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# Anther culture of elite rice hybrids for regeneration of doubled haploid lines

**Manmohan Sharma, Pooja, Mamta Sharma, RK Salgotra and Satish K Sharma**

**Manmohan Sharma**
School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu & Kashmir, India

**Pooja**
School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu & Kashmir, India

**Mamta Sharma**
School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu & Kashmir, India

**RK Salgotra**
School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu & Kashmir, India

**Satish K Sharma**
School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu & Kashmir, India

**Corresponding Author:**
**Mamta Sharma**
School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu & Kashmir, India

## Abstract
The present study was carried out to analyse the anther culture response of four commercial rice hybrids namely; DRRH3, PRH10, PHB7 and Indira Sona towards haploid induction with respect to cold pre treatment along with effect of growth hormones and amino acids on the callus induction and regeneration. $N_6$ medium augmented with different concentration and combinations of growth hormones and amino acids was used to record the response of genotypes for anther culture, calli regeneration, haploid production and root induction. It was observed that callus induction frequency increased with increase in 2,4-D concentration (1.5 to 2.5 mg $l^{-1}$). In addition, fortification of amino acids like tryptophan (25 mg $l^{-1}$) and cysteine (40 mg $l^{-1}$) showed a synergistic effect on callus induction ability. DRRH3 showed significantly higher scores of androgenic frequencies in comparison to other hybrids. Rooting was effectively induced in liquid half-strength MS medium. It was concluded that callus induction, regeneration, haploid production and androgenesis are genotype specific. However, slight manipulations in cold pretreatment prior to *in vitro* culturing and media composition can improve the response of genotype towards haploid induction.

**Keywords:** rice hybrids, anther culture, calli, haploid regeneration

## Introduction
Rice (*Oryza sativa* L.) is a highly explored self pollinating diploid (2n=2x=24) crop belonging to family Graminae (Poaceae) and is staple diet of 33 per cent of the global population. Rice is cultivated over an area of about 160.1 million hectare worldwide with production and productivity of around 730.0 million tonnes and 4.53 tonnes per hectare, respectively [1]. It is particularly an important crop in Asian continent where more than 90 per cent of the global rice is produced and consumed which accounts for as much as 75 per cent of the calorie intake of about 2 billion humans. India has the highest individual crop acreage of 44 million hectares under paddy with production and productivity of 111.00 million tonnes and 2.52 tonnes $ha^{-1}$, respectively [2] accounting for more than 40 per cent of total food grain production. In order to meet the demand of increasing population in India, the current production level needs to be increased to 130 million tonnes by the year 2025 [3] and can be done by developing high yielding rice varieties in shorter time duration utilizing molecular and tissue culture approaches. In India, rice hybrids have gained popularity over last two decades and 93 hybrids have been released for commercial cultivation [4]. In Jammu and Kashmir, rice hybrids PHB 71, Indira Sona, DRRH3 and PRH10 have been recommended for different agro climatic zones owing to superior yield in comparison to conventional pure line varieties.

Anther culture in rice is a technique which can be used to produce large number of haploids in a short span of time and induced to generate doubled haploid (DH) plants, thereby reducing the breeding cycle for varietal improvement [5]. Haploid plants are recognized by existence of only one set of chromosomes in their cells and have potential of accelerating the production of homozygous new varieties. Haploid plants derived from regeneration of cultured anthers followed by chromosome doubling, produce pure strains or double haploid plants [6]. DH plants carry two similar alleles of each gene so any recessive mutation becomes apparent and mutations or alleles with lethal effect are eliminated from the gene pool. Anther culture plays an important role in breeding programmes by providing homozygous plants with special agronomic characters like development of earliness, increased grain weight, superior grain, disease resistance dwarf plant type and abiotic stress tolerance [7]. The present study was carried out to analyze the anther culture response of four commercial rice hybrids namely; DRRH3, PRH10, PHB7 and Indira Sona towards haploid induction using cold pre treatment along with effect of growth hormones and amino acids.

## Materials and Methods

The plant material comprising of four commercial rice hybrids namely, DRRH3, PRH10, PHB7 and Indira Sona were used as mother plants for excising anthers. These were grown in pots filled with sterilized soil in polyhouse of School of Biotechnology, SKUAST-J, Chatha and were monitored regularly. Disease free panicles from primary tillers at boot stage of plant growth were collected during morning hours when distance between the auricle of flag leaf and penultimate leaf was 5-7 cm. At this stage pollen are assumed to be in the mid to late uninucleate stages. The panicles were washed thoroughly with tap water, sterilized with 70 per cent ethanol and were given chilling treatment at 4°C for 5 to 12 days.

