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Effect of growth regulators and chemical supplements on callus induction in *japonica* rice verities through anther culture

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Abstract: Anther culture is an efficient and convenient technique for rapid production of doubled haploids which are useful in crop breeding programs. The combination of different growth regulators with chemical supplements facilitates callus induction on N6 medium. Among the two varieties, highest callus induction frequency (44.44%) was recorded in treatment T_4 containing 1.5 mg/L 2, 4-D + 1 mg/L NAA + 5 mg/L silver nitrate in Azucena. However, in Moroberekan highest callus induction frequency (40.0%) was recorded in treatment T_{11} containing 1 mg/L NAA + 0.5 mg/L Kinetin + 500 mg/L casein hydrolysate + 250 mg/L L-Proline. The present study revealed that response of anther culture was better in Azucena (subsp. *japonica*) compared to the Moroberekan (subsp. *japonica*). Therefore, genotype, type of growth regulator with chemical supplements and their interaction plays significant role in order to achieve high callus induction.

Key words: Anther culture, Chemical supplements, Azucena, Moroberekan, Callus

Introduction

Haploid plants have the genotypic number of chromosomes that is a single set of chromosomes in sporophyte. Haploid can be induced by several techniques, the most promising and successful one being microspore androgenesis. Anther culture is a technique in which microspore produce callus after stress treatments, in specific callus induction media and androgenic callus produce plantlets in the another specific media for regeneration. The anther culture technique was first developed in rice by Niizeki and Oono, (1968). Anther culture has long been utilized as an important tool for producing haploids and DH (Double haploid) plants which bypass the inbreeding process (Germana 2011). It is the fastest method for DH production as it only takes between 8 to 9 months (Agache *et al*.,1989). Unfortunately, low percentages of both callus induction and plant regeneration are the main constraints in establishing successful anther culture in some rice varieties especially in *indica* rice since, these critical culturing responses are genotype dependent (Roy and Mandal, 2005). Consequently, the effective culture medium used for some rice varieties may not be appropriate for others, and the composition of culture media should be carefully selected when the anthers of particular rice variety was subjected to culture. Abe and Fustsuhara, (1986) tested 66 *indica* and *japonica* cultivars and reported that *japonica* varieties exhibited a higher rate of callus induction and regeneration than those of *indica*. Moreover, many agronomically valuable rice genotypes are recalcitrant to in *vitro* manipulation because of their poor callus production and regeneration ability. Lentini *et al.* (1995) reported that incorporation of $AgNO_3$ in the callus induction medium not only promoted anther response in *indica* rice but also the regeneration of green plants from the induced callus. Powell, (1990); Achar, (2002) have reported supplementation of madia with glutamine, casein, proline, biotin, inositol, coconut water, silver nitrate and polyvinylpyrolidone for better response of anther culture. Dewi *et al*. (2004) reported that putrescine is more efficient than spermidin and spermin in increasing callus induction and plant regeneration in anther culture. The different concentrations of silver nitrate (5, 10) mg/L promote good callus induction with growth regulator combination of 2, 4-D + kinetin and NAA + Kinetin in *japonica* rice (Bindeshwar, 2008). In recent years, silver ion in the form of nitrate has been extensively studied as it is related to the inhibition ethylene biosynthesis in anther culture. According to Beyer, (1976) silver nitrate is known to interfere with ethylene action and forming complexes which inhibits ethylene responses. Piyachai *et al*. (2011) studied the response of *indica* hybrid rice KDML 105 × SPR 1 for anther culture and found that the LS media supplemented with 10 µM KNO_3 + 2 mg/L 2,4-D + 2 mg/L NAA + 20% coconut water + 1 mg/L activated charcoal had induced high embryogenic callus.

There is a contrasting response of callus induction with growth regulators and chemical supplements among *japonica* varieties. Hence, there is further need to study the influence of factors such as auxin, cytokinin and chemical supplements in the media that favor the formation of high quality callus in a shorter time. With this view, the present investigation was to study the effect of growth regulators and chemical supplements on callus induction in *japonica* rice varieties.

