EFFECT OF COLD PRETREATMENT ON IMPROVING ANther CULTURE RESPONSE OF RICE (Oryza sativa L.)

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ABSTRACT

Present study was designed to evaluate the effect of cold pretreatment temperatures (4, 8 and 12 °C) and durations of treatment (5, 9 and 13 d) on anther culture efficiency among 13 genotypes. Anthers were cultured on N6 medium supplemented with 2, 4-D (2 mg/L) and Kinetin (0.5 mg/L). Anther inoculated media petri dish were incubated in the dark at 25 ± 1 °C for callus induction. When calli were 1-2 mm in diameter, they were transferred to MS regeneration medium supplemented with Kinetin (1 mg/L), NAA (1 mg/L) and BAP (2.5 mg/L) and were incubated at 25 ± 1 °C under 16 h/8 h light/dark condition. The cold treatment at 12 °C for 5 days was found to be the most effective in all experimental genotypes used for the study. Out of 13 genotypes evaluated, IR58025B (25eB) was highly responsive for both callus induction as well as green plant regeneration.

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KEYWORDS
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Cold pretreatment
Duration
Callus induction
Regeneration
1 Introduction

Doubled haploidy through anther culture is one of the most efficient and time saving innovative technology in varietal improvement (Chu et al., 2002). Improvement of an existing parental line by pyramiding or stacking of useful genes is a time consuming process through conventional breeding. However, the employment of doubled haploidy and Marker Assisted Selection (MAS) can enhance the efficiency of pyramiding or stacking process. Doubled haploidy allows rice breeders to stabilize desired traits in a single year, reducing the time required for new cultivar development by up to five years through conventional breeding. MAS involve indirect selection of traits in the regenerated plants by selecting the markers linked to gene of interest. Using MAS the selection efficiency is considerably increased as each plants contain a unique random recombination of the parental genomes and all the alleles of DHLs are fixed, which allows easy selection of targeted traits than in conventional populations.

Since the first report on haploid plant production in japonica rice (Niizeki & Oono, 1968), anther culture has been practised for production of DH plants in rice. However, the spectacular progress in this field has been made only in the recent past. Androgenesis is accomplished in two steps; callus induction from microspores and subsequent regeneration of plantlets from the calli (Bishnoi et al., 2000). As immature pollen is haploid (n), the plant developed is haploid as well. Haploid plants are sterile and cannot produce seed. However, spontaneous doubling of chromosomes during in vitro culture may produce fertile DH plant with two set of chromosomes that are mirror image of each other. The utilization of the technique depends on the efficiency of haploid plant regeneration from microspores and diploidization of these haploids to doubled haploids either spontaneously during tissue culture phase or induced thereafter. However, the exploitation of anther culture technique in breeding and genetics research in indica cultivars is still limited due to their poor androgenic response (Balachandran et al., 1999). Several efforts has been made to improve the efficiency of anther culture through improved culture techniques (Trejo-Tapia et al., 2002), formulation of new media compositions (Cha-Um et al., 2009), utilization of specialized chemicals for callus induction (Datta, 2005) and pretreatments of explants.

In most of the species, genotype is a deciding factor as different rice species, subspecies and varieties behave differently in their response (Ramakrishnan et al., 2005). Miah et al. (1985) reported that anther culture response varied from 42% for a japonica cultivar to 0% for an indica cultivar. The degree of response to anther culture in rice, in terms of frequency of callus induction and plant regeneration has been found to decrease in the following sequence japonica/japonica> japonica> indica/japonica> indica/indica> indica (Guiderdoni et al., 1992; Yan et al., 1996). However, components of tissue culture media and pretreatment can enhance the anther response (Asaduzzaman et al., 2003).

Maheshwari et al. (1980) stated that certain physical and chemical treatments given to flower buds or anthers prior to culture can be highly inductive for the development of microspore into plants. The most commonly used is cold shock treatment. Low temperature shock has been reported to enhance the androgenic response in several species including rice (Silva & Ratnayake, 2009; Gueye & Ndir, 2010; Sen et al., 2011). Some of the positive effects of cold pretreatment includes delay of anther wall senescence, increase of symmetric divisions of pollen grains and metabolic changes necessary for androgenesis. The possible role of low temperature prior to inoculation may presumably attribute to the elimination of weak or non-viable microspores (Lenka & Reddy, 1994). Besides, the delayed anther senescence due to cold pretreatment, it might be providing a suitable microenvironment to ensure supply of growth factors to trigger the embryogenesis from microspores (Cho & Zapata, 1988; Tsay et al., 1988; Datta et al., 1990; Chen et al., 1991). Also cold treatment increases the frequency of endo-reduplication leading to an increase of spontaneously developed DH plants (Amssa et al., 1980). Indica and japonica varieties are known to differ in their requirements for cold treatment (Zhao, 1983).