The selected spikelets were removed from leaf sheath and were surface sterilized with Tween 20 followed by sterilization with 70 percent ethanol and 0.1 percent mercuric chloride solution under laminar flow chamber. These were thoroughly washed with sterile distilled water to remove traces of mercuric chloride. These spikelets were transferred on sterilized filter paper and the anthers were separated by cutting the basal end of florets with scissors and dusting uniformly over the surface of $N_6$ medium [8] supplemented with maltose as carbohydrate source. The medium was augmented with different combinations and concentrations of growth regulators and amino acids as depicted in Table 1. The cultures were incubated in dark at 25±2°C and 60 percent humidity for 60-80 days. The callus induced was sub-cultured for further proliferation on the same callus induction media.

**Table 1:** Media composition used for callus induction and multiplication

| Treatments | $N_6$ Media composition |
|---|---|
| $TN_0$ | Basal medium |
| $TN_1$ | 2,4-D (1.5 mg $l^{-1}$) + Kinetin (0.5 mg $l^{-1}$) |
| $TN_2$ | 2,4-D (2.0 mg $l^{-1}$) + Kinetin (0.5 mg $l^{-1}$) |
| $TN_3$ | 2,4-D (2.5 mg $l^{-1}$) + Kinetin (0.5 mg $l^{-1}$) |
| $TN_4$ | $TN_3$ + Tryptophan (25 mg $l^{-1}$) |
| $TN_5$ | $TN_3$ + Cysteine (40 mg $l^{-1}$) |
| $TN_6$ | $TN_3$ + Tryptophan (25 mg $l^{-1}$) + Cysteine (40 mg $l^{-1}$) |

The callus regeneration was performed by sub-culturing small fragments of callus on MS medium [9] supplemented with sucrose (30 g $l^{-1}$) and different concentrations of growth regulators (BAP and Kinetin) and amino acids (tryptophan and cysteine) as presented in Table 2. The cultures were then incubated under cool white fluorescent light (3000 lux) in light/dark cycle for 16/8 h at 25±2 $^0$C and observed periodically for regeneration of callus.

**Table 2:** Media composition used for callus regeneration and haploid induction

| Treatments | MS Medium composition |
|---|---|
| $TM_1$ | BAP (1.5 mg $l^{-1}$) + Kinetin (0.5mg $l^{-1}$) + NAA (0.5 mg $l^{-1}$) |
| $TM_2$ | BAP (2.0 mg $l^{-1}$)+Kinetin (0.5mg $l^{-1}$)+NAA (0.5 mg $l^{-1}$) |
| $TM_3$ | BAP (2.5 mg $l^{-1}$)+Kinetin (0.5mg $l^{-1}$)+NAA (0.5 mg $l^{-1}$) |
| $TM_4$ | $TM_3$+Tryptophan(25 mg $l^{-1}$) |
| $TM_5$ | $TM_3$+ Cysteine (40 mg $l^{-1}$) |
| $TM_6$ | $TM_3$+ Tryptophan(25 mg $l^{-1}$)+ Cysteine (40 mg $l^{-1}$) |

Calli showing regeneration gradually developed green sectors and metamorphosed into bunches of minute plantlets. These green plantlets were separated from each other and sub-cultured on regeneration media and allowed to undergo complete organogenesis under *in vitro* conditions inside the culture vessels for one month. When the regenerated plantlets reached a height of 3-4 cm were excised and transferred to liquid rooting media without sucrose (RM1: MS full strength and RM2: half-MS medium) for further root development.

Data pertaining to anther culture frequency, calli regeneration frequency, haploid regeneration frequency was worked out [10]. Analysis of variance (AVOVA) was done using one factor completely randomly randomized design (CRD) for statistical analyses of data generation during *in vitro* studies with five replicates [11].

## Results and Discussion

Anther culture in rice is a rapid approach to attain homozygosity as it shortens the time required for developing new rice varieties as doubled haploids from $F_1$ hybrids [12]. Successful use of anther culture to produce double haploid plants is influenced by significant interaction among media composition and genotype [13]. In the present study four commercial rice hybrids namely, DRRH3, PRH10, PHB7 and Indira Sona were tested for their androgenic response towards induction of haploids and to analyze the effect of growth hormones and amino acids on their haploid induction frequency. The optimization of cold pretreatment and media components are two important factors that influence anther culture response of rice hybrids.

