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Optimization of Conditions for Callus Induction from BC_2F_1 Population (Ranbir Basmati x PAU148) through Anther Culture

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SKG, BKS and VS along with other authors designed the study problem and made changes as per requirement. Author Mridhu Sharma did practical work, managed the literature searches and wrote the first draft of the manuscript. Authors AKS, RKS and Manmohan Sharma reviewed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

With different culture conditions and concentrations of growth regulators and supplements, Anther culture technique can be easily employed for the production of haploids under *in vitro* conditions.
Aims: The present study was undertaken with the objective to optimize the development of doubled haploids using anthers for *in vitro* induction of callus on N_6 medium.
Place and Duration of Study: The samples (BC_2F_1 seeds) were raised previously in Skuast-J. From total degree program of 3 years, this work related to tissue culture technique was done in one year from January 2018 to January 2019.

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Methodology: The effect of levels of 2, 4-dichlorophenoxy acetic acid (2, 4-D) i.e. 0 to 3 mg/L in basal N_6 media was observed on callus induction frequency (CIF). The effect of duration of cold pre-treatment was observed on callus induction frequency at 2.5 mg/L of 2, 4-D by giving the cold pre-treatment at 4ºC from 8 to 12 days. Also the effect of different amino acids was checked on callus induction frequency.
Results: Highest callus induction frequency of 9.39 per cent was observed in N_6 medium fortified with 2.5 mg/L 2, 4-D and lowest callus induction frequency of 2.52 per cent at the concentration of 1.0 mg/L. The cold pre-treatment for 10 days gave highest callus induction frequency of 1.44 per cent and lowest callus induction frequency of 0.44 per cent was obtained at cold pre treatment for 8 days. The highest callus induction frequency of 12.55 per cent was observed in case of media supplemented with 25 mg/L tryptophan and 40 mg/L cysteine and lowest callus induction frequency of 7.18 per cent was observed when media was supplemented with 560 mg/L proline.
Conclusion: The cold pre-treatment of 10 days at 4ºC on media supplemented with 2.5 mg/L of 2, 4-D and combination of 25 mg/L tryptophan and 40 mg/L cysteine proves to provide best androgenesis conditions for anthers from BC_2F_1 population.

Keywords: Callus induction frequency; anther culture; 2,4- D; cold pre-treatments; amino acids.

1. INTRODUCTION

Anther culture is an important technique to develop homozygous lines by shortening the breeding cycle of new varieties and allows early expression of recessive genes. Beside the above advantages, anther culture also has disadvantages and constraints which include low efficiencies of callus production, low frequency of plant regeneration, and high proportion of albino plants [1,2]. Several factors like genotype of plant, concentration of growth regulator, time and temperature of pre-treatment and microscopic developmental stage affect the callus texture derived from *in vitro* anther culture of rice (Rukmini, et al. 2016). Cold pre treatment was reported to have promontory effect for androgenesis in several plant species. Pre-treatement under cold condition is very important to ensure green haploid plant regeneration [3]. Cold pre-treatment eliminates weak or non-viable microspores in culture [4], delays anther wall senescence, and increases the symmetric division of pollen grains and release of necessary substances (cold shock proteins and amino acids) for androgenesis [5]. The callus induction frequency irrespective of the media employed and prolonged treatment over the optimum proved to be inhibitory (Rukmini, et al. 2013). Competence of the plant during anther culture can be guessed by the texture and colours of the calli [6]. The embryogenic calli which were milky white in colour and compact in texture had excellent regeneration ability. However, friable calli had poor plant regeneration ability or did not respond at all. These results indicated that the callus induction medium has an influence on the morphogenic competence of the induced callus, determining its regeneration capability [7]. It was reported that there are conditions in which genotypes show high callus induction displayed poor regeneration ability and vice versa [4]. Application of higher dose of auxin sources can significantly increase the callus induction efficiency, however such calli are less in embryogenic and poor in green plant regeneration. Anthers of three rice cultivars *viz*, BR-3, BR-10 and BRRI Dhan 29 produced friable and compact callus texture with white in colour in Z2 media containing 2 mg/L 2, 4-D + 2.5 mg/l NAA + 0.5 mg/l Kinetin [8]. Many an embryogenic and non-embryogenic callus with multiple colours (white, yellow and brown) reported in rice cultivar Swat II on MS media containing different concentration of auxin and kinetin with Tryptophan [9].

