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# *In vitro* colchicine treatment to increase the plant regeneration from *Indica* rice anther culture

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**Abstract**
In anther culture, *in vitro* regeneration of green plants is not more than 5-10 percent in *indica* rice cultivars, which limits the application of anther culture worldwide. To address this problem, *in-vitro* colchicine treatment was employed on callus obtained from anther culture of indica rice. S x R cross was taken for colchicine treatment after callus induction from anther culture treatment of 100 and 500 mg/l of colchicine was given for 48 and 72 hours and then after shifted to regenerative media. The experiment was performed along with control (without colchicine treatment) in which green callus percentage and overall plant regeneration percentage increases by 1 to 2.5 fold in treated calluses compared to control. The treatment containing 100 mg/l of colchicine in regeneration media followed by 48 hrs of incubation has given highest green callusing percentage of 23.13 % as control having only 9.16 % of green calli induction in S x R cross. The addition of colchicine had no detrimental effects on the different anther culture efficiency parameters. The highest numbers of green calluses were achieved in cross (SX R) 23 % while the lowest plant regeneration obtained in the cross (SRP) 3.1 %. The whole process up to rooting required seven week time plants obtained are transferred to hydroponics in Yoshida medium for primary hardening for about 20-25 days in glasshouse and subsequently secondary hardening done in field. This work is an attempt for standardization of regeneration media along with addition colchicine for increasing double haploid generation in Rice.

**Keywords:** callus, hydroponics, colchicine, regeneration, DRP

## Introduction
Anther culture is a technology which can significantly reduce the time period required for development of new crop variety with the generation of double haploid (DH) population. Response ofanther culture differs with different varieties (Lee & Lee, 2002) [1] due to different genetic or environmental factors (Ozawa *et al.*, 2003) [2]. Apart from albino plant regeneration (Grewal *et al.*, 2011) [3] the early anther necrosis leads to poor callus proliferation in indica rice varieties (Chen *et al.*, 1991) [4]. Low efficiency of greening callus remains the problem in this technique to increase the greening percentage after callus induction colchicine can be used in media. According to Hansen & Anderson (1998) [12] microspores of two DH lines of wheat were treated with 8 different colchicine concentrations up to 3 mM for either 24 h or 48 h during microspore culture in which fertile plants among the regenerates was increased up to 53% with 1mM concentration. Anther culture technique was used to develop BLB and BLAST resistant rice lines. To increase the efficiency of anther culture by increasing the percentage of green plant regeneration colchicine was used. In Chhattisgarh Bacterial blight and BLAST incidence is problem. Conventional breeding approaches for obtaining resistant variety are limited. Anther culture for obtaining pure line is a better method for obtaining disease resistance rice within very short period of time.

## Materials and Methods
### Plant material
The plant material consisted of two recipient parents Safri-17 and Dubraj. Safri-17 released from JNKVV, Jabalpur using pure line selection from Safri lines and Dubraj is a local popular scented premium rice variety of Chhattisgarh State. Two donor parents RP-Bio226 and PR122 were used. RP-Bio226 was released by Directorate of Rice Research, Hydrabad, India. This was developed using MAS and has three BLB resistance genes (*Xa21, xa13 and xa5*). PR122 was developed from the cross PR108/IRRI-76//PR 106-P1 through pedigree method of selection and designated at Punjab Agricultural University. It carries blast resistance gene *Pi*1 and *Pi*2. The crosses were made to incorporate BLB and blast resistance in donor popular varieties of Chhattisgarh. The anthers from each cross (Table 1) were collected. The panicles were stored in 10°C for 10 days. The panicles were wrapped in moist whattmans filter paper to retain moisture. The panicles from cold pre-treatment were brought to the laminar air flow and

surfacesterilized by soaking them in 1% (v/v) sodium hypochloride solution for 5 min. The panicles were rinse with sterile distilled water 3 to 4 times.

**Table 1:** Details of crosses made for anther culture.

| Sr. no. | Crosses | Parent 1 Recipient | Parent 2 Donor | Parent 3 Donor | Abbreviation | Expected outcome in recipient parent |
|---|---|---|---|---|---|---|
| 1. | Safri 17 X RPBIO 226 | Safri 17 | RPBIO 226 | - | S X R | BLB resistance |
| 2. | Dubraj X RPBIO 226 | Dubraj | RPBIO 226 | - | D X R | BLB resistance |
| 3. | Safri 17 X PR122 | Safri 17 | PR122 | - | S X P | Blast resistance |
| 4. | Safri 17 X RPBIO 226 X PR122 | Safri 17 | RPBIO 226 | PR122 | SRP | BLB & Blastresistance |
| 5. | Dubraj X RPBIO 226 X PR 122 | Dubraj | RPBIO 226 | PR122 | DRP | BLB & Blastresistance |

