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Anther Derived Haploid Production in Rice and Identification of True Haploids by Markers and Flow Cytometry

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Abstract

Anther culture, an unconventional approach, could be a complementary technique along with conventional breeding for rice improvement, which induces homozygosity and developing true breeding lines whilst shortening the breeding cycle of new varieties.The present study was taken up to develop a high throughput and reproducible protocol for anther culture and development of haploids and doubled haploids from the F1 anthers of a rice hybrid, KRH-4. Among the media used for callus induction, the media which have basal N6 along with varying concentration of growth hormones has given the highest callus induction efficiency. Further, with respect to regeneration, no significant differences were observed between the two regeneration media R_1 and R_2 tried. The plantlets generated from anthers were segregated into haploids, diploids and doubled haploids based on polymorphic markers and flow cytometry analysis and the true haploids were treated with colchicine to produce doubled haploids.

Doubled Haploid Technology has the unique genetic property of producing completely homozygous lines from heterozygous parents in a single generation. Using pollenor ovules, the haploids can be generated and with colchicine treatment, chromosome doubling can be made. Anther culture in rice breeding can reduce breeding time, increase selection efficiency and also contributes to the development of doubled-haploid or di-haploid lines which are useful for developing mapping populations for molecular analysis (Lapitana *et al*., 2009). In fact, several doubled-haploids have been developed and selected for salinity stress tolerance following anther culture and are being extensively used in breeding programs (Senadhira *et al*., 2002).

Many factors are known to affect haploid induction and subsequent regeneration. Some of them are genotype of the donor plants, developmental stage of gametes, pre-treatment, composition of the culture medium, physical factors during tissue culture (Touraev *et al*., 1996; Datta *et al*., 1990). Therefore, a suitable and high throughput protocol has to be standardized for large scale production of haploids from anther. In this context, a research was initiated to develop a high throughput and reproducible protocol for anther culture and development of doubled haploids from the F1 anthers of a rice hybrid, KRH-4.

A popular rice hybrid, KRH-4 was sown in the field as well as in the greenhouse and the panicles was collected before their emergence and checked for their appropriate stage (uninucleate and early binucleate stage) under the microscope (Chen, 1976; Huang *et al*., 1986). The selected panicles were wrapped in aluminium foil and stored at a standardized temperature of 8°C for 8 days and then surface sterilized using 0.1 per cent mercuric chloride for 4-5 min followed by rinsing with distilled water for 2-3 times and again the anthers were inoculated in callus induction media (Plate 1-a).The inoculated anthers were incubated in dark at 25±1°C and the observation on the anther response to callus induction was recorded 3–4 weeks after inoculation (Rukmini *et al.*, 2011).

Five anther induction media (T_1 to T_5) were tried with N6 (Chu *et al*., 1975) as the basal medium (Table I). Sterilized anthers were inoculated in these media separately and the callus inducing response was observed.

The media containing kinetin gave significantly higher callus inducing response compared to the other media having BAP as a source of cytokinin. However, although treatment T_5 contained kinetin, the response was poor due to lack of nutritional supplements. Media T_4 which lacks NAA showed relatively less response

(5.41%) compared to T_1 (6.97%) and T_2 (7.44%) which had NAA in them (Figure 1a).

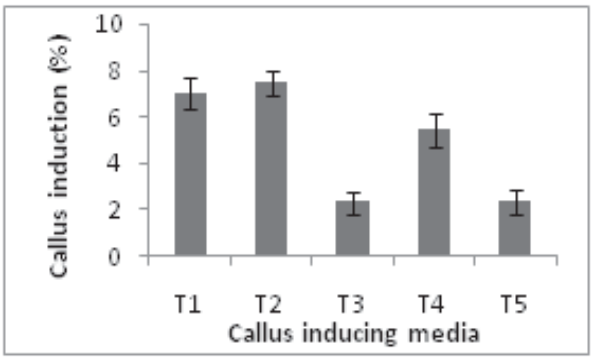
Embryogenic calli of 1-3 mm size (Plate 1-b) was used for the regeneration of green shoots where 2 different media R_1 and R_2were used (Table 1). The cultures were incubated with a 16-h light / 8-h dark regime at 25±1°C under artificial light (2000 lux) and observed for regeneration. Regeneration media, R_1 was found to be better as it has induced more green shoots (Plate 1-c) than R_2 (Figure 1b). In both the regeneration media, vigorous root development was noticed in R_1 and R_2, R_1 inducing better root development (Plate 1- d) compared to R_2 and relatively no other rooting media was used.

As the regenerated plantlets are originated from anther which has haploid (microspores) and diploid (anther wall) tissues, the plantlets derived from anthers may contain a mixture of haploid and diploid plants. Further in rice, spontaneous diploidization do occur to the extent of 72 per cent (Cho and Zapata, 1990). Towards segregating the plants and identifying true haploids, polymorphic SSR markers were used (Plate 1-e). With SSR markers, the haploids and doubled haploids have given out monomorphic bands, while, diploids have given out heterozygous bands. From this, the diploids were segregated and only the haploids and doubled haploids with monomorphic bands were retained. To separate the true haploids from doubled haploids, ploidy analysis was carried out using flow cytometer. The output from the flow cytometer is in the form of 1C for haploids and 2C for diploids and spontaneous doubled haploids. Based on these approaches (marker and flow cytometry data), true haploids and doubled haploids were identified (Table II).