**Effect of chilling pre-treatment**

The sterilized spikes were pre treated at 4°C for 5-12 days and cultured on $N_6$ basal medium without growth hormones. Analysis of variance (ANOVA) showed highly significant differences among genotypes with respect to anther culture frequency under chilling pre-treatments at 4°C for 10 and 12 days. The anthers responding to culture conditions turned brown and became swollen after 3-4 weeks of dark incubation and as evident from Table 3**,** DRRH3 was the only hybrid which responded to pre-chilling treatment with anther culture frequency of 0.42 per cent and 0.52 per cent at 10 days and 12 days pre-treatment respectively. Other genotypes *viz.* Indira Sona, PHB71 and PRH10 did not respond to any of the chilling pre-treatments when cultured on $N_6$ basal medium. Effectiveness of varying chilling pre-treatments on induction of callus in anthers of *indica* and *japonica* rice varieties and hybrids has been reported by several other research workers [14, 15, 16]. A pre-treatment at 10ºC for 7-9 days is reported to have a positive effect on *indica rice* hybrids [17]. Cold pre-treatment for 14 days at 8ºC was most effective for anther culture in selected varieties of *indica, japonica* rice and some inter sub-specific hybrids [18]. The chilling pre-treatment of anthers enhanced callus induction and regeneration response from rice anther, though chilling temperature and duration may vary from genotype to genotype. The influence of various stress pre treatment of anthers on callus induction response was studied [19] out of which cold treatment was reported to have a positive effect on callus formation as it inhibited gamete formation and delayed senescence of anther walls. However, prolonged pretreatment proved inhibitory

irrespective of media used resulting in formation of albinos due to degradation of chlorophyll [17].

**Table 3:** Effect of chilling pre-treatment (4 °C) on anther culture frequency of rice hybrids using $N_6$ basal medium

| S. No. | Genotypes | Anther culture frequency (%) on $N_6$ basal medium | | |
|---|---|---|---|---|
| | | 5 days | 10 days | 12 days |
| 1. | Indira Sona | 0 | 0 | 0 |
| 2. | PHB71 | 0 | 0 | 0 |
| 3. | PRH10 | 0 | 0 | 0 |
| 4. | DRRH3 | 0 | 0.42 | 0.52 |
| CV (%) | | - | 12.33 | 12.92 |
| CD | | - | 0.03 | 0.04 |
| SE (mean) | | - | 0.01 | 0.01 |

**Callus induction and multiplication**

The composition of culture medium plays a pivotal role in induction of microspore embryogenesis. $N_6$, MS, LS and SK1 solid media are commonly used for anther culture and $N_6$ was found to be more effective than any other media [20]. Better response of anther culture of indica rice was reported in presence of higher doses of nitrogen, phosphorus and potassium [6]. In case of anther culture of cereals ammonium nitrogen ($NH_4^+$) is required in low amounts and $N_6$ medium being high in NO3 - and contains half amount of $NH_4^+$ which proved very efficient for Japonica rice anther culture. In the present study, anthers were cultured on $N_6$ basal medium supplemented with maltose as carbon source and incubated in dark and started turning brown and slightly swollen in culture medium. Maltose is most preferred carbon source in rice anther culture as it degrades to release glucose molecules which are utilized rapidly thus enhancing androgenesis of rice microspores resulting in better callusing and regeneration response than sucrose [21]. Further, maltose also minimizes the chances of production of albinos in various cereal species [22, 23]. On contrary, a high ratio of green plantlets in comparison to albinos were obtained in presence of mannitol as carbon source in the callus induction medium for both Japonica and Indica types [24]. In present study, light cream coloured mass of cells started bursting out of the anther walls after 10-12 weeks of incubation from three hybrids namely, DRRH3, PRH10 and PHB7 in $TN_3$ treatment as depicted in Figure 1 where $N_6$ medium was supplemented with 2,4-D (2.5 mg $l^{-1}$) and Kinetin (0.5 mg$l^{-1}$) with maximum anther culture frequency of 1.99 per cent, 0.98 per cent and 0.13 per cent respectively; showing DRRH3 to be more responsive as depicted in Table 4. The treatment $TN_3$ was significantly superior over other combinations indicating that mean anther culture frequency increased linearly with increasing concentrations of 2,4-D from 1.5 mg $l^{-1}$ to 2.5 mg $l^{-1}$. Our results are supported by findings of Niroula and Bimb [25] who obtained high callus induction frequency in $N_6$ medium supplemented with 2,4-D (2.5mg $l^{-1}$) + Kinetin (0.5 mg $l^{-1}$) than $N_6$ + NAA (4 mg $l^{-1}$) + Kinetin (0.5 mg $l^{-1}$). The role of 2,4-D in callus induction of rice anthers has also well been highlighted [26] which validate the results of present investigation. 2,4-D and NAA alone or in combination with Kinetin in culture medium were the major determinants for embryogenic callusing from rice anthers which validate the possibility that the use of cytokinins like Kinetin and BAP is necessary for regeneration [27].