2. MATERIALS AND METHODS

The spikelets of BC_2F_1 plants at tillering stage were collected. The anthers from the top end and lowermost end were collected and pollen stage was identified under microscope with the help of 2 percent acetocarmine. The spikelets with pollens at uninucleate stage were collected and pre-treated at 4ºC for different number of days (8, 9, 10, 11 and 12 days) Table 2.

After the optimization of time of pre-treatment, the panicles were treated with Tween 20 for an hour and then treated under running water for 15 minutes. Then the panicles were treated two to three times with distilled water. These panicles were then taken into laminar air flow chamber where they were treated with 0.1 percent of mercuric chloride ($HgCl_2$) and then finally

washed in autoclaved distilled water almost three times.

With the help of the scalpel blade the panicles were cut at the basal end and holding at the tip of the panicles, the panicles were tapped at the rim of the test tube so that the anthers shed on the callus induction media (CIM) (Fig. 4). When the anthers in the media started to turn brown (Fig. 5) after a week or two, they then start to form callus (Fig. 6) which can be multiplied further on the same media compositions (Fig. 7).

For every treatment callus induction frequency was calculated by the formula:

No. of callus induced/No. of anthers inoculated x 100

The callus induction frequency was checked under different concentrations of 2,4-D. Different concentrations of 2,4-D (0 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L, 2.5 mg/L and 3.0 mg/L) (Table 1) were added in the callus induction media N_6 (3.97 g) with 4% maltose, 0.8% agar, 0.5 mg/L Kinetin and 1 mg/L NAA and its effect on callus was checked and the media was optimized for further studies. The optimized media was checked for callus induction frequency with the addition of different amino acids (proline-560 mg/L; glutamine-500 mg/L; tryptophan- 25 mg/L; cysteine- 40 mg/L and also the combination of tryptophan and cysteine) (Table 3).

All the trials of the experiments were repeated three times. The data recorded for different parameters were subjected to completely randomized design [10] with three replications each of six treatments of 2,4-D concentrations and five treatments each of cold pre-treatment and different amino acids by using OPSTAT.

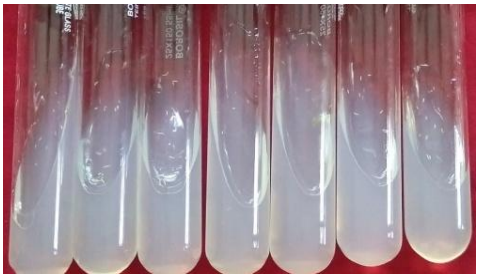

Fig. 1. Dusting of anthers on the media

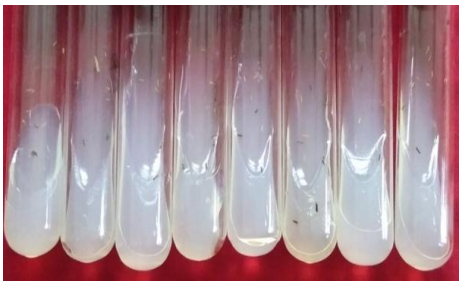

Fig. 2. Browning of anthers on the media

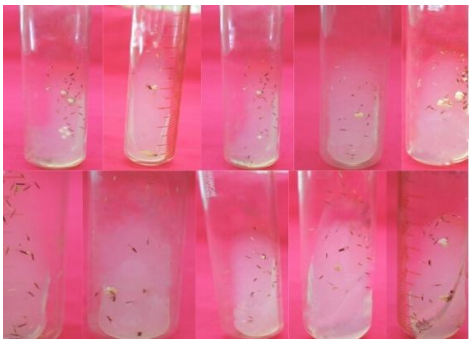

Fig. 3. Callus induction in N_6 media with different concentrations and compositions of amino acids and growth regulators