TABLE I

Composition of anther induction media and regeneration media

Media	Basic components	Hormones and other supplements
Anther induction media		
T_1	3.97g / L N6 + 0.5mg / L B5 Vitamins +	2mg/ L 2,4 D + 0.5 mg/ L Kinetin + 1mg/ L NAA + 4mg/ L $AgNO_3$
T_2	0.5g / L (Proline + Casamino acid +	2mg/ L 2,4 D + 0.5 mg/ L Kinetin + 1mg/ L NAA
T_3	Casein acid + Glutamine) + 30 g/ L	2mg/ L 2,4 D + 1mg/ L NAA + 0.5 mg/ L BAP
T_4	sucrose + 6g / L agar	2mg/ L 2,4 D + 2mg/ L Kinetin
T_5	3.97g / L N6 + 0.5mg/L B5 Vitamins + 30 g / L sucrose + 6g / L agar	2mg/ L 2,4 D + 1mg/ L Kinetin
Regeneration media		
R_1	3.97g / L N6 + 0.5 mg / L B5 Vitamins +	2.5mg/ L Kinetin + 0.2mg/L NAA
R_2	1 mg / L BAP + 30 g / L sucrose + 6g/ L agar	4 mg/ L Kinetin + 0.5mg/L NAA



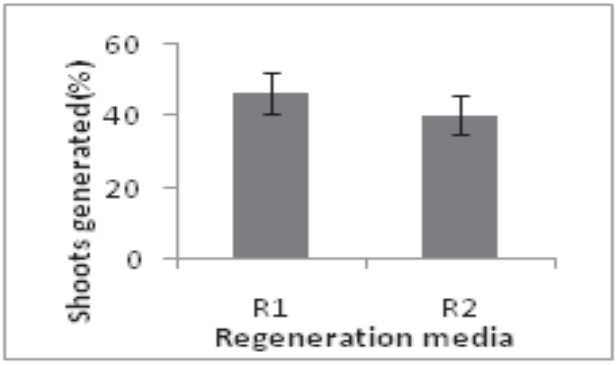


Fig. 1 (a) Callus induction efficiency in different callus inducing media (b) Regeneration efficiency of calli in two different regeneration media

TABLE II

Identification of true haploids and spontaneous doubled haploids based on Marker and Flow cytometry data

Sample no.	SSR Markers	Flow cytometer
Hybrid(KRH4)	II	2C
12	I	2C
13	I	2C
14	I	1C
15	I	2C
16	I	2C
17	I	1C
18	I	1C
19	I	2C
20	I	1C
21	I	1C
22	I	2C
23	I	1C

(I - Homozygous bands (Haploids/ Doubled haploids); II - Heterozygous bands (Diploids); 1C - Single set of chromosome (haploid); 2C - Set of double chromosome (doubled haploid/ diploid)

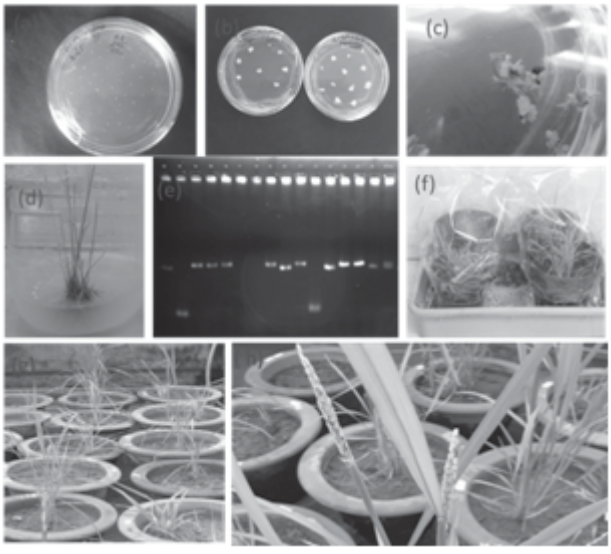


Plate. (a) Anther inoculation in N6 media, (b) Callus regenaration, (c) Shoots formation, (d) Plantlets in rooting media, (e) SSR markers analysis of plantlets (f) Hardening process, (g) Anther derived plants in green house (h) Panicle emergence in anther derived plants

The true haploids confirmed through marker studies and by flow cytometry analysis were treated with colchicine. Colchicine is the most frequently used chemical for chromosome doubling in plants (Ouyang *et al*., 1994; Soriano *et al*., 2007). Colchicine treatment @ 0.25 mg / 100 ml DMSO (2% v/v) was given to the young haploid plants at 3 to 5 tillering stage for 2-3 hours for inducing doubling of the chromosome. Later, plants were hardened (Plate1-f) and incubated for gradual acclimatization and then shifted to the greenhouse (Plate 1-g) for further development (Plate1-h).

From the above observations, it is evident that the doubled haploidy has the potential to be a very efficient tool for the production of completely homozygous lines from heterozygous donor plants in a single step.

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