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Exploring factors affecting anther culture in rice (*Oryza sativa* L.)

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ABSTRACT

Anther culture is an important *in vitro* culture technique for the production of double haploids. It is largely species and genotype specific. Asian-cultivated rice (*Oryza sativa,* ssp. indica) is recalcitrant to anther culture which limits its practical application in rice breeding. Several researchers tried to optimize the medium recipes and culture techniques for callus induction and plantlet regeneration. Negligible response to callus induction and recovery of a higher frequency of albino plants are the major hindrance for the use of the technique in crop improvement. Shortening the culture period and sexual hybridization of rice subgroups (japonica/indica) may be adopted to improve green plant regeneration from anther-derived callus in indica rice. However, genetic transformation technology using individual genes related to different aspects of anther culture could be a more direct approach. Besides, the role of genotypes, physiological status of donor plant, developmental stage of pollen, pre-treatment, culture media, phytohormones, and culture conditions for successful anther culture have been discussed.

1. INTRODUCTION

Rice (*Oryza sativa* L., 2*n* = 24, Family Poaceae) provides food to over half of the global population [1]. Rice is being highly self-pollinated; development and selection of pure breeding lines with manifested superior phenotype are the ultimate objective for desired genetic improvement. In this context, anthers of F_1 hybrids can serve as excellent breeding material for raising pollen-derived homozygous plants (double haploids [DHs]). Such DH plants are individually genetically unique due to gene shuffling (during micro-gametogenesis) which paves the way for the selection of desirable plants with high yield potential and biotic/abiotic stress tolerance. Besides, anther culture offers a greater chance of recovery of desirable recessive genes compared to conventional breeding. Asian cultivated rice (*O. sativa* ssp. indica) is recalcitrant to anther culture which limits its practical application in rice breeding. However, the benefit of this technique can be successfully harnessed if a reproducible protocol for anther culture is available. Several researchers tried to optimize the media recipes and culture techniques for callus induction and plantlet regeneration. Negligible response to callus induction and recovery of a higher frequency of albino plants are the major hindrance for the use of the technique in crop improvement. In this pursuit, we present the finding of our extensive reviews on factors determining successful anther culture response in rice.

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2. ANTHER CULTURE IN RICE

Anthers, in general, are very sensitive to *in vitro* culture. The accidental discovery of androgenic haploidy in Datura by Guha and Maheswari [2] led several researchers to attempt such novel technique in a wide range of crop plants. Haploid plant production was first reported in rice through anther culture by Niizeki and Oono [3]. Anther culture is a two-step process [Figure 1]. First step is the initial development of callus that leads to the second step, i.e., regeneration of green plants from callus. Limited morphogenetic potential of anther-derived calli and a higher percentage of regenerated albino plants [4] seem to be major constraints for DH breeding. Gueye and Ndir [5] reported the recovery of a total of 93 regenerants, of which 79 were albinos. Therefore, optimization of culture variables is often required for the successful use of anther culture.

3. FACTORS AFFECTING ANTHER CULTURE

The success of anther culture depends on exogenous and endogenous factors such as maturity of the donor plant, genotype, microspore developmental stages, panicle pretreatment, temperature and duration of pretreatment, culture media, and growth conditions.

3.1. Genotype

Response to anther culture varies differently within species, subspecies, or varieties. *Oryza glaberrima* is more potent for callusing and regeneration than *O. sativa* [5]. Japonica types are *in vogue* more responsive to microspore embryogenesis than indica types in rice. Many researchers have reported genotypic specificity within indica subspecies using improved media [4]. Indica cultivars show poor

