male sterile cytoplasm which is similar to cytoplasmic male sterile (CMS) lines, thus reducing the cyto-nuclear conflict (Kumar *et al*. 2017). Moreover, similar type of cytoplasm that iso-cytoplasmic lines and CMS lines have rules out the necessity of test crosses for assessment of restoration potential. The genetic relationship among most of the popular Indian hybrids is termed as half-sib, having 50 percent in commonality, as IR58025A being the common CMS line. Therefore, the iso-cytoplasmic restorer lines serve as a better tool for restorer line diversification. We did not observe any recombinants among the regenerated DHLs as no heterozygous loci were amplified with a set of eight hyper-variable SSRs indicating that the DHLs were regenerated as a result of chromosomal doubling of normal reduced gametes.

Evaluation of DHL population for three consecutive seasons indicated that the lines, SMB-1, SMB-2 and SMB-3 showed significantly higher yield levels compared to the hybrid and the parental lines. SMB-1 showed yield increment of 19.6%, followed by SMB-2 (10.4%) and SMB-3 (7.3%) as compared to KRH-2. As the number of productive tillers and biomass in these three high yielding DHLs were slightly more than KRH-2, the yield increase may be justified. Also, slightly more number of GP and higher TGW of SMB-1 (RP6301-189-17-2) than KRH-2 might have contributed to the latter's higher percentage of YLD heterosis. All the hybrid and varietal checks used in this study are of medium duration, high-yielding with wide cultivation across the country. These commercially released hybrids showed a mean grain yield of 5-7 t /ha with an average yield increment of 15-20% over the checks of the same duration. Our results demonstrated that SMB-1, SMB-2 and SMB-3 showed a positive standard heterosis with respect to KRH-2 which is a high yielding and one of the popular hybrids in India. Further, SMB-1 showed the highest percentage of standard heterosis of 19.61% over KRH-2 which may be considered for commercialization.

## Conclusions

The present study analyzed the effect of four different callus induction media and percentage of callus induction in the elite rice hybrid, KRH-2 and established that N6 medium was the most efficient providing for higher green plant regeneration (when plant regeneration was carried out in MS regeneration medium) and lowest albino frequency. Using this optimized protocol, a total of 125 DHLs were developed in this study. Three promising DHLs (SMB-1 (RP6301-189-17-2), SMB-2 (RP6300-188-23-5) and SMB-3 (RP RP6301-189-27-3-2) have been identified which recorded equivalent or superior yield and agro-morphological parameters as compared to KRH-2.

## References


Baenziger PS, Russell WK, Graef GL and Campbell BT. 2006. Improving Lives: 50 years of crop breeding, genetics and cytology. *Crop Science,* 46 :2230-2244.

Baenziger PS, Wesenberg DM, Smail VM, Alexander WL and Schaeffer GW. 1989. Agronomic performance of wheat doubled haploid lines derived from cultivars by anther culture. *Plant Breeding,* 103, 101-109.

Bagheri N, Jelodar NB. 2008. Combining ability and heritability of callus induction and green plant regeneration in rice anther culture. *Biotechnology Journal*. 7(2): 287-292.

Balachandran SM, Sarma NP, Siddiq E. 1999. Inheritance of anther culture response in rice. *Current Science,* 77: 962-964.

Ball ST, Zhou H, Konzak CF. 1993. Influence of 2, 4-D, IAA and duration of callus induction in anther cultures of spring wheat. *Plant Science,* 90: 195-200.

Chen CC, Tsay HS, Huang CR. 1991. Factors affecting androgenesis in rice (*Oryza sativa* L.) In: Bajaj YPS (ed) Rice Biotechnology in Agriculture and Forestry Springer Verlag, Berlin 14: 193-211.

Chani E, Veilleux RE, Boluarte Medina T. 2000. Improved androgenesis of inter-specific potato and efficiency of SSR markers to identify homozygous regenerants. *Plant Cell Tissue and Organ Culture,* 60:101–112.

Cho MS, Zapata FJ. 1988. Callus formation and plant regeneration in isolated pollen culture of rice *Oryza sativa* L. cv. Taipei 309. *Plant Science,* 58: 239-244.

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Scientia Sinica,* 16: 659-688.

Collard BCY, Jahufer MZZ, Brouwer JB and Pang ECK. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts, *Euphytica*, 142: 169–196.

Dellaporta SL, Jonathan W, James BH. 1983. A plant DNA mini preparation: Version II. *Plant Molecular Biology Reporter,* 1 (4), pp 19-21.

Forster BP, Thomas WTB. 2005. Doubled haploids in genetics and plant breeding. *Plant Breeding Reviews,* 25: 57–88.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research,* 50: 151-158.

