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Time utilization for Callus induction and regeneration from *japonica* rice anthers with different growth regulator treatments

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ABSTRACT
The present experiment was conducted to evaluation of the time utilization of different growth regulator treatments on callus induction and regeneration from anthers of two japonica rice cultivars i.e. Azucena and Moroberekan. Panicles with a distance of 12-13 cm between flag leaf and subtending leaf for Azucena and 14-15 cm for Moroberekan were selected because at this stage of panicle development microspores were in the mid-uninucleate stage. Time utilization was observed in 16 growth regulator treatments of callus induction and 18 growth regulator treatments of regeneration. Data of callus induction and regeneration were analyzed with factorial completely randomized design (FCRD). The lowest time for callus induction was recorded in Moroberekan with callus induction treatment T_{16} containing 2,4-D 2 mg/L + NAA 2 mg/L + Kinetin 1 mg/L (7weeks) and longest time for callus induction was recorded in Azucena with callus induction treatment T_1 containing 2,4-D 1 mg/L + Kinetin 0.5 mg/L (20weeks). However, earliest regeneration was observed in regeneration treatment T_{10} (28 and 21 days respectively) for both Azucena and Moroberekan. The longest time for regeneration was recorded in Moroberekan in regeneration treatment T_5 containing Kinetin 2 mg/L + NAA 0.5 mg/L) (77days). The above findings will be of immense value in the application of in vitro androgenesis for rice improvement.
Key words: *Anther, growth regulator treatments, japonica varieties, callus, regeneration*

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INTRODUCTION

Time performs significant role in rice anther culture. It contributes in panicle collection, cold pretreatment, callus induction, regeneration and hardening, also involves in many growth regulator treatments that helps in reducing breeding cycle in development of homozygous haploid plants. Many authors are already explaining about the callus induction and regeneration of rice with growth regulator treatments. They are discussing about time taken by growth regulator treatments in callus induction and regeneration of rice such as, The anthers of F_1 hybrids of *indica* × *japonica* were observed callus induction after 4-8weeks with NAA + Kinetin and regeneration after 2-8weeks with Kinetin + IAA + NAA (1). Again, the growth regulator combination of 2,4-D + NAA + Kinetin took 6 weeks for callus induction and BAP + Kinetin + NAA growth combination took 4-6weeks in *indica* rice cultivar (2). Callus induction and regeneration were also observed after 6weeks from 2,4-D + Kinetin; Kinetin + NAA by F_1 hybrids of *indica* × *japonica* (3). Callus treatment NAA + Kinetin and regeneration treatment Kinetin + NAA respond after 30days in *japonica* rice genotypes (4). Anthers of F_1 and BC_1F_1 hybrids of wide compatibility trait × recurrent parent produced callus with 2,4-D + NAA + Kinetin at 4-5 weeks and regeneration with BAP +

Kinetin + NAA after 5-15 days (5). Panicles of long duration rice hybrid induced callus with 2,4-D + Kinetin after 3-4 weeks and regeneration with BAP + Kinetin + NAA after 14 days (6). So, time depends on growth regulator treatments that produce plant at short period in tissue culture. With this background, the present investigation was carried out to the "time requirement for upgrading callus induction and regeneration from *japonica* rice anthers by different growth regulator treatments".

MATERIALS AND METHODS

Two *japonica* rice cultivars i.e. Azucena and Moroberekan were employed for this study. Panicles of each cultivar were harvested between 6.00 to 9.00 am on sunny days. The panicles with boot leaf sheath were washed thoroughly in tap water and spread with 70% (w/v) ethanol (7).

They were covered with moist tissue paper, kept in polyethylene bag and cold shocked at 4 °C for 8 days in a refrigerator. Panicles with a distance of 12-13 cm between flag leaf and subtending leaf for Azucena and 14-15 cm for Moroberekan were selected because at this stage of panicle development microspores were in the mid-uninucleate stage. On the day of culture, selected spikelets were surface sterilized in tissue culture bottles with 0.2% freshly prepared $HgCl_2$ solution for 10 minutes (8). The $HgCl_2$ was drained off and the panicles were washed four times in sterile distilled water.

