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Optimization of *In vitro* Androgenic Protocol for Development of Haploids and Doubled Haploids in Indica Rice Hybrid, KRH 4

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ABSTRACT

In vitro androgenesis is an attractive biotechnological technique that provides rapid transformation of heterozygous hybrids into homozygous lines and thus, significantly reducing the duration of breeding cycle. Due to difficulty in generating embryogenic calli, low plant regeneration and frequent albino plant regeneration, the prospective benefits of doubled haploid technology have not been exploited fully in indica rice hybrids. The present study, therefore aims at optimizing a previously standardized *in vitro* androgenic protocol to improve callus and shoot regeneration efficiency in an indica rice hybrid, KRH 4. Accordingly, the most potential anthers for androgenesis were obtained from boots with flag leaf to penultimate leaf distance of 10-12 cm. In particular, anthers at 4/6th position (ratio of length of stamen to the glume) within a spikelet were shown to have the highest callus induction efficiency. Further, callusing was advanced by 18 days and callus induction was improved by 13 per cent when sucrose was replaced with maltose a carbon source to the previously standardized callus induction media for KRH 4 rice hybrid. Finally, among the optimized callus induction media, T_3 media containing N_6 basal media supplemented with 2, 4-D (2 mg/L), NAA (1 mg/L), kinetin (0.5 mg/L), maltose (3%) and clerigel (0.2%) and maintained at a p^H of 5.8, had the highest callus induction efficiency and also produced more embryonic calli. Similarly, shoot regeneration efficiency of embryonic calli (2 mm) was enhanced by inoculating embryonic calli in R_2 regeneration media containing N_6 basal media supplemented with kinetin (3 mg/L), NAA (0.1 mg/L), BAP (1 mg/L), sucrose (3%) and clerigel (0.2%) maintained at a p^H of 5.8 and kept under absolute dark for 48 hrs followed by transferring them to BOD incubator maintaining 16-h light/8-h dark regime at 27 ± 2 °C.

Keywords : Callus induction efficiency, Doubled haploid technology, Embryogenic calli, Haploids, *In vitro* androgenesis, Indica rice, Shoot regeneration efficiency

DOUBLED haploid (DH) technique serves as one of the main breeding methods used for development of new varieties in many crops. It reduces the breeding time significantly by reducing the selection cycles for obtaining desirable homozygosity from heterozygous parents in a single generation, the cornerstone of hybrid seed production. Being much faster technique, DHs are useful for genetic mapping of complex qualitative traits (Pooja and Sheshshayee, 2017) for linkage studies and estimation of recombination fractions, to unmask recessive mutants, to avoid transgenic hemi-zygotes, or for reverse breeding (Dunwell, 2010; Dwivedi *et al*., 2015). As DH technology has lot of applications and offers several advantages, it becomes one of the most exciting areas in the crop improvement programmes. The production of doubled haploid lines is now routine for a number of important crop plants. For many of the crop species, large populations of doubled haploids can be obtained by utilizing interspecific crosses and / or embryo rescue (Lazaridou *et al*., 2011; Pauls *et al*., 2013 and Chaudhary *et al.,* 2020) or tissue culturing of gametic cells (EL-Hennawy *et al*., 2011; Herberle-Bors *et al*., 2012). In rice, the androgenic method of producing doubled haploids is widely employed due to its inherent advantages. However, *in vitro* androgenesis is genotype-dependent and studies showed that, japonica rice varieties / hybrids are more responsive to androgenesis than indica rice varieties / hybrids because of recalcitrant nature of indica rice (Grewal *et al*., 2011). In this regard, several attempts have been made in the past to standardize the protocol for

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improving *in vitro* culturing efficiencies in indica rice with fairly a good success (Mishra *et al*., 2011 and Rout *et al*., 2016). Apart from genotypic response, many other factors such as physiology of donor plant, stage of pollen, pre-treatment of anthers, media composition, anther wall factors, microspore density and culture conditions (light and temperature) have shown to influence the anther culturability in rice (Mishra *et al*., 2011). However, manipulation of the limiting factors related to androgenesis in indica rice have been shown to improve the androgenic response (Samantaray *et al*., 2021). Therefore, it is inevitable to create congenial conditions for effective androgenesis in indica rice through several possible measures.