Grewal RK, Lulsdorf M, Croser J, Ochatt S, Vandenberg A, Warkentin TD. 2009. Doubled-haploid production in chickpea (*Cicer arietinum* L.): Role of stress treatments. *Plant Cell Reports,* 28: 1289 –1299.

Han H, Huang B. 1987. Application of pollen-derived plants to crop improvement. *International Review of Cytology,* 107:293–313.

Herath HMI, Bandara DC. 2011. Anther culture performance in selected high yielding *indica* (of Sri Lanka) and *japonica* rice varieties and their inter sub-specific hybrids. *Journal of National Science Foundation,* Sri Lanka. 39: 149-154.

Hooghvorst Isidre, Ramos-Fuentes Eduardo, López-Cristofannini, Camilo, OrtegaMirari, Vidal Raimon, Serrat Xavier, Nogués Salvador. 2018. Antimitotic and hormone effects on green double haploid plant production through anther culture of Mediterranean japonica rice. *Plant Cell Tissue and Organ Culture,* 134(2): 205–215.

Hu H. 1985. Use of haploids for crop improvement in China. *Genetic Manipulation Crops Newsletter,* 1: 11-23.

Huang HG, Ling HS, Tseng PL, Shien YL, Shi P. 1978. Studies of medium component in anther culture of rice (*Oryza sativa* L.) subsp Hsien by mathematical methods. Proceedings Symposium, *Plant Tissue Culture,* Science Press, Peking pp. 244-246.

IRRI. 2002. Standard Evaluation System for Rice (SES). Los Banos. Philippines: International Rice Research Institute (IRRI). 4 ed., pp. 15–16.

Jahne A, Lorz H. 1995. Cereal microspore culture. *Plant Science*, 109: 1-12.

Jaikishan I, Rajendrakumar P, Ramesha M, Viraktamath B, Balachandran S, Neeraja C, Sujatha K, Rao K, Natarajkumar P, Yadla H, Kannabiran S, Ramaprasad A, Sundaram R. 2009. Prediction of heterosis for grain yield in rice using 'key' informative EST-SSR markers. *Plant Breeding*, 129. 108 - 111. 10.1111/j.1439-0523.2009.01633.x.

Kaushal L, Balachandran SM, Ulaganathan K, Vinay Shenoy. 2014. Effect of culture media on improving anther culture response of rice (*Oryza sativa* L.) *International Journal of Agriculture Innovations and Research,* 3 (1): 218-224.

Kaushal L, Balachandran SM, Ulaganathan K, Singh Akhilesh Kumar, Priyadarshi R Shenoy V. 2015. Auxin to improve green plant regeneration of rice anther culture. *International Journal of Agriculture and Crop Sciences,* 8(1): 15-26.

Khalequzzaman M, Haq N, Hoque ME, Aditya TL. 2005. Regeneration efficiency and genotypic effect of 15 *indica* type Bangladeshi rice (*Oryza sativa* L.) landraces. *Plant Tissue Culture & Biotechnology,* 15: 33-42.

Mishra R, Rao RN, Rao GJN. 2011. Anther culture response of *indica* rice hybrids. *Oryza,* 48(4): 375–377.

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. *Journal of Experimental Biology and Agricultural Sciences,* 1(2): pp. 69-76.

Mishra R, Rao GJN, Rao RN, Kaushal P. 2015. Development and characterization of elite doubled haploid lines from two *indica* rice hybrids. *Rice Science,* 22(6): 290-299.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum,* 15: 473-497.

Naik N, Rout P, Ngangkham U, Verma R L, Katara J L, Sahoo K K , Singh O N, Samantaray S. 2016. Development of doubled haploids from an elite *indica* rice hybrid (BS6444G) using anther culture. *Plant Cell, Tissue and Organ Culture,* 128: 679-689.

Olufowote JQ, Xu Y, Chen X, Park WD, Beachell HM, Dilday RH, Goto M and Mccouch SR. 1997. Comparative evaluation of within-cultivar variation of rice (*Oryza sativa* L.) using microsatellite and RFLP markers. *Genome,* 40 (3): 370-378.

Paterson AH, Lan TH, Reischmann KP, Chang C, Lin YR, Liu SC, Burow MD, Kowalski SP, Katsar CS, DelMonte TA, Feldmann KA, Schertz KF, Wendel JF. 1996. Toward a unified genetic map of higher plants, transcending the monocot-dicot divergence. *Nature Genetics,* 14: 380-382.