**Media**

The anthers were plated on artificial media supplemented with maltose, agar and 2, 4-D. The pH of the media was maintained at 5.8 using sodium hydroxide and hydrochloric acid. After 25-30 days of anther inoculation, callus was generated. The calluses were kept in different concentration of colchicine for 48 and 72 hours as given in Table 2. The generated callus was transferred to greening medium containing BAP, Kinetin, NAA and sucrose. This treatment was only given to Cross S X R for increasing the percentage of callus greening and for more green plant regeneration. Four different treatments T1, T2, T3 and T4 along with control were used. After colchicine treatment the callus were transferred to fresh media. The shoots were regenerated within 25 days of transferring. The shoots were shifted to media containing NAA for rooting. The primary rooted plants are then transferred to greenhouse condition for primary hardening in Yoshida medium (Yoshida, 1976) [10].

$$\text{Callusing percentage} = \frac{\text{Number of callus observed}}{\text{Total no of anthers inoculated}} \text{ X } 100$$

**Table 2:** Treatments of Colchicine

| Treatments | Colchicine (mg/l) | Colchicine treatment (hrs) |
|---|---|---|
| Control | 0 | 0 |
| T1 | 100 | 48 |
| T2 | 100 | 72 |
| T3 | 500 | 48 |
| T4 | 500 | 72 |

**Result and Discussion**

**Calli induction from anther culture**

The anthers were cultured on the callus-inducing artificial medium supplemented with 2, 4- D (Fig 1A). The anther culture response of 4 different cross of rice has shown significant variable calli induction frequency. Callusing percentage varied from 5.1 % in DRP to 12.9 % in S X R (Fig 1B) (Table 3). Similar results were obtained by Bagheri & Jelodar, 2008 as they obtained different calli induction from 4.01 % in Amol-2 to 22.26 % in Rashti cultivars of rice. Rita Chopkar *et al.*, 2016 [13] also found that highest callus induction percentage showed by IBD-1 (27.06%) and the lowest number of calli induced in variety Samleshwari (1.78%) rice varieties. The variable callus induction may be due to different genetic constitution of genotypes as different genotype respond differently under *in-vitro* conditions.

**Effect of colchicines treatment on callus greening percentage**

For increasing the percentage of callus greening and for obtaining more green plants, cross S X R was subjected to *in vitro* colchicine treatment. Four different treatments T1, T2, T3 and T4 with control observed for greening. The greening medium prepared with two concentrations of colchicine 100 mg/l and 500 mg/l with BAP + KIN + NAA and sucrose for callus greening and 23.13 % of calli responded in the media containing 100 mg/l colchicine with 48 hrs of incubation. Then after 48 hrs of incubation callus were transferred to fresh media. The control callus, without colchicine treatment, has responded less with 9.16 % of callus greening (Table 3). Similar results were obtained by Alemanno (1994) [6] as plating rice anthers on a semisolid induction medium containing 250 or 500 mg/l colchicine for 24 or 48 hrs of incubations followed by transfer to colchicine-free medium that of standard anther culture procedures resulted in overall 1.5 to 2.5 fold increase in doubled haploid green plant productions compared to control anther cultures. Zamani (2008) [11] also done anther culture of the three genotypes of wheat which were treated with 0.03% colchicine for 3 days at the beginning of microspore induction regenerants obtained from the colchicine-supplemented induction media produced significantly higher percentages of fertile plants in all genotypes of wheat. Pickens (2006) [9] winter Rose leaf sections were placed on various media supplemented with either colchicine or oryzalin at various concentrations for 1 to 4 days. On various colchicine-containing media, prolific calluses were produced and adventitious shoot formation was observed. Bohus obert (2004) also applied colchicine 0.02% during first 3 days of culture which stimulate embryogenesis in anther culture of maize. Navarro-Alvarez, 2006 [7] found that with addition of colchicine to wheat anther culture media responds in increase of doubled haploid plant Production. Therefore media containing colchicine was used in all other crosses for higher greening percentage. The 100 mg/l colchicine treatment has increased greening percentage by 2-2.5 folds. Increase in greening percentage may be due to in vitro chromosome doubling. As per review and our findings, it is recommended to treat the anther cultured callus with colchicines to increase the green plant production thereby increasing the efficacy of anther culture.