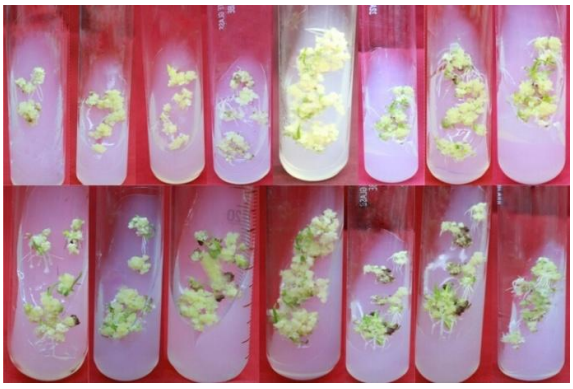

Fig. 4. Multiplication of callus induced

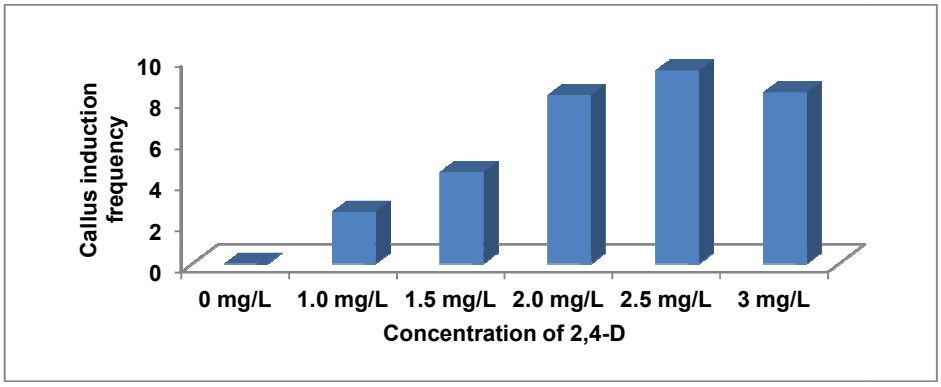

Fig. 5. Effect of levels of 2, 4-D on callus induction frequency

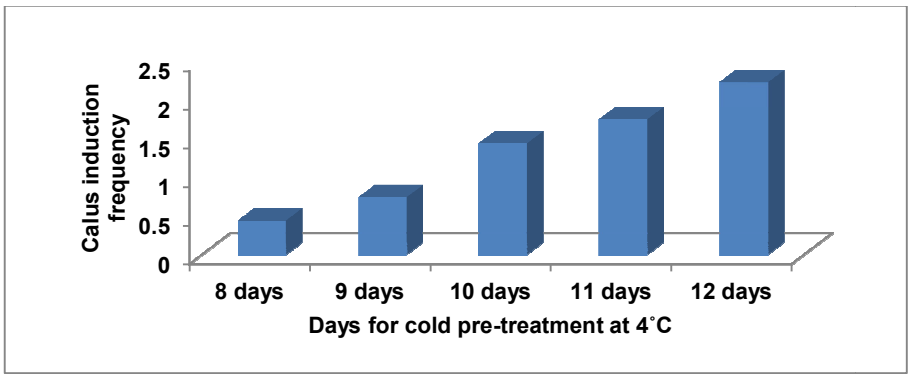

Fig. 6. Effect of cold pre treatment on callus induction frequency

Table 1. Effect of levels of 2,4-D on callus induction frequency

Concentration of 2,4-D	Anther inoculated	Callus induced	Callus induction frequency (%)
0 mg/L	220	0	0
1.0 mg/L	198	5	2.52
1.5 mg/L	224	10	4.46
2.0 mg/L	230	19	8.24
2.5 mg/L	266	25	9.35
3 mg/L	240	20	8.31
Total	1378	79	5.48± 0.015
C.D. (P = 0.046) CV = 0.46			