Identification of responsive anthers (right stage of microspores) for callus induction is one of the key challenges in anther culture (Afza *et al*., 2001). There are a very few reports on the use of morphological indicators such as panicle length, spikelet position, anther colour and size and anther position in the spikelet on callus induction efficiency. The distance between the flag leaf and the penultimate leaf as well as the late uni-nucleate and early bi-nucleate pollen stages have been utilized as markers for callus induction with varying degree of success (Sarao and Gosal, 2018). The optimal microspore stage for greatest callus induction in indica rice varieties is the mid uni-nucleate stage, which corresponds to spikelets of yellowish green colour with anthers reaching the centre of the spikelet (Shahjahan *et al.*, 1992). Upon selecting most responsive anthers, they should be cultured on most effective tissue culture media. Improving the composition of tissue culture medium, particularly, by modifying the plant growth regulators improves the success rate of *in vitro* androgenesis in indica rice hybrids significantly by the enhancing the callus induction and green plantlet regeneration efficiency.

Earlier, an attempt was made by Debina *et al*. (2016) to standardize the anther culture protocol for the generation of haploids and doubled haploids in an indica rice hybrid, KRH 4 at the Dept. of Crop Physiology, UAS, GKVK, Bengalore. They were successful in generating a small number of DH lines due to lower callusing and regeneration efficiency (Debina *et al*., 2016, Debina, 2019). However, the success of any DH programme depends on generation of a large number of haploids/doubled haploids through increased efficiency of callusing and regeneration. Therefore, in the present study, an attempt was made to improve the previously defined anther culture protocol of Debina *et al*. (2016) for the KRH 4 rice hybrid, primarily to make indica rice anther culture more convenient and efficient.

Material and Methods

Plant Material and Determination of Anther/ Microspore Development Stage

An indica rice hybrid, KRH 4 obtained from Hybrid Rice Section, Zonal Agricultural Research Station (ZARS), VC Farm, Mandya was employed in this investigation. Plants of KRH 4 rice hybrid were raised in pots inside the greenhouse during *kharif* 2018. At booting stage (panicles along with the boot leaves in which they are still enclosed), panicles of different lengths *i.e.,* 5, 8, 10, 12 and 15 cm (Fig. 1B) from flag leaf to penultimate leaf were collected to identify the right stage of microspore for anther culture (Fig. 1D-G). In fact, in many systems, it has been reported that, the uni-nucleate to early bi-nucleate staged microspores give good response upon callusing (*Afza et al*., 2001). Keeping this in view, after collecting the panicles of different lengths, they were separated into three sections *viz.,* top, middle and lower part. Five spikelets in each section were used to assess the position effect of spikelets on anther culturability and efficiency.

To determine the microspore developmental stage, two or three anthers from different positioned spikelets were crushed and stained with 2 per cent aceto-carmine and examined under 40 x optical microscope. Here the length of anthers from the base of spikelets also matters as far as anther response is concerned (*Afza et al*., 2001). Accordingly, the anthers which are near to the base of spikelet, approximately, at a distance of 1mm were concluded as 1/6th position