The temperature and duration of the cold pretreatment varies with the genotypes (Kiviharju & Pehu, 1998). The present study was undertaken with an aim to determine the optimum cold pretreatment temperature and time duration required for maximum anther culture efficiency in rice experimental genotypes used in this study.

2 Materials and methods

2.1 Plant materials

Seeds of thirteen experimental genotypes (seven parents and six F1s) were grown at Barwale Foundation farm, following the standard agronomic practices (Table 1).

2.2 Methods

Panicles were collected from primary tillers of the selected genotypes in the morning hours (9 am to 10 am) when the distance between flag leaf and penultimate leaf was 5-7 cm, as described by Croughan (1998). Selection of the spikelet was done based on cytological observations or position of the anthers. Pollen with late uni-nucleate to early bi-nucleate stage as well as early to mid uni-nucleate stage (Jahne & Lorz, 1995) or anther occupying 1/3 to 1/2 of the spikelet length (Lapitan & Villegas, 1999) is the most suitable stage for anther culture. Boots were wrapped in muslin cloth and sealed in the polythene bags separately. Forty five panicles for each genotype were stored in the cold temperatures of 4°, 8° and 12° C. Five panicles per each genotype at each cold pretreatment temperature were taken out at interval of 5, 9 and 13 d. Following pretreatment, the panicles were surface sterilized with 0.1% mercuric chloride for 5 minutes under aseptic conditions and rinsed thoroughly with sterilized distilled water.
Individual spikelet was cut at the base to expose the anthers. Anthers were plated onto the N6 medium (Chu et al., 1975) supplemented with 2, 4-D 2 mg/L and Kinetin 0.5 mg/L. One petri dish constitutes one replicate and an average of 4 replicates was cultured for each treatment. The cultures were incubated in complete dark at 25 ± 1 °C for 4-5 weeks for callus induction. The cultured plates were examined periodically to observe the progress with respect of callus formation and data on percentage of callus induction was recorded. When Calli was at least 1-2 mm diameter, they were transferred to MS regeneration medium (Murashige & Skoog, 1962) supplemented with 2.5 mg/L BAP, 1 mg/L Kinetin, 1 mg/L NAA and maintained at 25 ± 1 °C under 16 h/8 h light/dark period. The cultures were examined weekly and data on percentage of calli regenerating green and/or albino plants was recorded. The regenerated shoots were transferred to hormone free, half strength MS medium for rooting. Well developed plants with profuse roots were assigned numerical numbers as 1, 2, 3 and so on and finally acclimatized in greenhouse under standard agronomic practices.

Frequency of responded anther was recorded after 60-80 days of anther plating. The callus induction and regeneration frequencies was calculated as : callus induction frequency (%) = number of anthers producing calli/number of anthers plated x 100; green or albino plant frequency (%) = number of green or albino regenerating calli/ number of calli transferred x 100. Analysis of variance (ANOVA) was conducted using CROPSTAT computer software for statistical analysis of the data generated during the present study.

Table 1 Rice genotypes used in the study.

<table>
<thead>
<tr>
<th>Code</th>
<th>Genotype</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>Samba Mahsuri Sub-1</td>
<td>Donor for Sub-1 gene</td>
</tr>
<tr>
<td>PB</td>
<td>IR-64 Sub-1</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>BR-11 Sub-1</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>TDK-1 Sub-1</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>Swarna Sub-1</td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>Dular</td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>IR58025 with eui (25eB)</td>
<td>Recurrent parent with eui gene</td>
</tr>
<tr>
<td>SA</td>
<td>25eB x Samba Mahsuri Sub-1</td>
<td>F1 with eui x Sub-1 genes</td>
</tr>
<tr>
<td>SB</td>
<td>25eB x IR-64 Sub-1</td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>25eB x BR-11 Sub-1</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>25eB x TDK-1 Sub-1</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>25eB x Swarna Sub-1</td>
<td></td>
</tr>
<tr>
<td>WF</td>
<td>25eB x Dular</td>
<td></td>
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</tbody>
</table>

Figure 1 (a) Anther (b) Callus induction (c) Green spots formation (d) Albino shoot regeneration (e) Plantlet development (f) Acclimatization

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3 Results

Callus induction commenced almost three weeks from culture establishments. It was observed that the anthers became necrotic prior to formation of calli (Figure 1a). Calli formed during this experiment were mostly yellowish in colour and compact in texture (Figure 1b), except for Dular (PF) and 25eB x Dular (WF) in which calli was of friable type.