Materials and Methods

Two *japonica* rice varieties i.e. Azucena and Moroberekan were grown in the *Kharif* season with recommended fertilizers in the field condition. Panicles were collected at the early flowering stage, when young panicles were still enclosed within the leaf sheath. Panicle distance of 10-15 cm between flag leaf and subtending leaf were harvested from the plants at 6:00-9:00 am and then washed with tap water. Harvested panicles were soaked in 70 % ethanol for 1 min and rinsed two times with sterilized water (Dalpat *et al*., 2014). Surface sterilized panicles were kept for cold pre-treatment at 4°C for one week. The anthers with mid uninucleate stages were first determined by cytological test using acetocarmine staining technique. On the day of inoculation, panicle was surface sterilized by in 70% (v/v) ethyl alcohol for 2 seconds followed by 0.2% $HgCl_2$ for 10 minutes (Gioi and Tuan, 2004).The treated panicles were rinsed 3-4 times with sterile distilled water. Anthers were isolated from spikelet avoiding any mechanical damage, followed by inoculation onto the callus induction medium which consisted of basal N6 medium containing different combinations of growth regulators like 2, 4-D, NAA and Kinetin with chemical supplements: silver nitrate, casein hydrolysate and L-proline (Table 1). The pH of the media for callus induction was adjusted to 5.8 with 1 N HCl or 1 N NaOH before adding agar with 3% maltose and 0.8% agar. The cultures were sealed with parafilm and placed in the dark at 23±2 °C with relative humidity 60-70%. The treatments were replicated thrice. The observation of percent callus induction frequency was recorded on the 10-20 weeks after inoculation.

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

Data analysis: The callus induction frequency was analyzed by factorial completely randomized design (FCRD) of square root transformation with correction factor 0.5%. ANOVA was performed for callus induction frequency.

Results and Discussion

Anther culture is one of the most intensively studied areas in rice tissue culture. However, most of the advances and achievements that have been made from *japonica* rice, since anthers of *indica* cultivers show low response to androgenesis.Various tissue culture techniques are being applied for varieties development of cereal crops (Dorosieve, 1996). Among these techniques, anther culture, protoplast fusion, leaf culture, root culture and dehusked seed culture are important in rice tissue culture to exploit somaclonal variation for the creation of novel rice varieties (Roly *et al.,* 2013).

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 Moroberkaen were selected because at this stage pollen was mid uninucleate. Similar kind of observations were 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 (Gupta and Borthakur, 1987) and panicle with a distance between subtending leaf and the flag leaf of 7–13 cm (Afza *et al*., 2000); 7–10 cm (Suriyan *et al*., 2009); 5-10 cm (Gueye and Ndoye, 2010);

Table -1: Treatments used for callus induction

Treatments	2, 4-D (mg/L)	NAA (mg/L)	Kinetin (mg/L)	Silver nitrate (mg/L)	Casein hydrol ysate (CH) (mg/L)	L-Proline (mg/L)
T_0 (Control)	0	0	0	0	0	0
T_1	1	1	0	5	0	0
T_2	1	1.5	0	5	0	0
T_3	1	2	0	5	0	0
T_4	1.5	1	0	5	0	0
T_5	1.5	1.5	0	5	0	0
T_6	1.5	2	0	5	0	0
T_7	2	1	0	5	0	0
T_8	2	1.5	0	5	0	0
T_9	2	2	0	5	0	0
T_{10}	0	1	1	0	500	250
T_{11}	0	1	0.5	0	500	250
T_{12}	0	1.5	0.5	0	500	250
T_{13}	0	1.5	1	0	500	250
T_{14}	0	2	0.5	0	500	250
T_{15}	0	2	1	0	500	250

Table-2: ANOVA for callus induction frequency

Source of variance	Degree of freedom	MSS
Varieties	1	6.52**
Treatments	15	9.80**
Varieties × Treatment	15	4.07**
Error	64	0.08
Total	95	
Grand mean	2.91	
SEM	0.29	
CV	10.24	
CD (1 %)	0.71	

** Significant P <0.01

Table-3: Effect of growth regulators with chemical supplements on callus induction in *japonica* rice varieties

Treatments	No. of anthers inoculated		%callus induction	
	Azucena	Moroberekan	Azucena	Moroberekan
T_0 (Control)	28.33	38.66	0.00 (0.70)	0.00 (0.70)
T_1	34.00	25.00	5.88 (2.51)	0.00 (0.70)
T_2	56.66	52.66	0.00 (0.70)	17.09 (4.18)
T_3	69.00	38.66	2.89 (1.82)	7.75 (2.85)
T_4	27.66	36.66	44.44 (6.70)	19.09 (4.42)
T_5	38.66	47.66	10.34 (3.23)	6.29 (2.60)
T_6	40.33	53.33	9.91(3.20)	13.12 (3.68)
T_7	53.00	45.00	5.66 (2.48)	2.22 (1.64)
T_8	35.66	47.00	11.21(3.41)	10.68 (3.29)
T_9	38.33	54.66	0.00 (0.70)	16.46 (4.11)
T_{10}	47.00	38.33	2.12 (1.60)	2.60 (1.75)
T_{11}	38.66	37.00	18.10 (4.31)	40.54 (6.40)
T_{12}	36.00	41.66	5.55 (2.45)	4.80 (2.29)
T_{13}	63.66	43.33	3.14 (1.87)	16.15 (4.07)
T_{14}	33.33	43.33	12.00 (3.53)	13.84 (3.77)
T_{15}	53.66	46.00	9.31 (3.11)	17.39 (4.22)

4 to 8 cm (Abbasi *et al*., 2011) 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 (Sopory and Munshi, 1996; Goeresckae*t al.*, 2005). However, the early-uninucleate to mid-uninucleate stage of microspores were found to be best suited for androgenic response (Datta and Potrykus, 1998; Jahne and Lorz, 1995).