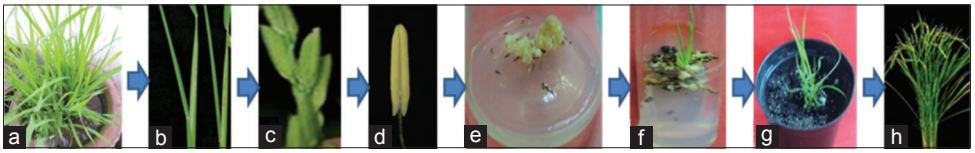

Figure 1: Anther culture-derived plantlet production in rice. (a) Rice plant as source of explants, (b) boots, (c) spikelets, (d) anther, (e) callus induction from anthers, (f) regeneration of plantlet, (g) plant establishment in pot mixture, and (h) field grown plants at panicle stage

callus growth, poor regeneration ability, and low percentage of albino plants [6]. Only 5 of 18 indica cultivars showed pollen callusing and 4 calli differentiate into plants [7], whereas only 1 of 35 indica cultivars exhibited pollen callusing [8]. Tran and Vuong [9] also recorded low response for callus induction (3.53%) and plantlet regeneration (1.12%) frequency in indica rice. However, combining high yielding indica rice with high anther culture responding japonica genotype may improve anther culture response [10]. Thuan *et al*. [11] reported high callus induction frequency from anthers of F_1 plants derived from four crosses of aromatic and improved rice cultivars. Calli from a F_1 hybrid (Bg 90-2: Indica rice/Hu Lo Tao: Japonica rice) showed a higher frequency of green plantlet regeneration than their parents. High callus induction frequencies (30–34%) and low regeneration response in F_1s were obtained by Mishra *et al.* [12], while positive relationship for both was noticed by Javed *et al*. [13] and Shahnewaz *et al.* [14]. Dash *et al.* [15] reported a callus induction frequency of as high as 37.83 % from anther culture of a cross CRMS31B/CRMS24B. However, genetic transformation technology using individual genes related to different aspects of anther culture could be a more direct approach to improve green plant regeneration.

3.2. Physiological Status of Donor Plant

Anthers collected at the beginning of the flowering period respond better, and it declines with the age of plants [16]. Field-grown plants show superiority over to those grown in the glasshouse or pots [17]. Plants with excessive vegetative growth are not desirable, and therefore, second application and/or foliar spray of nitrogen is avoided. Besides, a favorable day (34°C) and night (25°C) temperatures at booting stage seem to be a determining factor for androgenic embryogenesis [18]. Anthers from the primary tillers are *in vogue* more responsive than secondary tillers [19]. Besides, physiological status of anthers from the middle portion of the panicles in boot stage seems to be favorable for callusing and regeneration [20].

3.3. Developmental Stage of Pollen

The developmental stage of pollen can enhance anther culture efficiency. In rice, the most suitable stage is the early to mid-uninucleate pollen stage [21], while the older pollens at tetrad stage and after the first pollen mitosis do not respond to culture due to starch deposition leading to differentiation into male gametophyte [22]. Such above appropriate condition corresponds to the 3–4 cm distance between the collar of the flag leaf and ligule of the penultimate leaf [23]. Therefore, it is important to optimize the anther culture response in rice by assessing the optimum developmental stage of pollen grain in the anther by acetocarmine staining.

3.4. Pre-treatment

Anthers exposed to various kinds of stresses (cold, heat, osmotic stress, sugar starvation, gamma irradiation, and chemical treatment) before *in vitro* culture are reported to induce androgenesis [24] and inhibit callus induction from somatic origin (anther wall and tapetum). However, the type and duration of pre-treatments vary with the species and variety of rice [25].

3.4.1. Cold pre-treatment

A low-temperature stress has been reported to induce and ensure continuance of sporophytic mode of the development of microspores [26] instead of follow-up gamete formation. It triggers embryogenesis from microspores [27] and increases the frequency of spontaneous development of DH plants [28]. Green plant regeneration is reported to be enhanced by 6 days of cold pretreatment to the panicles [29]. Pre-treatment of anthers at 10°C for 11–12 days [30] results in better callusing response but led to albino plantlet regeneration, while pretreatment at 12°C for 5 days gives best regeneration response [31]. Besides, cold pre-treatment eliminates weak or non-viable microspores in culture [32], 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 [33].

