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Optimization of Pre-treatment Incubation Period on Callus Induction Response in Anthers of Selected Rice Genotypes

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ABSTRACT

Background: The microspores of five tropical *japonica* and five *indica* rice genotypes were subjected to androgenic studies. The effect of growth regulators on callus induction were studied to improve the anther culture efficiency.

Methods: The cold pre-treatment of panicles at 10°C were done at different days of intervals *viz*., 5, 8, 10 and 12 days. The microspores at uninucleate stage were selected and dusted after pre-treatment. The anthers were cultured in N6 basal media supplemented with casein hydrolysate (250 mg/L), proline (250 mg/L), silver nitrate (100 mg/L), maltose (50 g/L) and growth regulators.

Result: The 8 days of cold pre-treatment initiated calli in most of the ten genotypes. The days taken for callus induction varied with genotype from 32-55 days. The callus induction frequency ranged from 1.41 to 5.12%. The responsive genotypes (Azucena, Palawan, Nira) on callus induction were studied for their regeneration potential.

Key words: Anther culture, Callus initiation, Cold pre-treatment, *Indica*, Plantlet regeneration, Tropical *japonica.*

INTRODUCTION

To sustain the future demand on rice production for growing population, re-orientation of breeding programs should be done to improve varieties for drastically changing environments, biotic and abiotic stress (Tripathy, 2021). To accelerate the crop improvement, different conventional and biotechnological approaches are combined and used. Among them, for rapid development of crops, haploid technology (anther culture) is adopted in plant breeding for various crops (Savenko *et al*., 2021).

Doubled haploid lines, with complete homozygosity have been produced through anther or microspores, by spontaneous or induced chromosome duplication in shorter time. The haploid plant production from anther culture was first reported by Niizeki and Oono (1968) in rice. The two step process involves induction of haploids and doubling of chromosome. This technique helps in shortening the breeding cycle (one meiotic recombination) and rapid attainment of homozygous lines compared to conventional methods. The technique widens the genetic variability and suitable for genetic manipulations. Recessive genes can be recovered by this technique (Tripathy *et al*., 2019). The developed doubled haploid plants neglects the inbreeding process and forms new lines with unique gene combination (Tripathy, 2021). It improves genetic gain of crops with limited time.

The differences in androgenic response and doubled haploid production in rice had been mainly due to genotypic differences, where *indica* genotype exhibited low androgenic response compared to *japonica* due to its recalcitrant nature. The major difficulty in production of haploids was low induction of embryogenic calli, lesser green plant generation and high number of albino plantlets. These can be improved by making cross combination between selected parents (Mohiuddin *et al*., 2014; Hooghvorst *et al*., 2018).

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To trigger the androgenic embryo development the microspores were exposed to cold stress and osmotic stress. Cold pre-treatment was found to help in delayed anther wall senescence and increased the microspore division. Diffusion of nutrients was facilitated through anther wall for inducing sporophytic division and also filtered excess concentration of nutrient from medium. The pollen development was suggested to be hindered and haploids developed *via* formation of callus. During this process of callus development genetic recombination may occur and results in genetically unique lines (Maharani *et al*., 2020).

Several factors was suggested to be influenced by genotype of the plant like pre-treatment of panicle, condition

of anther, development of microspores, composition of media like macro and micro elements, Plant Growth Regulators (PGR), carbon source, organic supplements, number of sub cultures and external growing condition. The amount of Cu^{2+} and Ag^{+} in the media influenced the embryogenesis, shooting, rooting and multiple shoot induction by preventing necrosis. For effective induction, anthers at middle uni-nucleate pollens were used (Sammour *et al*., 2015). The most frequently used medium for androgenic induction was N6 medium (Chu *et al*., 1975). The ratio of KNO_3 and (NH_4) SO_4 in N6 media enhanced the response of genotypes for callus induction (Kaushal *et al*., 2014). The combination of plant growth regulators like BAP, IAA, NAA and kinetin improved green plant regeneration from microspore derived calli (Lantos *et al*., 2022).

Hence, the current study focused on pre-treatment temperature, incubation period effect on panicles and effect of plant growth regulators on callus induction in selected rice genotypes.