Anthers of individual cultivars were inoculated onto the N6 basal medium (9) supplemented with different concentrations of growth regulators (2,4-D 0, 1, 2 mg/L), (NAA 0, 1, 2 mg/L), Kinetin (0, 0.5,1 mg/L) and Maltose 3% (w/v). The cultures were sealed with parafilm and kept in dark at 23±2 ˚C, with a relative humidity of 50-60%. The jam jar bottles were examined periodically at weekly interval for 10-20weeks, to observe the progress in respect of callus formation.

Embryogenic calli were transferred into the MS basal medium (10) supplemented with different concentrations of growth regulators (Kinetin at 0, 0.5, 1, 2 mg/L), (BAP at 0, 1, 2 mg/L), (NAA at 0, 0.5, 1 mg/L) and Sucrose 3% (w/v). The cultures were again incubated at 23±2 °C with a relative humidity of 50-60% and 16 hour photoperiod at a photon flux density of 3000 lux from white cool fluorescent tubes. Regeneration was observed after 21-77days of incubation.

Data analysis: Figures of callus induction and regeneration were analyzed by factorial completely randomized design (FCRD) of square root transformation with correction factor 0.5%.

RESULTS AND DISCUSSION

The early development of rice *in vitro* anther culture requires not only growth regulator treatments but also panicle harvesting stage, microspore stage and culture conditions. It helps in reducing breeding time and this creates novel rice varieties.

Panicle harvest stage: Panicles were harvested at the early flowering stage, when young panicles were still enclosed within the leaf sheath. Panicles with a distance of 12-13 cm between subtending leaf and the flag leaf for Azucena and 14-15 cm for Moroberekan was selected because at this stage pollen was mid uninucleate. Similar kind of observations was also depicted by different researchers related to rice anther culture. They have pointed out that panicles should be excised when it is enclosed by the sheath (11) and panicle with a distance between subtending leaf and the flag leaf of 7-13 cm (12); 4 to 8 cm (13) have been used for successful callus induction in different rice varieties. Pollen grains at uni-nucleate to early bi-nucleate stages are considered to be optimum for the anther culture in many species (14; 15). However, the early-uninucleate to mid-uninucleate stage of microspores were found to be best suited for androgenic response (16; 17).

Cold pre-treatment and dark incubation: For induction or directing the microspores towards sporophytic pathway rather than natural gametophytic pathway is intensely subjected by stress treatment of the anthers before culture establishment. The response to cold or heat treatment is also genotype dependent. In graminaceous crops, it has been reported that cold pre-treatment of young spikes/panicle was effective for anther culture (18). The cold pre-treatment variation was noticed among the *indica* and *japonica* rice genotypes (19). They also pointed out that when cold treatment duration exceeded a certain limit, the induction frequency decreased substantially. Therefore, in the present study cold

pre-treatment was given to selected panicles at 4°C for 8days. The cultures were incubated in dark for 10-20 weeks for callus induction.

The observations of time requirement for callus induction with different growth regulator treatments are presented in Table-1. Mean of callus induction treatments ranged from 6 to 13 weeks. Azucena 8 to 20 weeks and Moroberekan 7 to 13 weeks took for callus induction. Among the callus induction treatments, earliest callus induction was observed in T_9 treatment 2,4-D 1 mg/L + NAA 1 mg/L + Kinetin 0.5 mg/L took less time for callus induction (6weeks). In the interaction between variety and callus induction treatment, least time for callus induction was recorded in Moroberekan in callus induction treatment T_{16} 2,4-D 2 mg/L + NAA 2 mg/L + Kinetin 1 mg/L (7weeks). Application of 2,4-D and NAA in combination with Kinetin could lead to an increase of the early formed calli. The combination of growth regulator concentrations incurs reciprocal effect that abates the time of callus induction. Equal proportion of concentration produces callus in less time period. The combination of 2, 4-D with kinetin was more effective in producing embryogenic and or organogenic calli, when addition of NAA could enhance the quality of the initiated callus, while cytokinin may increase the growth rate of pre embryogenic masses (20). Callus induction was observed in F_1 hybrids of *indica* × *japonica* between 4 to 8 weeks (21). Three thai rice cultivars KDML105, HJ, and PT1 induced callus on N6 media containing 2,4-D + NAA + Kinetin at 6 weeks (22). Anthers of *japonica* genotype IKP were observed callus induction on N6 media containing 2,4-D + NAA + Kinetin at 4-6 weeks (23). The callus induction was reported in *japonica* variety Azucena on N6 media containing 2,4-D + NAA + Kinetin at 6 weeks and 7 weeks (24).