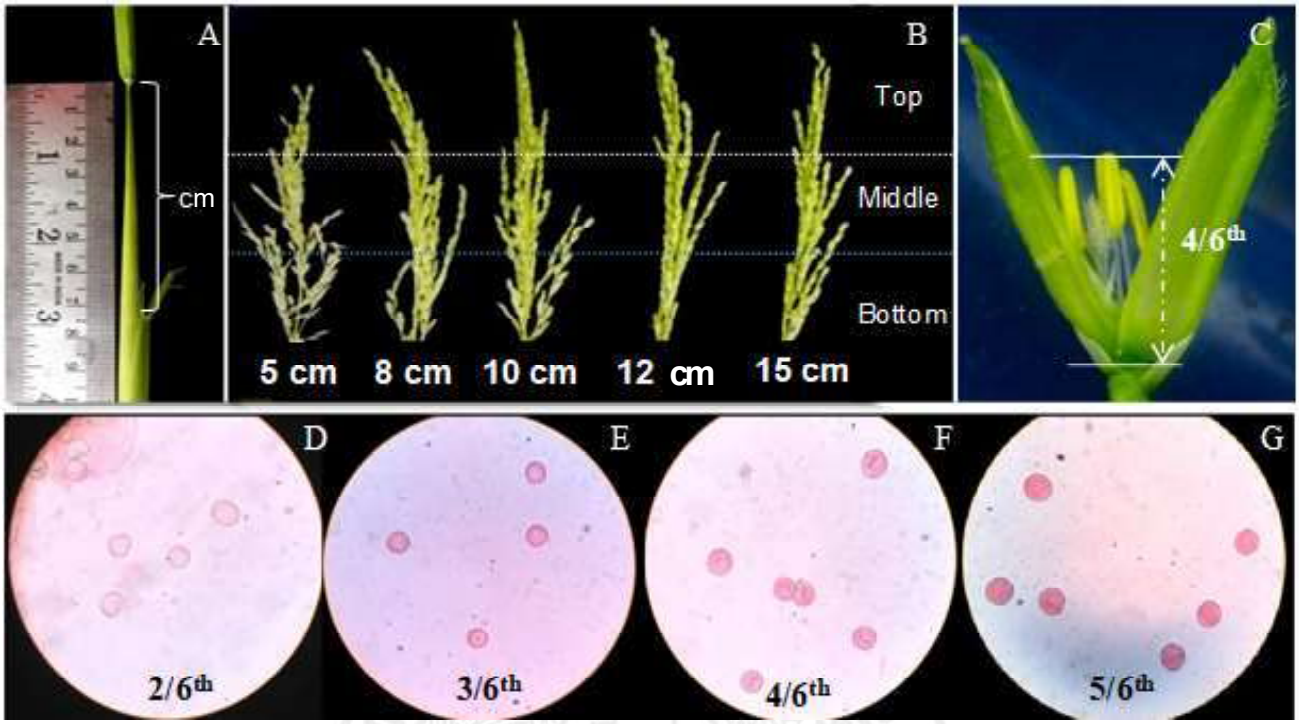

Fig. 1: Determination of anther/microspore development stage in an indica rice hybrid, KRH 4. A) Boot depicting flag leaf to the penultimate leaf distance (cm); B) Panicles having flag leaf to the penultimate leaf distance of 5-15 cm.; C) Spikelet having anthers at 4/6th position. (D-G) Microspore stage identification using 2 per cent acetocarmine; Lighter stain (2/6th positioned anthers) to indicate early uni-nucleate stage, medium stain (3/6th and 4/6th positioned anthers) mid to late uni-nucleate stage and darker stain (5/6th positioned anthers) to represent bi-nucleate microspore stage

(as the approximate length of spikelet was 6 mm), while the anthers near to the tip of spikelet at a height of 5 mm were denoted as 5/6th positioned anthers (Fig. 1C). Microspores with lighter stain (obtained from 2/6th positioned anthers) depict early uni-nucleate stage (Fig. 1D), medium stain (obtained from 3/6th and 4/6th positioned anther) depict mid to late uni-nucleate stage (Fig. 1E and 1F) and darker stain (obtained from 5/6th positioned anther) depict bi-nucleate stage (Fig. 1G).

To evaluate the rate of responding anthers, spikelets from all the three sections were wrapped and stored separately at a standardized temperature of 10 ^{0}C for 7-8 days for cold pretreatment (standardized in the previous experiment). Further, pretreated spikelets were surface sterilized in 0.1 per cent $HgCl_2$ for five minutes inside the laminar hood and inoculated in callus induction media (T_2) with altered media composition (Table 1) and kept in complete darkness. After 4-5 weeks of dark incubation, number of responding anthers under each treatment was recorded. The rate of anther response was denoted by a '+' sign (Table 3). Further, the influence of anther position within the spikelet on per cent callus induction was determined by counting the number of anthers at specified position within spikelet responding to callus formation to the total number of anthers inoculated.