Cold pre-treatment and dark incubation: The induction of microspores to sporophytic pathway instead of gametophytic pathway is strongly influenced by some kind of stress treatment of the anthers before culture. The response to chilling 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 (Zhou and Cheng, 1982). Zhou *et al*., (1983) also noticed that variation in the requirement of cold pre-treatment among the *indica* and *japonica* rice genotypes. 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 8 days. The cultures were incubated in dark for 7-8 weeks for callus induction.

According to Sunderland *et al.* (1984), cold pretreatment promoted senescence of the anther wall, destroying the close association between tapetum and pollen. This might disturb the programmed microsporogenesis and allow embryogenesis to be initiated. Nitsch, (1974) reported that cold shock triggers pollen to undergo asymmetric mitotic division which is a pre-requisite for callus induction. The combination of cold pretreatment and dark incubation had synergistic effect on callus induction frequency (Badigannavar, 1995).

Callus induction: In the present study N6 (Chu *et al*., 1975) basal medium were used with 3% maltose as a carbohydrate source. Usage of N6 medium was the best for callus induction from anthers of rice have been reported by several earlier reports (Afza*et al*., 2000; Islam *et al*., 2004; Gueye and Ndoye 2010). The role of maltose to enhance the anther culture ability of cereals because of its slow degradation which results in stabilization of medium osmolarity, later on promoting microspore division and callus formation in *japonica* rice varieties (Xie *et al*., 1995 and Sengsai *et al*., 2007). The most crucial constituents in the rice anther culture medium were auxins and cytokinins. Auxins have been essential plant growth regulator for the induction of callus from anthers of cereals and the type and level of the auxin present in culture medium regulate the callus formation (Germana, 2011). Furthermore, not only growth regulator, but chemical supplements also influence on callus induction. In the present investigation there was significant differences among the varieties and treatments for callus induction and interaction between varieties and treatments (Table-2) with different chemicals supplements. The frequency of callus induction ranged from 2.12% to 44.44% in Azucena and 2.22% to 40.54% in Moroberekan (Table-3). Among the varieties, highest callus induction frequency (44.44%) was recorded in treatment T_4 containing 1.5 mg/L 2, 4-D + 1 mg/L NAA + 5 mg/L silver nitrate in Azucena. These findings supporting to several earlier reports that the addition of $AgNO_3$ in N6 basal medium, the frequency of callus induction of rice was doubled (Lentni *et al*. 1995). The combination of 2, 4-D + NAA induced callus but it is less capable of plant regeneration due to inhibitory effect of 2, 4-D, but silver nitrate with combination of 2, 4-D + NAA in medium will doubled up the production of callus (Huang *et al*. 1985). Silver nitrate as an ethylene inhibiting agent to delay anther senescence. Faruq *et al*. (2014) also reported highest callus induction in *japonica* genotype E7 (IR 77734-93-2-3-2), E11 (IR 78554-145-1-3-2) and *indica* genotype Garib and E13 (IR 77512-2-1-2-2) in N6 containing 2, 4-D + NAA + silver nitrate.

However, in Moroberekan highest callus induction frequency (40.0%) was recorded on treatment T_{11} containing 1 mg/L NAA + 0.5 mg/L Kinetin + 500 mg/L casein hydrolysate + 250 mg/L L-proline. Several results showed the role of different chemical supplements in anther culture of rice. Lenka and Reddy, (1994) reported that combination of NAA + Kinetin promotes faster growth of the early formed calli. Casein hydrolysate (CH) was found to be beneficial for generation of embryogenic calli in *japonica* rice (Toki, 1997). The use of proline in the medium has been reported to be effective for the initiation and maintenance of embryogenic calli (Achar, 2002). Sengsai *et al*. (2007) also reported callus induction BC_1F_1 anthers of KDML 105 × IRBB5 in N6 media containing NAA + Kinetin + Casein hydrolysate + L-Proline. The beneficial effect of CH might be due to the influence of undefined organic nitrogenous compounds in CH, which generally favour embryogenic callus induction. However, the dose of CH added to the medium may vary according to the rice genotypes (Roy and Mandal, 2005).

In conclusion, the present investigation indicates that response of anther culture was better in Azucena (subsp. *japonica*) compared to the Moroberekan (subsp. *japonica*). Therefore, genotype and types of growth regulator along with chemical supplements need to be considered in order to achieve high callus induction. These findings will be of immense value in the application of *in vitro* androgenesis for rice crop improvement.

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