Table 1: Effect of growth regulators on time requirement for callus induction from anthers in *japonica* rice cultivars

Treatments	2,4-D (mg/L)	NAA (mg/L)	Kinetin (mg/L)	Time taken for callus induction (weeks)		Mean of treatment
				Azucena	Moroberekan	
T_0 (Control)	0	0	0	0	0	0.0
T_1	1	0	0.5	20	0	10.0
T_2	1	0	1	0	13	6.5
T_3	2	0	0.5	17	9	13.0
T_4	2	0	1	17	8	12.5
T_5	0	1	0.5	13	8	10.5
T_6	0	1	1	10	9	9.5
T_7	0	2	0.5	10	11	10.5
T_8	0	2	1	10	8	9.0
T_9	1	1	0.5	0	12	6.0
T_{10}	1	1	1	8	11	9.5
T_{11}	1	2	0.5	16	9	12.5
T_{12}	1	2	1	11	9	10.0
T_{13}	2	1	0.5	16	8	12.0
T_{14}	2	1	1	10	10	10.0
T_{15}	2	2	0.5	13	11	12.0
T_{16}	2	2	1	8	7	7.5

The observations of time requirement for regeneration in the two *japonica* rice varieties Azucena and Moroberekan with different growth regulators is presented in Table 2. Mean of regeneration treatment 14 to 63 days was recorded. Among the regeneration treatments, earliest regeneration time (14 days) was recorded in T_3 regeneration treatment with Kinetin 1 mg/L + NAA 0.5 mg/L. The appropriate concentration of growth regulator may produce early embryo. Plant regeneration in limited time depends on genuine callus viability, callus texture, callus colours and culture condition. Precede gene regulation of callus also may develop early embryo. Plant regeneration was observed in *japonica* rice cultivar Tainan 5 on MS media containing Kinetin + NAA at 10-14 days (25). Anther derived callus of *japonica* rice varieties Nipponbare and Hayahishiki was reported regeneration on MS containing Kinetin + NAA at 14 days (26). Plant regeneration was observed to rice cultivar on MS media containing Kinetin + NAA at 14 days (27).

In interaction between variety and regeneration treatment, in both Azucena and Moroberekan regeneration was induced earliest in regeneration treatment T_{10} (28 and

21days respectively). The combination of Kinetin and BAP found to be more effective for regeneration compared with TDZ and inclusion of NAA in shooting medium enhanced the regeneration frequency and number of shoots developed per explant (28). The callus regeneration has resulted in deepwater rice cultivar on MS media containing Kinetin + BAP + NAA after 20-25 days (29). Androgenic callus of rice cultivar has reported regeneration on MS containing Kinetin + BAP + NAA at 28 days (30).

Table 2: Effect of growth regulators on time requirement for regeneration in *japonica* rice cultivars

Treatment	Kinetin (mg/L)	BAP (mg/L)	NAA (mg/L)	Days to callus regeneration		Mean of treatment
				Azucena	Moroberekan	
T_0 (Control)	0	0	0	0	0	00.0
T_1	0.5	0	0.5	61	0	30.5
T_2	0.5	0	1	0	0	00.0
T_3	1	0	0.5	0	28	14.0
T_4	1	0	1	0	35	17.5
T_5	2	0	0.5	0	77	38.5
T_6	2	0	1	35	0	17.5
T_7	0.5	1	0.5	0	0	00.0
T_8	0.5	1	1	66	40	53.0
T_9	0.5	2	0.5	0	0	00.0
T_{10}	0.5	2	1	28	21	24.5
T_{11}	1	1	0.5	0	0	00.0
T_{12}	1	1	1	56	0	28.0
T_{13}	1	2	0.5	0	0	00.0
T_{14}	1	2	1	0	0	00.0
T_{15}	2	1	0.5	0	0	00.0
T_{16}	2	1	1	0	35	17.5
T_{17}	2	2	0.5	57	69	63.0
T_{18}	2	2	1	0	0	00.0

CONCLUSION

The *in vitro* anther culture of *japonica* rice varieties depends on various factors as growth regulator combinations at different concentrations, choice of variety, maturity stage of anther explant, stress treatments prior to media inoculation, incubation conditions and various other relative conditions . The choice of the most favourable culture conditions combination can only be the answer to produce haploid rice plants from anthers in lesser time. Moreover, time efficiency also should be considered while developing protocols for variety specific culture conditions.

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