Table 2. Effect of cold pre treatment on callus induction frequency

Cold treatment (In days)	Anther inoculated	Callus induced	Callus induction frequency (%)
8 days	680	3	0.44
9 days	672	5	0.74
10 days	694	10	1.44
11 days	682	12	1.75
12 days	670	15	2.23
Total	3398	45	1.32±0.017
C.D. (P = 0.055) C.V. = 2.36			

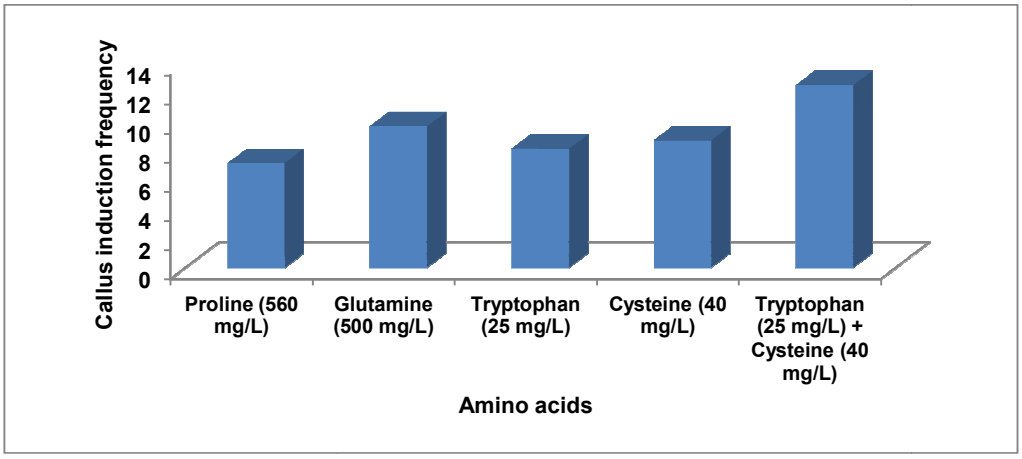

Fig. 7. Effect of amino acid supplemented media on callus induction frequency

Table 3. Effect of amino acid supplemented media on callus induction frequency

Amino acids	Anther inoculated	Callus induced	Callus induction frequency (%)
Proline (560 mg/L)	195	14	7.13
Glutamine(500 mg/L)	206	20	9.70
Tryptophan(25 mg/L)	232	19	8.19
Cysteine (40 mg/L)	240	21	8.74
Tryptophan(25 mg/L) + Cysteine (40 mg/L)	239	30	12.55
Total	1112	104	9.28±0.020
C.D. (P = 0.064) C.V. = 0.377			

3. RESULTS AND DISCUSSION

3.1 Effect of Levels of 2, 4-D on Callus Induction Frequency

The anthers showed browning indicating that the wall of anther has dehisced so that the callus is not formed by anther wall and within a week started to show the callus induction under the influence of 2, 4-D. It was seen that with increased concentration of 2, 4-D the callus induction frequency also increased till 2.5 mg/L then decreased with increasing concentration. The maximum callus induction frequency of 9.39 percent was observed at 2.5 mg/L concentration of 2, 4-D and minimum callus induction frequency of zero percent was observed when no 2,4-D was added to the media followed by 2.52 percent when the concentration 2,4-D was increased to 1.0 mg/L (Table 1, Fig. 1). From our study we observed highest callus induction frequency of 10.07% when the concentration of 2,4-D was 2.5 mg/L, that of Kinetin was 0.5 mg/L and NAA was 2 mg/L. Similarly, the highest callus induction frequency was observed in Azucena rice variety on N6 medium supplemented with 2 mg/L 2, 4-D and 1 mg/L Kinetin; 1 mg/L 2, 4-D and 2 mg/L NAA and in Moroberekan rice variety on N6 medium supplemented with 1 mg/L 2, 4-D, 2 mg/L NAA and 0.5 mg/L Kinetin and 2 mg/L 2, 4-D and 2 mg/L NAA and 1 mg/L Kinetin [11]. The high callus induction frequency of 6.66percent in Azucena rice variety on N6 medium supplemented with 1 mg/L 2, 4-D and 2 mg/L NAA and 0.5 mg/L Kinetin was observed by Lal, et al. [12]. Similarly, the callus induction frequency of 4.24 percent was obtained by Gueye and Ndoye [13] in IKP (*Japonica*) variety on N_6 medium supplemented with 1 mg/L 2, 4-D and 2 mg/L NAA and 0.5 mg/L Kinetin.