**Table 4:** Effect of media on anther culture frequency of rice hybrids

| S. No. | Genotype | Anther culture frequency (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $TN_0$ | $TN_1$ | $TN_2$ | $TN_3$ | $TN_4$ | $TN_5$ | $TN_6$ |
| 1. | Indira Sona | 0 | 0 | 0 | 0 | 0.45 | 1.03 | 1.81 |
| 2. | PHB71 | 0 | 0 | 0 | 0.13 | 3.12 | 3.92 | 5.79 |
| 3. | PRH10 | 0 | 0 | 0.25 | 0.98 | 5.02 | 5.34 | 7.83 |
| 4. | DRRH3 | 0.52 | 0.66 | 1.05 | 1.99 | 8.82 | 9.52 | 11.56 |
| CV (%) | | 14.92 | 13.34 | 11.16 | 9.86 | 8.49 | 7.73 | 8.56 |
| CD | | 0.04 | 0.05 | 0.07 | 0.18 | 0.79 | 0.71 | 0.77 |
| SE(mean) | | 0.01 | 0.02 | 0.03 | 0.06 | 0.26 | 0.24 | 0.26 |

**Fig 1:** Calli mass bursting out from anthers in response to culture conditions

In order to further enhance anther culture frequency scores, $TN_3$ medium was supplemented with tryptophan (25 mg $l^{-1}$) and/or cysteine (40 mg $l^{-1}$) and it was found that anther culture frequency scores for different genotypes showed improvement over the respective anther culture frequency scores in medium without amino acids. Table 4 shows a significant increase in anther culture frequencies of all genotypes when both amino acids i.e. tryptophan (25 mg $l^{-1}$) and cysteine (40 mg $l^{-1}$) were supplemented in $TN_3$ medium ($TN_6$). In $TN_6$ medium, maximum anther culture frequency was observed in DRRH3 (11.56%) followed by PRH10 (7.83%), PHB71 (5.79%) and Indira Sona (1.81%). It highlights the role of amino acids as organic nitrogen source that triggered anther callusing in the genotypes. Role of amino acids in enhancing callusing in rice anthers and regeneration have been already been reported [28, 14, 15].

**Callus regeneration frequency**

MS medium is most commonly used for plant regeneration instead of using $N_6$ media due to higher concentration of NH4+. In the present study, the shoot regeneration from calli was performed on MS medium fortified with BAP or Kinetin and lower concentration of NAA) which is corroborated with the findings of Rout *et al.*, [29] who reported that MS is better than $N_6$ on shoot regeneration in anthers of *indica* rice. Successful green plantlet regeneration has been reported from calli on MS medium supplemented with sucrose and growth regulators [30, 31]. In the present study the regeneration media was supplemented with sucrose (30 g$l^{-1}$), Kinetin (0.5 mg$l^{-1}$), NAA (0.5 mg$l^{-1}$) and varying concentrations of BAP (1.5, 2.0

and 2.5 mg $l^{-1}$) which showed significantly different response to callus regeneration. The responsive calli of two genotypes i.e. DRRH3 and PRH10 turned green after 12-15 days of inoculation and showed regeneration as evident from Figure 2. Several experiments conducted by different researchers have also shown that application of polyamines promotes the plantlet regeneration [32]. The callus regeneration frequencies scores in these two genotypes increased linearly with increase in dose of BAP from 1.5 mg $l^{-1}$ in $TM_1$ to 2.5 mg $l^{-1}$ in $TM_3$ medium treatment as shown in Table 5. Maximum callus regeneration frequency was observed in DRRH3 (6.22%) followed by PRH10 (3.22%) in $TM_3$ medium. These observations are supported by the findings of Mishra *et al.*, [17] who reported the positive influence of Kinetin: NAA: BAP in ratio of 1:1:3. The green plant regeneration was 32-36.0% in Rajalaxmi (CRHR 5) and Ajay (CRHR 7), elite *indica* hybrids when the growth regulators were used in same ratio. However, the incidence of albinos was also higher in both the genotypes in this media and the callus regenerated on hormone free media had shown very less regeneration frequency. The regeneration of green plantlets in MS medium with Kinetin: NAA: BAP (1:1:3) were obtained by Chopkar *et al.,* [12].