Calli of 1-2mm diameter was transferred to MS media for regeneration. Within two to three weeks time, the transferred calli started differentiating into clumps of green spots first and then into green shoots (Figure 1c). Some of the calli instead of forming green spots induced white shoot like structures which developed subsequently in to albino plants (Figure 1d). The green plantlets were further transferred into rooting media for root development (Figure 1e). Well developed plants with profuse roots were acclimatized in green house (Figure 1f).

A significant genotypic difference in callus induction was observed among the 13 genotypes by ANOVA (p<0.05). In this study, parents showed lower callus induction compared to F1 except for genotype PG. The data on callus induction revealed that level of response is not solely attributing the main effects of genotype, temperature and duration, but also their interaction (Figure 2a, 2b and 2c). From the results, it was evident that callus was induced in all the genotypes, but the frequency varied from 1.29% to 30.44% depending upon the genotype. Of the 13 genotypes evaluated, PG was more responsive with 30.44% callus induction at 12 ºC x 5 d, whereas SB found to be poor with 1.29% at 4ºC x 13 d. Callus induction frequency increased with temperature (4ºC< 8ºC<12ºC).

A maximum callus induction was observed at 12 ºC (5.60%-30.44%), followed by 8 ºC produced moderate level (1.34% to 19.45%) and 4 ºC which induced lowest frequency (1.29%-15.60%) in most of the genotypes. However, another response was inversely proportional to duration (5 d> 9 d>13 d). Duration of pretreatment for 5 d gave best the induction and reduced with increase in duration. With 5 d maximum calli induced ranged from 3.88% to 30.44%, followed by 9 d with moderate level (3.64%-21.89%). Whereas, 13 days treatment produced lowest percentage of callus (1.29%-11.17%). It implies that cold treatment at 4ºC and 13 days of pretreatment was not adequate for callus induction. The best response was observed at 12 ºC for 5 d of pretreatment with a frequency of 10.07% to 30.44%.

Similarly, significant genotypic difference for green plant regeneration was observed among the genotypes by ANOVA (p<0.05). The data on green plant regeneration revealed that genotype, pretreatment temperature, duration and their interaction significantly contributed to variation (Figure 3a, 3b and 3c). The green plant regeneration also followed the same trend of callus induction with increase in temperature (4ºC< 8ºC<12ºC). However, with respect to duration the response varied. Among the 13 genotypes evaluated, eleven showed high frequency of green spots formation with 5 d (2.76% to 29.19%). Plant regeneration of F1 was higher when compared to that of parents except for PG. The lowest response was observed at 4 ºC with a frequency of 0% to 12.13%. However, highest green plant regeneration was observed at 12 ºC x 5 d which varied from 2.76% for PF to 29.19% for PG, followed by 12 ºC x 9 d as the frequencies gradually decreased ranging from 2.58% to 24.99% and least at 12 ºC x 13 d (0% to 9.31%).
It implies that cold treatment at 4°C and 13 d of pretreatment duration was not adequate for green plant regeneration. Occurrence of albino plants (shoot without chlorophyll) seems to be a common phenomenon in anther culture of cereals (Clapham, 1977).

Albino regeneration was observed in WF as compared to other genotypes at all three pretreatment temperatures.

In case of untreated anthers (control), even although callus formation was observed in all experimental genotypes except for SB, but the frequency was very less (1.07% to 5.72%), compared to frequency achieved at 12°C (Figure 5a).