3.2 Effect of Cold Pre Treatment on Callus Induction Frequency

The callus induction frequency increased with increasing number of days of cold pre-treatment (Table 2, Fig. 2). The highest callus induction frequency of 4.17 percent was observed in anthers given cold pre-treatment at 4ºC for 10 days and lowest callus induction frequency of 1.03% was observed in cold pre-treatment at 4ºC

for 8 days. The results of chilling pre-treatment given by the other authors are somewhat similar to our study. Xie, et al. [14] investigated the effect of cold pre-treatment of panicles for 12–35 days on the isolated microspore culture and observed that 12 days of cold pre-treatment at 6 ±1ºC were necessary for callusing. The panicles collected from Safri 17 x Pb 3 cross and Safri 17 x RYT3275, kept in 10ºC for 10, 11 and 12 days and 10, 11,12,13,14 days respectively. The result showed the maximum callus induction frequency of 6.1 percent when the pre-treatment of 10ºC was given for 12 days in case of Safri 17 x Pb 3 and that of 1.43 percent when the pre-treatment of 10ºC was given for 11 days in case of Safri 17 x RYT3275. The callus induction and plant regeneration in *indica* rice varieties were seen to be affected by genotypes and the length of cold pre-treatments [3]. The varying temperature and duration of chilling pre-treatments in callusing and regeneration response in anthers of various genotypes of *indica* and *japonica* rice varieties and hybrids has been reported by several research workers [15,16,17]. The pre- treatment at 10ºC for 11–12 days was observed to give good callusing response but poor regeneration response as they resulted in more albinos, while the temperature of 12ºC for 5 days gave best regeneration response [6]. Therefore, the cold-pre treatment given at temperature 11-12 days at any temperature is best for callus induction response.

3.3 Effect of Amino Acid Supplemented Media on Callus Induction Frequency

Addition of amino acid supplements increased the average callus induction frequency than average callus induction frequency with only 2, 4-D. When individual amino acids were added, the highest callus induction frequency of 9.17percent was observed when glutamine (500 mg/L) was added to the media. The callus induction frequency increased to 12.55 percent when tryptophan (25 mg/L) and cysteine (40 mg/L) were added together (Table 3, Fig. 3). Also the amino acids triggered the callusing of anther as it acts as an organic nitrogen source. 25 rice cultivars were observed for regeneration when supplemented with specific amino acids and different combinations of phytohormones by Khatun, et al. [15]. In *Indica* rice varieties, microspore cultures have been observed to increase callus formation when in addition to growth regulators are supplemented with Tryptophan and Cysteine (amino acids) [18].

4. CONCLUSION AND RECOMMENDATION

The best cold pre-treatment for later generations in anther culture is at 4ºC for 10 days. From the different concentrations of 2, 4- D, the media supplemented with 2.5 mg/L of 2, 4-D was observed to respond the best. Further supplementation of media with amino acids with the combination of 25 mg/L tryptophan and 40 mg/L cysteine proves to provide best androgenesis conditions for anthers from BC_2F_1 populations.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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