**Table 5:** Effect of media on calli regeneration frequency of rice hybrids

| S. No. | Genotypes | Calli regeneration frequency (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | $TM_1$ | $TM_2$ | $TM_3$ | $TM_4$ | $TM_5$ | $TM_6$ |
| 1. | Indira Sona | 0 | 0 | 0 | 0.99 | 1.04 | 2.07 |
| 2. | PHB71 | 0 | 0 | 0 | 2.01 | 2.60 | 3.69 |
| 3. | PRH10 | 1.58 | 2.47 | 3.22 | 3.32 | 3.98 | 7.08 |
| 4. | DRRH3 | 3.97 | 4.88 | 6.22 | 7.01 | 7.01 | 9.95 |
| CV(%) | | 20.9 | 13.85 | 10.53 | 16.01 | 15.12 | 6.52 |
| CD | | 0.39 | 0.34 | 0.34 | 0.71 | 0.74 | 0.49 |
| SE(mean) | | 0.11 | 0.11 | 0.11 | 0.24 | 0.25 | 0.17 |

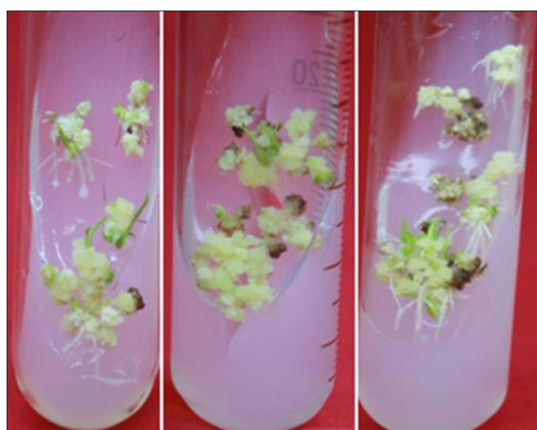

**Fig 2:** Callus regeneration on selective media combinations

In order to observe the effect of amino acids $TM_3$ medium was supplemented with tryptophan (25 mg $l^{-1}$) and/or cysteine (40 mg $l^{-1}$). Inclusion of both the amino acids i.e. tryptophan (25 mg $l^{-1}$) and cysteine (40 mg $l^{-1}$) to $TM_3$ medium significantly improved callus regeneration frequency scores in comparison to those where these were supplemented singly (Figure 3). Maximum value for callus regeneration frequency in this medium was observed for DRRH3 (9.95 %) followed by PRH10 (7.08%), PHB71 (3.69%) and Indira Sona (2.07) as shown in Table 5. The present observations along with findings reported earlier [33, 14, 15] validate that augmentation of amino acids in induction and regeneration media leads to high callus regeneration.