MATERIALS AND METHODS

This experiment was conducted in the tissue culture laboratory of the Department of Genetics and Plant Breeding, Tamil Nadu Agricultural University, Coimbatore during the year 2022. The plant materials selected for the study consisted of five tropical *japonica* (Azucena, Palawan, Nira, Pato and, Iguapecateto) and five *indica* (CB 174R, CB 87R, TRY 2, TRY 3 and ADT 53) rice genotypes. All the selected genotypes were grown under field condition for collection of explant (anther).The disease free panicles were collected from the primary tillers during 7-9 a.m. The internode distance from flag leaf auricle to next leaf is around 5-7cm, where the microspores were at uninucleate to binucleate stage. The collected panicles were sterilized with 70% ethanol and wrapped tightly with germination paper followed by polythene cover. Then the wrapped panicles were stored in 10°C for cold pre-treatment in different days of intervals *viz*., 5, 8, 10 and 12 days.

After cold pre-treatment panicles were taken and florets from the middle were selected for sterilization. The selected florets were initially surface sterilized with 70% ethanol for two minutes followed by 0.1% mercuric chloride for ten minutes and rinsed with sterile distilled water for three times.

After sterilization, florets were cut at the base (below anthers) using scissors, then the florets tip were picked up using forceps and tapped on the rim of test tubes containing callus induction media. Around 250 anthers were dusted in an induction media. The media used for callus induction consisted of N6 basal media supplemented with casein hydrolysate (250 mg/L), proline (250 mg/L), silver nitrate (10 mg/L), maltose (50 g/L) and plant growth regulators (PGR) 2,4-D (2.0 and 2.5 mg/L), Kn (0.25 and 0.5 mg/L) and NAA (0.25 and 0.5 mg/L). Then pH was adjusted to 5.8 and solidified with 0.8% Agar. After dusting, the tubes were incubated in dark at 25±1°C for callus induction. The observations made were callus induction rate, number of days taken for callus induction at pre-treatment of 10°C.

Callus induction percentage (%) -

$$\frac{\text{No. of anthers showing callus induction}}{\text{No. of anthers dusted in the media}} \times 100$$

RESULTS AND DISCUSSION

The five tropical *japonica* genotypes and five *indica* genotypes were chosen for the experiment to optimize the cold pre-treatment temperature, incubation period and plant growth regulator concentration influencing the callusing ability.

Effect of cold pre-treatment on callus initiation

The androgenic response of selected genotypes was evaluated in various incubation periods of 5, 8, 10 and 12 days at pre-treatment temperature of 10°C. The obtained results showed significant difference for callus induction percentage of different pre-treatment incubation days. The callusing ability of selected genotypes was observed between 8-12 days of pre-treatment at 10°C. The genotypes Azucena, Palawan, Pato, CB174R, ADT 53, TRY 2 and TRY 3 initiated calli in 8 days cold pre-treated anthers. The 10 days cold pre-treatment incubation, initiated calli in Nira. The incubation period of 12 days, initiated calli in the genotypes of CB 87R and Iguapecateto in cold pre-treated anthers. None of the genotypes responds to callus induction less than 8 days of incubation. The cold pre-treatment effect was suggested to stimulate androgenesis in several genotypes (Dash *et al*., 2022; Sharma *et al*., 2021; Patnaik *et al*., 2020; Win *et al*., 2018). In previous studies, the cold pre-treatment temperatures, differed from 4-10°C.. Especially, the cold pre-treatment temperature of 10°C induced calli, irrespective of genotypes in majority studies. Based on the previous reports, the incubation temperature was regulated to 10°C in our study. The factor, important in deciding the callus induction percentage was pre-treatment incubation period. The incubation period of 8-10 days initiated calli in our study and it correlated well with the observations of Dash *et al*. (2022), Win *et al*.(2018), Cristoffanini *et al*. (2018) and Hooghvorst *et al*. (2018). The cold pre-treatment was found to delay the degradation process and protect the microspores and found to increase the free amino acids, helping the anthers to adapt for metabolic changes. Further, increase in incubation period, resulted in decreased callus induction, degradation of chlorophyll and albino plantlet production (Sharma *et al*., 2021). From the reports it can be concluded that, the optimum cold pre-treatment temperature had an effect on callus induction and plantlet regeneration.