PG showed highest callus induction of 5.72% among all 13 genotypes. However, the green plant regeneration was very less in control (0% to 14.29%), as compared to treated one varied from 0% to 29.19% (Figure 5b). Out of 13 genotypes evaluated, green spots formation was observed only in PG with 14.29%.
4 Discussion

Doubled haploidy plays an important role in rapid production of homozygous or true breeding lines in the immediate generation, shortens time period for cultivar development. Degree of difference in response was found among 13 genotypes. This finding agree with the earlier observations (Ratheika & Silva, 2007; Talebi et al., 2007). In present study, F$_1$ hybrids showed higher response for anther culture than parents except for PG (recurrent parent), which was the most responsive among the genotypes evaluated. This implies that PG might have an anther culture response enhancing gene/s and the higher response of F$_1$ was due to transfer of this gene/s from recurrent parent to F$_1$ hybrid. These findings were consistent with the previous report (Herath & Bandara, 2011), stating that the difference between indica parents for anther culture response also affected its F$_1$ hybrids response. The genotypic difference for anther culture response varied from 6.07% to 15.58% was observed among the 13 genotypes. This indicates that genotype is the deciding factor for anther culture. Similar observations were found from previous studies stating that the genotype is the most critical factor for rice anther culture response (Datta et al., 1990, Quimio & Zapata, 1990; Guideroni et al., 1992; Bagheri & Jelodar, 2008). The effect of genotype and pretreatment temperature, duration of pretreatment and their interaction were significant (at less than
Green plant regeneration followed the same pattern of callus induction, such as response increased with temperature (4°C< 8°C< 12°C) and decreased with duration of the pretreatment (5 d> 9 d> 13 d). The callus induction capabilities of the calli decreased gradually beyond sixth day of culture. In the present study, the least response was observed at 4°C and the response decreased with long duration (13 d) of pretreatment. However, these findings do not support the beneficial effect of 5°C cold pretreatment reported by Croughan & Chu (1991), but agree with the statement of the induction potential decreased when immature inflorescences were pretreated for long time. This observation indicates that, each genotype requires specific pretreatment temperature and incubation period for optimum callus response. Prolonged treatment over optimum was proved to be inhibitory (Lenka & Reddy, 1994). Thus, the temperature 12°C and duration of 5 d, favoring efficient callus induction was evident from the present study.

Since generation of high number of green plants is the prerequisite for use in the breeding programs, green plant regeneration needs more attention than callus induction.

Both PF and WF showed higher callus induction frequencies (15.55% and 22.83% respectively) at 12°C x 5 d, but lower green plant regeneration rates (2.76% and 4.26% respectively). The higher callus induction observed in this study might be due to higher doses of auxin in the induction medium. Liang (1978) reported that hormone requirement is genotype specific. Therefore, optimum level of auxin in the callus induction media required some degree of compromise between callus induction and regeneration frequency. Whereas, the poor quality of callus (friable texture) in the present study might be one of the reason behind poor green plant regeneration. The present results were in agreement with earlier studies stating that the friable callus considered being of poor quality with a low potential for regeneration in wheat (Moris & DeMacon, 1994). In the case of un-treated anthers (control) callus induction was observed in all the genotypes except for SB. But the frequency was very less as compared to the treated one. This indicates that cold treatment is essential for improved callus induction. However, these findings do not support the statement of no callus induction for un-treated anthers (Sen et al., 2011). Green spots formation was observed only in one (PG) among the genotypes tested for un-treated anthers, whereas green spots formation was observed in all genotypes for the case of treated one. This indicates that cold treatment is essential for improved anther culture response and manipulation of pretreatment has the ability to improve the callus induction and subsequent green plant regeneration. Cold temperature treatment was reported to have promootery effect for androgenesis in several plant species. Cold pretreatment not only slows down the degradation process in the anther tissues, but also protects the microspores from toxic effect of decaying anthers (Duncan & Heberle, 1976). Sunderland & Roberts (1979) reported that cold pretreatment assures survival of a greater proportion of the embryogenic pollen grains. In cold treated anthers, the total content of free amino acids is increased, which might be conducive for adaptation of microspores to the metabolic changes and embryogenesis induction (Claparols et al., 1993; Xie et al., 1997).

The results in the present study indicate that suitable pretreatment temperature and duration can improve the responsiveness of both genetically high and low responsive genotypes. Cold treatment at 12°C for 5 d gave the best performance for callus induction and plant regeneration in our study. This finding will accelerate the large scale production of doubled haploid lines through anther culture and speed up the stacking and pyramiding processes.

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References


Herath HMI, Bandara DC (2011) Anther culture performance in selected high yielding indica (of Sri Lanka) and japonica