Optimization of Callus Induction Media

To maximize the callus induction efficiency in KRH 4 rice hybrid, the standardized callus induction media of Debina *et al*. (2016) was optimized by fine tuning the culture media components such as carbon source, hormone (auxin and cytokinin) source and their concentration (Table 1). Spikelets from boots (10-12 cm) with anthers positioned at 4/6th were selected and dark incubated at 25 ± 2 °C in different optimized callus induction media (length of the panicle and position of anthers standardized in the previous experiment of the present study). The

TABLE 1

Composition of callus induction media for an indica rice hybrid, KRH 4

Treatments	Basal media	Carbon sources	Hormones and other supplements	Gelling agents
		Standardized callus induction media for indica rice hybrid, KRH 4 (Debina *et al.,* 2016)		
T_1	N_6	Sucrose (3%)	2,4-D (2 mg/L) + NAA (1mg/L) + Kinetin (0.5 mg/L)	Agar (0.6%)
		Optimized callus induction media for indica rice hybrid, KRH 4		
T_2	N_6	Maltose (3%)	2,4-D (2 mg/L) + NAA (1mg/L) + Kinetin (0.5 mg/L)	Agar (0.6%)
T_3	N_6	Maltose (3%)	2,4-D (2 mg/L) + NAA (1mg/L) + Kinetin (0.5 mg/L)	Clerigel (0.2%)
T_4	N_6	Maltose (3%)	2,4-D (2 mg/L)	Clerigel (0.2%)
T_5	N_6	Maltose (3%)	2,4-D (2 mg/L) + NAA (1mg/L) + BAP (0.5 mg/L)	Clerigel (0.2%)

Note: Other supplements such as proline (0.5 g/L) + Casamino acid (0.5 g/L) + Glutamine (0.5 g/L) + $AgNO_3$ (4 mg/L) were included in all treatments

observations such as days to callus initiation, per cent callus induction and embryonic callus formation were all recorded.

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of anther producing callus}}{\text{No. of anthers plated}} \times 100$$

$$\text{Embryonic calli (\%)} = \frac{\text{No. of embryonic calli produced}}{\text{Total number of calli produced}} \times 100$$

Optimization of Shoot Regeneration Media

Previously standardized shoot regeneration media for an indica rice hybrid, KRH 4 (Debina *et al*., 2016) was optimized to improve shoot regeneration efficiency. The white and friable calli, also known as 'embryonic' calli, with a diameter of 1-2 mm were sampled and transferred to optimized medium combinations including different growth regulators and gelling agents (Table 2). Unlike in the previous case as reported by Debina *et al.* (2016), we have kept the embryonic calli under complete darkness for two days after inoculation but before transferring them to photoperiodic condition. In addition, we have also replaced agar with clerigel as a gelling medium in our new protocol. After inoculation, the embryonic calli inoculated for regeneration were kept under absolute dark for 48 hrs followed by transferring them to BOD incubator maintaining 16-h light / 8-h dark regime at 27±2 °C. Incubated embryonic calli were monitored periodically for greening of calli, green shoot regeneration and albino plant regeneration. The shoot regeneration

TABLE 2

Composition of shoot regeneration media for an indica rice hybrid, KRH 4

Treatments	Basal media	Carbon sources	Hormones	Gelling agents
		Standardized shoot regeneration media for indica rice hybrid, KRH-4 (Debina *et al.,* 2016)		
R_1	N_6	Sucrose (3%)	NAA (0.2 mg/L) + Kinetin (2.5 mg/L) + BAP (1 mg/L)	Agar (0.6%)
		Optimized shoot regeneration media for indica rice hybrid, KRH 4		
R_2	N_6	Sucrose (3%)	NAA (0.1 mg/L) + Kinetin (3 mg/L) + BAP (1 mg/L)	Clerigel (0.2%)
R_3	N_6	Sucrose (3%)	Kinetin (2 mg/L) + BAP (0.5 mg/L)	Clerigel (0.2%)
R_4	N_6	Sucrose (3%)	NAA (0.5 mg/L) + Kinetin (1 mg/L) + BAP (2 mg/L)	Clerigel (0.2%)

Note: 1-2 mm embryonic calli were incubated at 27 ± 2 °C in BOD incubator maintaining 16-h light / 8-h dark regime

frequency was calculated by the following formula (Islam *et al*., 2005).

$$\text{Shoot regeneration frequency (\%)} = \frac{\text{No. of regenerated plantlets}}{\text{No. of calli plated for regeneration}} \times 100$$

Results and Discussion

Optimized Callus Induction Protocol to Enhance Callus Induction Efficiency In an Indica Rice Hybrid, KRH 4

In rice breeding programmes, the prospective applicability of anther culture technique has been limited due to factors such as high genotypic dependency, poor callus induction and green plant regeneration frequencies and high albino plant regeneration (Mishra *et al*., 2016). Therefore, a genotype-independent increment in anther culture efficiency demands greater exertion. In this regard, manipulating the non-genetic factors like culture medium components and pre and post-culture conditions appear to improve rice anther cultarability.