**Plant regeneration**

The cross S X R which was treated with colchocine respond more for greening callus than other crosses which were not treated with colchicine. Highest greening of 23 % was observed in S X R cross and D X R has greening 14.5 % while S X P 8.8 % (Fig 1C). While double crosses SRP and DRP are having 3.1 and 7.2% of greening respectively (Graph 1). Green callus were shifted to shooting medium (Fig 1D). The elongated shoots were then transferred to MS media having NAA for rooting (Fig 1E). The rooted plants are then transferred to hydroponics with Yoshida media for primary hardening (Fig 1G). S X R cross produce highest 983 plants while D X R and S X P produced 129 and 10 plants respectively. While the double crosses SRP and DRP

involving three parents produced 23 and 38 plants (Table 4).) The plants were grown in field till maturity for obtaining seeds. Collected seeds replanted for evaluation and selection of lines with BLB and Blast resistance.

**Table 3:** Effect of colchicine treatment on greening percentage of S X R cross

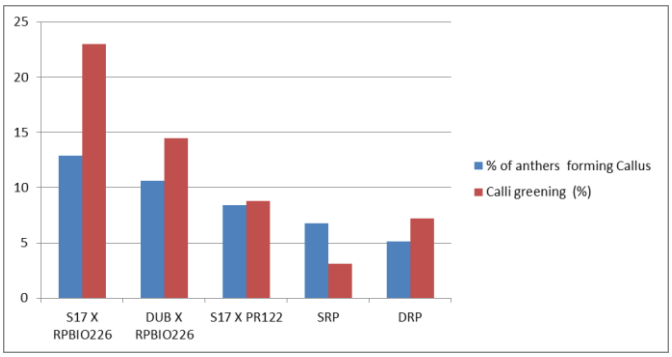
| Treatments | Colchicine (mg/l) | Colchicine treatment (hrs) | No. of callus inoculated | No. of green callus observed (*) | Frequency of callus greening (%) | Fold increase in greening % of callus |
|---|---|---|---|---|---|---|
| Control | - | - | 131 | 12 | 9.16 | |
| T1 | 100 | 48 | 134 | 31* | 23.13 | 2.52 |
| T2 | 100 | 72 | 127 | 22 | 17.32 | 1.89 |
| T3 | 500 | 48 | 139 | 25 | 17.98 | 1.96 |
| T4 | 500 | 72 | 145 | 18 | 12.41 | 1.35 |

*Highest green callus observed

**Table 4:** Anther culture capabilities of five different hybrids

| Hybrid | No. of Anthers | No ofcallus | % of Callusing | Calli greening | Calli greening (%) | Number of green plants generated |
|---|---|---|---|---|---|---|
| S17 X RPBIO226 | 6700 | 864 | 12.9 | 199 | 23* | 983 |
| DUB X RPBIO226 | 2790 | 296 | 10.6 | 43 | 14.5 | 129 |
| S17 X PR122 | 5250 | 441 | 8.4 | 39 | 8.8 | 10 |
| SRP | 33600 | 2285 | 6.8 | 71 | 3.1 | 23 |
| DRP | 13900 | 709 | 5.1 | 51 | 7.2 | 38 |

*Colchicine treated




Graph 1

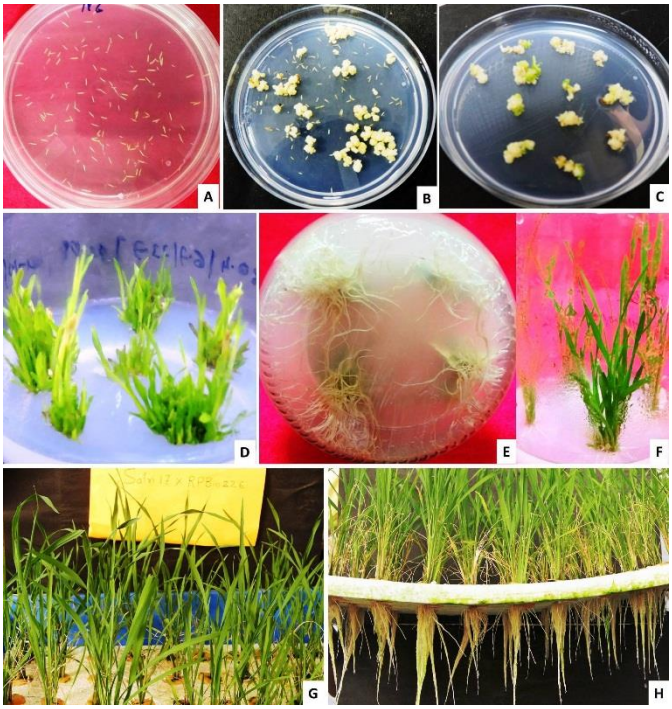



**Fig 1:** *In vitro* induction of double haploid plants through anther culture with their subsequent stages (A) Anthers plated on N 6 medium (B) Callusing of anthers (C) Greening observed in callus (D) Shoot in shoot elongation medium (E and F) Rooting of plantlets (G) Plants in hydroponics medium (H) Roots grown during hydroponics

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