Raina SK, Zapata FJ. 1997. Enhanced anther culture efficiency of *india* rice (*Oryza sativa* L.) through modification of the culture media. *Plant Breeding,* 116: 305-315.

Ratheika V, Silva TD. 2007. A study on the anther culture response in varieties of rice (*Oryza sativa* L.) subspecies *indica*. In: Proceedings of the 27th Annual Sessions of the Institute of Biology, Sri Lanka: 28.

Shahid MQ, Chen FY, Li HY, Wang SZ, Chen PF, Lin SQ, Liu XD, Lu YG. 2013. Double-Neutral Genes, *Sa-n* and *Sb-n*, for Pollen Fertility in Rice

to Overcome *Indica* × *Japonica* Hybrid Sterility. *Crop Science,* 53(1):164–176.

Senadhira D, Zapata-Arias FJ, Gregorio GB, Alejar MS, De La Cruz HC, Padolina TF, Galvez AM. 2002. Development of the first salt tolerant rice cultivar through *indica*/*indica* anther culture. *Field Crops Research,* 76: 103–110.

Snape JW. 1989. Doubled haploid breeding: Theoretical basis and practical applications, in: A. Mujeeb-Kazi and L.A. Stich (Eds.), Review of Advances in Plant Biotechnology, 1985-88: 2nd International Symposium on Genetic Manipulation in Crops, CIMMYT, Mexico D.F., Mexico and IRRI, Manila, Philippines. pp. 19-30.

Sundaram RM, Vishnupriya MR, Biradar SK, Laha GS, Reddy GA, Rani NS, Sarma NP, Sonti RV. 2008. Marker assisted introgression of bacterial blight resistance in Samba Mahsuri, an elite *indica* rice variety. *Euphytica,* 160: 411–422.

Suriyan C, Bootsaya S, Aussanee P, Chalermpol K. 2009. An efficient procedure for embryogenic callus induction and doubled haploid plant regeneration through anther culture of Thai aromatic rice (*Oryza sativa* L.subsp. *indica*). *In Vitro Cellular & Developmental Biology – Plant*, 45(2): 171–179.

Tang F, Tao Y, Zhao T, Wang G. 2006. *In vitro* production of haploid and doubled haploid plants from pollinated ovaries of maize (Zea mays). *Plant Cell Tissue and Organ Culture,* 2006 84 (2):233–237.

Tinker NA, Mather DE, Rossnagel BG, Kasha KJ, Kleinhofs A, Hayes PM, Falk DE, Ferguson T, Shugar LP, Legge WG, Irvine RB, Choo TM, Briggs KG, Ullrich SE, Franckowiak JD, Blake TK, Graf RJ, Dofing SM, SaghaiMaroof MA, Scoles GJ, Hoffman D, Dahleen LS, Kilian A, Chen F, Biyashev RM, Kudrna DA, and Steffenson BJ. 1996. Regions of the genome that affect agronomic performance in two-row barley. *Crop Science,* 36: 1053-1062.

Wang BN, Huang Z, Shu LH, Ren X, Li XH, He GC. 2001. Mapping of two new brown plant hopper resistance genes from wild rice. *Chinese Science Bulletin,* 46: 1092–1095.

Wu X, Chang X, & Jing R. 2012. Genetic insight into yield associated traits of wheat grown in multiple rain-fed environments. *PLOS ONE,* 7(2): e31249.

Wu J, Shahid MQ, Chen L, Chen Z, Wang L, Liu X, Lu Y. 2015. Polyploidy enhances $F_1$ pollen sterility loci interactions that increase meiosis abnormalities and pollen sterility in autotetraploid rice. *Plant Physiology,* 169(4):2700–2717.

Yashitola J, Thirumurugan T, Sundaram RM, Naseerullah MK, Ramesha MS, Sarma NP and Sonti Ramesh V. 2002. Assessment of purity of rice hybrids using microsatellite and STS markers, *Crop Science,* 42: 1369-1373.

Yuan LP. 1994. Increasing yield potential in rice by exploitation of heterosis. In: Virmani SS (ed) Hybrid rice technology: new developments and future prospects. IRRI, Manila, pp 1–6.

Zapata-Arias FJ. 2003. Laboratory protocol for anther culture technique in rice. In: Mauszynski M., K.J. Kasha, Forster BP. Doubled haploid production in crop plants, a manual. Kluwer Academic Publishers, Dordrecht: 109-116.

Zhang ZH. 1989. The practicability of anther culture breeding in rice. In: MujeebKazi A &Sitch LA (Eds.) Review of advances in plant biotechnology, 1985–1988, CIMMYT, Mexico & IRRI Manila. pp 31–42.