Callus induction efficiency is directly proportional to the number of callus producible anthers (with the appropriate microspore development stage) in the culture media. Therefore, selecting appropriate stage of anthers with uni-nucleate microspores is a pre-requisite for increasing callus induction efficiency in rice anther culture (Zapata, 2003). In the previous study by Debina *et al*. (2016), it was reported that, the KRH 4 rice hybrid anthers obtained from panicles of 8-12 cm from flag leaf to penultimate leaf (Fig. 1A) showed efficient culturability. However, in the extended study, we identified the most potential anthers from different positions of a panicle for callus induction by establishing a relationship between panicle lengths, spikelet position on panicle, anther position in spikelet (Table 3). Boots with a flag leaf to penultimate leaf distance of 10-12 cm had the most responsive anthers

Table 3

Relationship between panicle length, spikelet position on panicle and anther position in spikelet on anther response

Panicle elongation stage	Length of panicle from flag leaf to penultimate leaf (cm)	Spikelet position on panicle	Anther position in spikelet*	Anther length (mm)	Anther colour	Anther response
Early boot stage	5 cm	Top	2/6th	1	Pale Yellow	-
		Middle	2/6th	1	Pale Yellow	-
		Bottom	2/6th	1	White	-
Middle boot stage	8 cm	Top	4/6th	2	Yellow	+
		Middle	2/6th	1	Pale Yellow	-
		Bottom	2/6th	1	White	-
	10 cm	Top	4/6th	2	Yellow	++
		Middle	3/6th	2	Yellow	+++
		Bottom	2/6th	1	White	-
	12 cm	Top	4/6th	2	Yellow	++
		Middle	4/6th	2	Yellow	+++
		Bottom	3/6th	2	Yellow	+++
Late boot stage	15 cm	Top	5/6th	2	Yellow	+
		Middle	5/6th	2	Yellow	+
		Bottom	4/6th	2	Yellow	++

Note: The '+' sign symbolizes the rate at which anthers responded. ; + : 1-10 anthers responded out of 60 anthers ; ++ : 10-20 anthers responded out of 60 anthers ; +++ : 20-30 anthers responded out of 60 anthers