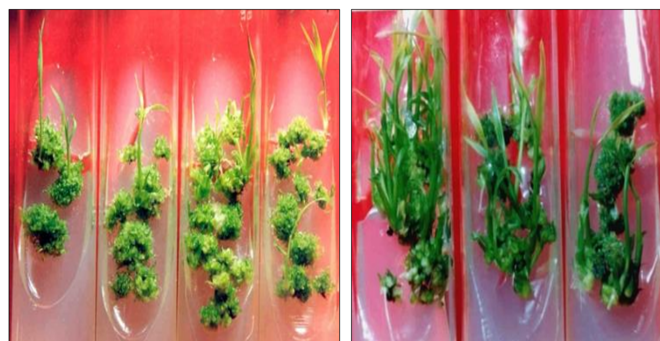

**Fig 3:** Regenerated calli masses on selective media combinations

## Haploid regeneration frequency

The green plantlets were produced from calli showing regeneration. Out of four genotypes only DRRH3 and PRH10 showed haploid regeneration and the frequency scores increased linearly with increasing doses of BAP. Hybrid DRRH3 showed maximum haploid regeneration frequency of 6.98 percent followed by PRH10 (4.20%) in $TM_3$ medium. Haploid regeneration was observed in all the genotypes in $TM_3$ medium supplemented with tryptophan (25 mg $l^{-1}$) and cysteine (40 mg $l^{-1}$) alone or in combination; with haploid regeneration frequency scores in the order of DRRH3>PRH10>PHB71>Indira Sona as depicted in Table 6. However, supplementation of both amino acids in $TM_3$ medium resulted in significantly higher haploid regeneration frequency values (Figure 4). Maximum haploid regeneration frequency values being 17.27 percent in DRRH3 followed by 14.45 percent in PRH10, 8.07 percent in PHB71 and 3.94 in Indira Sona supported by findings of Sharma *et al.*, [15] and Siddique [20].

**Table 6:** Effect of media on haploid regeneration frequency of rice hybrids

| S. No. | Genotypes | Haploid regeneration frequency (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | $TM_1$ | $TM_2$ | $TM_3$ | $TM_4$ | $TM_5$ | $TM_6$ |
| 1. | Indira Sona | 0 | 0 | 0 | 2.80 | 2.48 | 3.94 |
| 2. | PHB71 | 0 | 0 | 0 | 5.26 | 5.29 | 8.07 |
| 3. | PRH10 | 3.10 | 3.29 | 4.20 | 9.04 | 9.56 | 14.45 |
| 4. | DRRH3 | 5.16 | 6.34 | 6.98 | 14.27 | 13.21 | 17.27 |
| CV(%) | | 13.13 | 12.31 | 11.49 | 19.35 | 16.61 | 7.21 |
| CD | | 0.36 | 0.39 | 0.43 | 2.03 | 1.70 | 1.06 |
| SE(mean) | | 1.21 | 0.13 | 0.14 | 0.67 | 0.56 | 0.35 |

**Fig 4:** Regenerated haploid plants on selective media combinations

## *In vitro* rooting of regenerated shoots

*In vitro raised* haploid shoots transferred to full strength MS basal liquid medium ($RM_1$) and ½ MS basal MS medium

($RM_2$) in absence of sucrose for root induction. As evident from Table 7, highest number of roots per plant (3.60±0.45) was induced in rice hybrid DRRH3 while the lowest number of roots (2.40±0.38) per plant was recorded in Indira Sona in $RM_1$ medium. As shown in figure 5, starvation of cultures in ½ MS liquid medium (RM2) resulted in significant increase in number of roots per plant for each genotype in comparison to respective values in $RM_1$ medium which is in agreement with the observations reported by Bagheri *et al.*, [34] and Herath and Bandara [5].

**Table 7:** Effect of media on induction of roots in rice hybrids

| Genotype | No. of Roots per plant | |
|---|---|---|
| | $RM_1$ | $RM_2$ |
| Indira Sona | 2.40±0.38 | 3.60±0.28 |
| PHB71 | 2.60±0.24 | 3.80±0.14 |
| PRH10 | 3.20±0.17 | 3.80±0.14 |
| DRRH3 | 3.60±0.45 | 4.80±0.56 |
| CD | 0.70 | 0.79 |
| CV(%) | 17.77 | 14.70 |
| SE(m) | 0.23 | 0.26 |

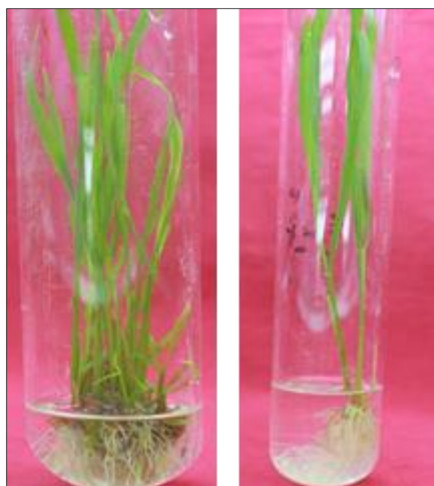

**Fig 5:** *In* vitro rooting of haploids

## Conclusion

In conclusion, callus induction, callus regeneration, haploid production and androgenesis are genotype specific. However, media constituents and *in vitro* conditions can be manipulated to improve the response of genotypes towards haploid induction through anther culture. Cold pre treatment and augmentation of specific amino acids as organic source of nitrogen can play an important role in improving the overall androgenic response of cultured anthers/microspores.

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