Days taken for callus initiation

The anthers of selected ten genotypes were plated in callus induction medium. Individual anthers in the media initiated visible calli at different days after plating. The days taken for callus induction ranged from 32-55 days and varied with genotypes (Table 1). The variation in days observed for callus induction was affected by genotype. In tropical *japonica* lines Azucena, Palawan, Pato, Nira and Iguapecateto, the calli initiated at 41, 38, 45, 32 and 40 days

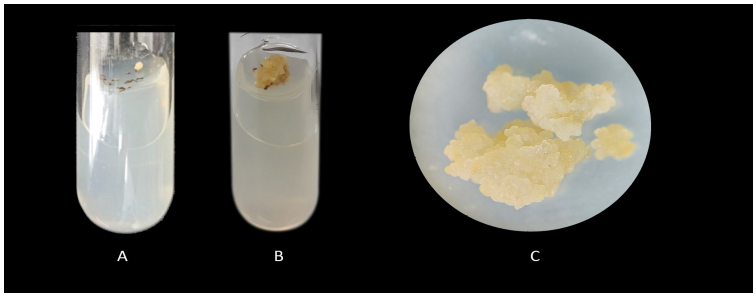
respectively after inoculation, respectively. In *indica* genotypes, callus was initiated after 35 days of inoculation in CB 174R, CB 87R, TRY 2, TRY 3 and in ADT 53, 45 days after inoculation. For callus initiation among the selected ten genotypes, Nira initiated callus at the earliest of 32 days and maximum of 45 days taken for ADT 53. Initially, the plated anthers turned brown, further the anthers got swelled up and busted to initiate calli from the middle of anther. In previous studies, the asynchronous initiation of calli around 3-6 weeks after anther plating was reported. The results of the current study confirms with the findings of Dash *et al*. (2022), Mon *et al*. (2020) and Dewi *et al*. (2019). Silva and Ratnayake, (2009) reported 8 weeks for callus initiation in Bg 250 rice genotype. The maximum of 59 days were taken for callus initiation in Hnankar rice genotype (Win *et al*., 2018). Therefore, it was inferred that, the days taken for callus induction was genotype dependent.

Response of genotypes on callusing ability

While comparing the selected genotypes for their callusing ability, the response percentage varied was tabulated in Table 1. The callus induction frequency varied from 1.41 to 5.12%, subjective to cold pre-treatment and genotype of explant (Table 1). The significant differences were observed among genotypes for callus induction. Silva and Ratnayake, (2009) studied the anther culture ability of *kuruluthuda* and BG 250 local rice genotypes on N6 and SK-I medium. The maximum callus induction frequency of 3.6-17.2% in *kuruluthuda* and 1.4% in Bg 250 variety was obtained in N6 medium. The callus induction frequency was higher in N6 media (16.35%) when compared with MS (6.7%) and SK1 (2.43%) media, supplemented with BAP (0.5 mg/L), 2,4-D (2.0 mg/L) and Maltose (30 g/L) (Rout *et al*., 2016). Based on the previous reports, N6 basal medium was used to determine the callusing efficiency.The callusing ability of all genotypes were achieved in N6 medium supplemented with 2,4-D (2.5 mg/L) + Kn (0.5 mg/L) + NAA (0.5 mg/L). Further, the media was additionally added with 250 mg/L of casein hydrolysate and proline, 10 mg/L of silver nitrate with 5% maltose. Among the genotypes, a tropical *japonica* genotype Palawan expressed the highest callus induction of 5.12% (Fig 1), followed by Azucena 4.20%. The *indica* genotype CB87R observed lowest callus induction of 1.41 % and showed least responsiveness.

Comparing the two sub species, tropical *japonica* genotypes performed better for callus induction in N6 medium. The high response of *japonica* rice genotypes in N6 media was due to uptake of inorganic nitrogen as nitrate and ammonium ions. whereas, *indica* genotypes requires low ammonium ions as nitrogen source for their response.Among the 10 rice genotypes evaluated, *tropical japonica* genotypes responded well than *indica* genotypes, where sucrose and maltose used separately as carbon source. The response to callus induction is high, when maltose used as a carbon source than sucrose by Win *et al*. (2018). In anther culture the maltose is used as a carbon source than sucrose, due to slow decomposition rate (Mishra *et al*., 2016; Rukmini *et al*., 2013). Therefore in our study maltose is used as a carbon source. The combination of cold pre-treatment with osmotic stress has promotive effects in the callus induction percentage (Ali *et al*., 2021; Kaushal *et al*., 2014). Roy and Asit, (2005) improved androgenic response in *indica* varieties by supplementing casein hydrolysate to N6 media. To enhance the embryogenesis and reduce early senescence of anthers, an ethylene inhibitor silver nitrate, to the optimum amount is added to the media. Where, it blocks the effect of endogenously synthesised ethylene (Ali *et al*., 2021; Kaushal *et al*., 2014).