* Anther position in spikelet keep increasing from early boot to late boot stage

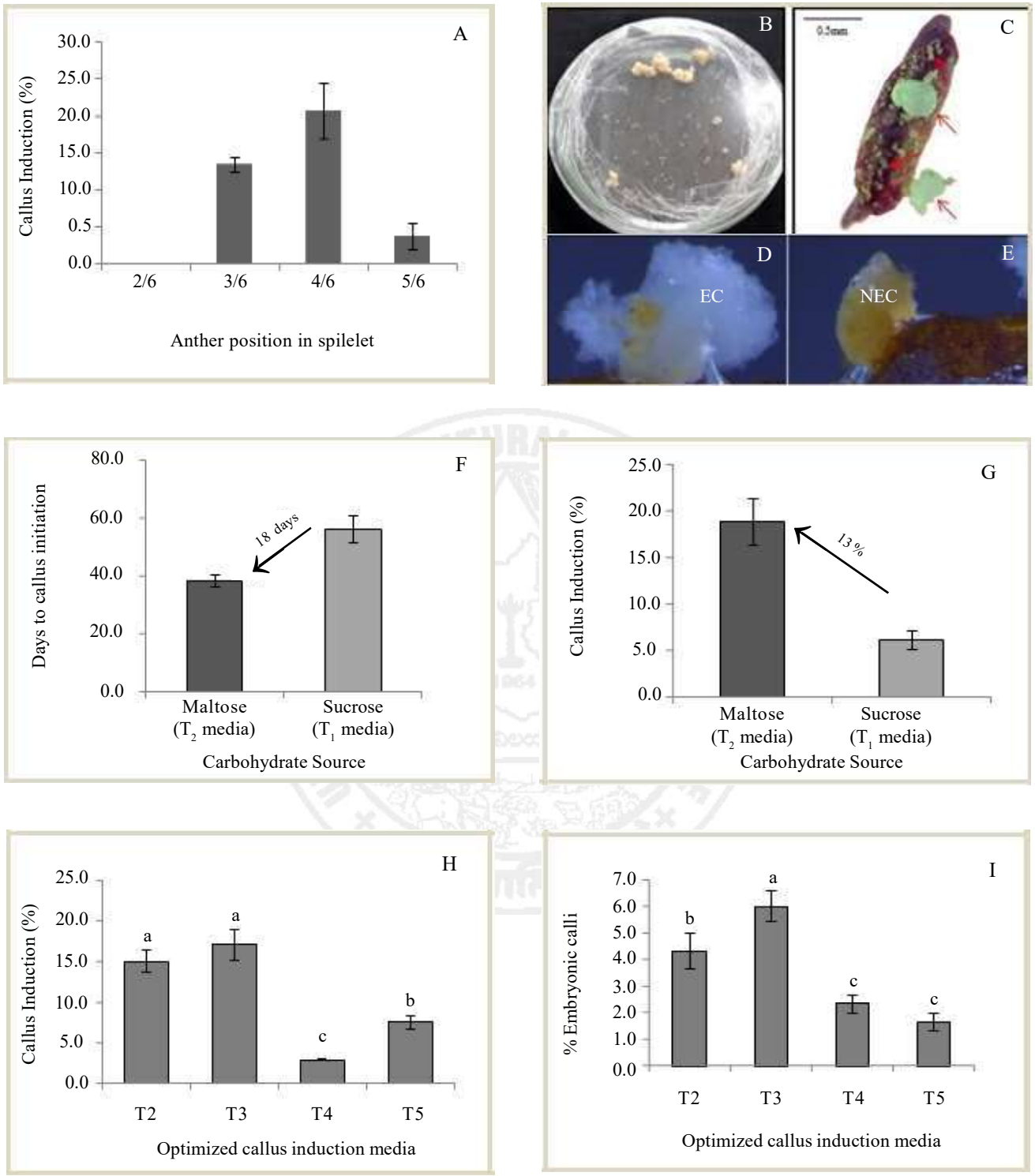

Fig. 2. Callus induction from anthers of indica rice hybrid, KRH-4. (A) Position of anthers in the spikelet and its influence on per *cent* callus induction in rice. (B) Calli derived from dark-induced anthers at 25±2°C. (C) Induction of multiple callus from a single anther (arrow head). (D) Embryonic calli (EC). (E) Non-embryonic calli (NEC). (F & G) Effect of carbon source on days to callus initiation (~0.5 mm calli) and per *cent* callus induction. (H & I) Effect of optimised callus induction media on per cent callus induction and embryonic calli formation, respectively.

for callus induction. Further, anthers positioned at a distance of 3/6th and 4/6th within the spikelet had a higher response rate in culture than anthers positioned 2/6th and 5/6th within the spikelet (Table 3).

Callus induction frequency (%) among anthers at different positions (2/6th, 3/6th, 4/6th and 5/6th) within the spikelets was determined and it was higher in 4/6th positioned anthers (20.8%) followed by 3/6th (13.5%) and 5/6th (3.8%) (Fig. 2A and 2B). In 2/6th positioned anthers, callus induction was not at all observed (Fig. 2A). Afza *et al.* (2001) have also reported that, anthers at different positions possess different callusing ability. According to the present findings, 4/6th positioned anthers (Fig. 1C) from 10-12 cm panicle length should be employed in rice anther culture to achieve higher efficiency and hence, maximize the utility of this technology in rice breeding programmes (Table 3).

Culture media plays a pivotal role in the androgenic response in indica rice types. The culture medium not only provides nutrition to the microspores but also directs the pathways of embryo development (Kaushal *et al*., 2014). A carbohydrate source is essential in anther culture because of its osmotic and nutritional effects (Bishnoi *et al*., 2000). Maltose has been demonstrated to be a superior carbohydrate supplement for androgenesis in numerous species including cereals when compared to sucrose (Mostafiz and Wagiran, 2018). When sucrose (3%) was replaced with maltose (3%) in the callus inducing media used by Debina *et al*. (2016), the callus formation was early by 18 days (Fig. 2F) and callus induction percentage was increased by 13 per cent (Fig. 2G). This can be explained by the fact that, sucrose is hydrolyzed rapidly to glucose and fructose by the highly abundant invertase enzyme whereas, maltose is degraded more slowly and probably also serves as better osmolyte. Further, removal of sucrose from the medium and adding maltose in its place may reduce the toxic effect to the microspores and therefore, induce better callusing (Mostafiz and Wagiran, 2018).

To improve callusing responsiveness in KRH 4 rice hybrid, the standardized callus medium (Debina *et al*., 2016) was further improved by modifying hormone and gelling agent composition (Table 1). Among the optimized callus induction media used in the present study, T_3 media containing N_6 basal media supplemented with 2, 4-D (2 mg/L), NAA (1 mg/L), kinetin (0.5 mg/L), maltose (3%) and clerigel (0.2%) and maintained at a pH of 5.8 showed higher callusing efficiency which was on par with T_2 media containing basal media supplemented with 2, 4-D (2 mg/L), NAA (1 mg/L), kinetin (0.5 mg/L), maltose (3%) and agar (0.6%) maintained at a pH of 5.8 (Fig. 2H). In addition, the percentage of embryonic calli (which generally responds for shoot regeneration) was also significantly higher in T_2 and T_3 treatments compared to rest of treatments (Fig. 2I). However, between T_2 and T_3, treatments, T_3 had higher callus induction percentage with high percentage of embryonic calli. Treatments T_2 and T_3 differ in the gelling agent where, T_2 had agar while T_3 had clerigel as gelling agent. This data reveals that, the gelling agent do influence the callus induction, with clerigel had a higher percentage of embryonic calli formation than agar. Clerigel has been reported to yield better results than agar in of somatic embryos, callus growth and callus regeneration and shoot multiplication (Owen *et al*., 1991). As compared to agar, clerigel do not significantly reduce the pH of the media after autoclaving (Owen *et al*., 1991) and hence, it is likely that, it may have a role in improving the callus induction efficiency.

Optimized Shoot Regeneration Protocol to Enhance Shoot Regeneration Efficiency in an Indica Rice Hybrid, KRH 4

Successful green shoot regeneration from embryonic calli depends on exogenous supply of plant growth regulators (Trejo-Tapia *et al*., 2002). The growth regulators that have been mostly used in *in vitro* anther culture are 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and cytokinin which can be manipulated at different concentrations in the culture media for improved shoot regeneration of embryonic calli obtained from potential anthers (Ali *et al*., 2021). Thus, optimizing plant growth regulators in shoot regeneration media attains lot of significance in enhancing the regeneration efficiency in indica rice

hybrid. A good quality embryogenic calli (1-2 mm) obtained from treatment T_3 were transferred to optimized regeneration medium (R_2-R_5) to investigate their potential role for shoot regeneration compared to previously standardized shoot regeneration media (R_1) of Debina *et al*. (2016). Plant regeneration efficiency of embryonic calli was highest when incubated on N_6 basal media supplemented with 3 mg/l kinetin, 0.1 mg/l NAA, 1 mg/l BAP, 3 per cent sucrose and 0.2 per cent clerigel (pH 5.8) (R_2 media; Table 2) and kept under absolute dark for 48 hrs followed by transferring them to BOD incubator maintaining 16-h light / 8-h dark regime at 27 ± 2 °C (Fig. 3). In R_2 media, per cent shoot regeneration was about 33.3 per cent, which was approximately 4.4 per cent more than in R_1 media (28.9%) (Fig. 3A). Greening of the callus was observed between 7 and 15 days on regeneration media. Calli with delayed greening have never initiated shoots and as a result, such calli have gone brown for unknown reasons. Shoot initiation from green calli (Fig. 3B) took 15-45 days from the incubation of embryonic calli. Subsequently, 1-2 cm length shoots were transferred to rooting media (Fig. 3C) (Standardized by Debina *et al*., 2016) and we got fairly a good root induction in the same media as reported by Debina *et al*. (2016). Well rooted *in vitro* anther cultured plantlets (Fig. 3E) were further acclimatized under green house conditions.