Similar to our results, the maximum callus induction percentage of 19.22% observed by Win *et al*. (2018) in tropical *japonica* genotype (Paw San Taung Pyan Hmwe) inoculated in N6 + 2,4-D (2 mg/L) + Kn (0.5 mg/L) from 19 rice genotypes studied. Likewise, Mon *et al*. (2020) also observed that, the callus induction of 2.6% in Yar-8 and 0.5% in Htat Yin genotypes in N6 medium containing 2 mg/L of 2,4-D and 0.5 mg/L of Kn. The maximum callus induction of 11.56% was observed in DRRH3 hybrid by Sharma *et al*., (2021) in N6 medium containing 2.5 mg/L of 2,4-D ,0.5 mg/L of Kn and additionally added with aminoacids tryptophan (25 mg/L) and cysteine (40 mg/L). Dash *et al*. (2022) also found callus induction in six *indica* rice lines, in N6 medium supplemented with 2,4-D and Kn at 2.0 mg/L, 0.5 mg/L respectively. The callus induction of 7.66% and 4.18% were



A); Callus initiation from anther, B); Callus proliferation after 20 days of subculture, C); Callus proliferation after 45 days of subculture.

Fig 1: Callus initiation from tropical *japonica* Palawan anthers.

observed in *japonica* genotypes NRCV 980385 and H 28 by Hooghvorst *et al*., (2018) in N6 medium with combination of 2,4-D (2.0 mg/L) and Kn (0.5 mg/L). In BS 6444G genotype, the spikes were pre-treated for 7-8 days in 10°C. for callus induction. The maximum callus induction percentage of 27.57% was initiated in N6 basal medium supplemented with 2,4-D (2.0 mg/L), BAP (0.5 mg/L) with $AgNO_3$ (5 mg/L) studied by Naik *et al*., (2017).

The callus induction frequency of different genotypes can be improved by manipulating the components of media (Raina and Zapata, 1997). In addition to media components, externally added auxin and cytokinin influences the callus formation. The increase of 2,4-D from 1.5 to 2.5 mg/L, increases the frequency of callus induction in rice hybrids were reported by Sharma *et al*. (2021). They further validated, the combination of 2,4-D, NAA and Kn in the media

Table 1: Callus induction from anthers of ten rice genotypes.

Genotype	Period of cold Pre-treatment at 10°C. (in days)	N6 media + 2,4-D (2.5 mg/L) + Kn (0.5 mg/L) + NAA (0.5 mg/L) + 50 g/L Maltose, Casein hydrolysate (250 mg/L), Proline (250 mg/L), Silver nitrate (100 mg/L)	
		Callus Induction percentage (%)	Days taken for callus initiation
Azucena	5	-	-
	8	4.20	41-54
	10	-	-
	12	1.77	41-54
Palawan	5	-	-
	8	3.68	38-45
	10	-	-
	12	5.12	38-45
Pato	5	-	-
	8	1.65	45-55
	10	2.29	45-55
	12	-	-
Nira	5	-	-
	8	-	-
	10	3.17	32-45
	12	-	-
Iguapecateto	5	-	-
	8	-	-
	10	-	-
	12	2.15	40-45
CB174R	5	-	-
	8	3.30	35-50
	10	3.04	35-50
	12	-	-
CB87R	5	-	-
	8	-	-
	10	-	-
	12	1.41	35-45
ADT53	5	-	-
	8	2.05	45-55
	10	1.93	45-55
	12	-	-
TRY2	5	-	-
	8	1.91	35-40
	10	-	-
	12	-	-
TRY3	5	-	-
	8	2.00	35-45
	10	-	-
	12	-	-

enhancing the embryogenic callus induction from anthers. The exogenously supplied plant growth hormones influences the androgenesis efficiency with specific genotype (Lantos *et al*., 2022). So, genotype specific media standardization is needed to improve androgenic plants.

CONCLUSION

The incubation period of cold pre-treatment and days taken for callus induction varied from genotype to genotype.The callusing ability of all genotypes were achieved in N6 medium supplemented with 2,4-D (2.5 mg/L) + Kn (0.5 mg/L) + NAA (0.5 mg/L). Among the genotypes, a tropical *japonica* genotype Palawan expressed the highest callus induction of 5.12%, followed by Azucena 4.20%. The *indica* genotype CB 87R observed lowest callus induction of 1.41%. Further, standardization of media components will improve the callus induction ability of genotypes.

Conflict of interest: None.

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