Albino plant regeneration is one of the limiting factors that makes anther culture in indica rice inefficient compared to japonica rice (Shahzad *et al*., 2017). Reports show that the frequency of albino regeneration ranges from 10 to 100 per cent in indica rice. Thus, successful indica rice anther culture requires the development of effective shoot regeneration media with increased green shoot and lower albino shoot generation. In the present study, *Per cent* albino shoots regeneration in R_2 media (5.8 %) was lower compared to R_1 media (17.3 %) (which was standardized by Debina *et al*., 2016) (Fig. 3A and 3D). Why and how the albino plants percentage reduced significantly with the new protocol of ours is not clear. However, it is speculated that, as the greening per cent has increased, the number of plants turning albinos might have reduced in the new media. This however needs to be examined thoroughly.

To increase the success rate of callus induction and shoot regeneration in indica rice hybrid, there is a need to overcome the gap between the essential steps determining the effective media composition and each component's concentration in the *in vitro* anther culture protocol. Maltose can be used as a source of carbon to achieve significantly higher callus induction rate. Present study also reveals that, the gelling agent

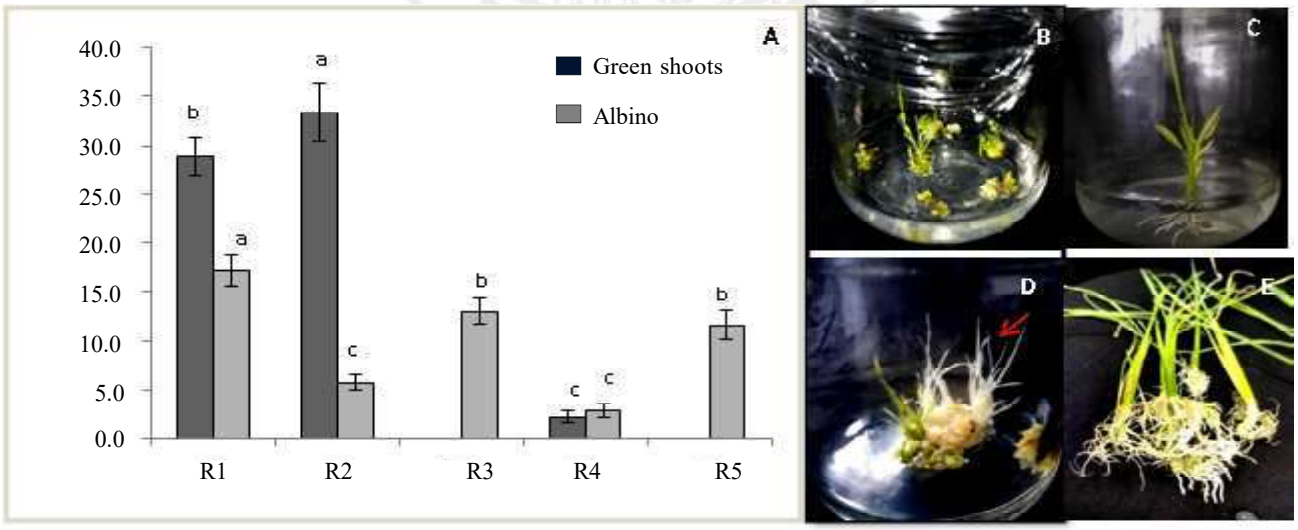

Fig. 3: Shoot regeneration from embryogenic calli of indica rice hybrid, KRH 4. A) Effect of optimized shoot regeneration media on per cent green shoot and albino regeneration; B) Initiation of shoot regeneration from embryonic calli; C) Shoot elongation; D) Albino shoot regeneration (red arrow) from calli and E) Rooting of green shoots

do influence the callus induction with clerigel had a higher percentage of embryonic calli formation than agar. Optimization of regeneration protocol although improved green shoot regeneration, generation of albino plantlets from embryonic calli needs to be minimized, which is a formidable obstacle in utilization of rice anther culture for indica rice improvement. The findings of this study, reiterates the importance of introducing minor changes in callus induction and shoot regeneration media to improve their efficiency leading to the production of haploids and doubled haploids more effectively in indica rice types.

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