3.4.2. Heat shock treatment

Heat temperature pre-treatment synchronizes physiological states of microspores and stimulates embryoid formation by disrupting the cytoskeleton at the initial stage of microspore development [34].

3.4.3. Osmotic stress

Osmotic stress, for example, mannitol can offer as a substitute or replace the pre-cold treatment as it enables physiological isolation microspores [35] leading to embryoid formation. It improves the sugar uptake causing increased glucose level in the anther tissue. Treatment of anthers in 0.4 M mannitol was useful to enhance androgenesis in anther culture of indica cultivar IR 43 from 3% to 33%. However, cold treatment combined with mannitol-induced osmotic stress had no added advantage [36] or even detrimental.

3.4.4. Sugar starvation

Sugar starvation contributes to the promotion of high-frequency embryogenesis and plantlet regeneration from microspores isolated from anthers of indica and japonica rice and also in isolated microspores [37]. Sugar starvation of anthers of indica rice variety IR 43 for 2 days at the beginning of culture caused a 12-fold increase in the androgenic response in this variety [38]. However, cold treatment was superior to sugar starvation. Touraev *et al*. [39] reported better anther culture response by starvation of anthers in sugar-free medium in wheat.

3.4.5. Gamma radiation

Low irradiation pre-treatment is known to promote embryogenesis in anther culture. Chen *et al*. [40] reported significant stimulatory effect of 20 kR gamma ray treatment on the regeneration of green plantlets. Similarly, Mkuya *et al*. [41] observed prolific DH production and green plant regeneration following gamma-irradiation of indica rice line TM7–5.

3.4.6. Chemical treatment

Ethrel and ethephon (ethylene releasers) have pronounced effect in haploid production in various plant species. Plants are sprayed

with 4000 ppm ethrel just before meiosis in pollen mother cells which result in multinucleated (4–6) pollen with fewer starch grains. Parthenogenetic embryoids may be induced due to additional mitosis from such pollen grains in culture [42]. Wang *et al.* [43] reported enhancement in anther culture response with 4000 ppm ethrel (2-chloroethyl phosphonic acid) treatment for 48 h at 10°C. Besides, androgenic response of rice anthers was enhanced if the inflorescences were pre-treated with ethrel. Similarly, Wang *et al*. [44] reported a positive effect of ethephon on mitotic activity of microspores.

3.5. Culture Media

Haploid plants can be produced either through embryo formation [45] or callusing [3] by varying medium composition. N6 [46], MS [47], and SK_1 [48] solid media are *in vogue* used for anther culture and the former is being more potent than two later media [49] and even compared to MO19 [50], Blayde's [51], MSN1, SK8, and R2 (modification of MS medium) for callus induction [50] and green plant regeneration [12]. Frequency of callus formation was better in N6 medium as compared to MS medium (11.9% and 7.95%, respectively) [11]. Lentini *et al*. [8] favored liquid media for callus induction as it provides greater access to nutrients and hormones, and there are chances of more rapid dispersion of toxic substances [6]. Choice of media proves to be genotype specific [4]. Khanna and Raina [52] indicated significant genotype x culture media interaction for regeneration response in three indica rice cultivars. N6 media respond better to anther culture in japonica than indica [8]. Kaushal *et al.* [53] reported the highest callus induction, green plant regeneration, and least albino plant development of 40.64%, 40.93%, and 3.72%, respectively, in He-2 medium [54].

Higher doses of nitrogen, phosphorus, and potassium in the media led to better anther culture response in indica rice [22]. In general, ammonium nitrogen (NH_4^+) is required in low amount for anther culture in cereal. N6 medium being high in NO_3^- and low in (NH_4^+) has proved to be very efficient for japonica rice anther culture [55]. Sucrose and iron are crucial for pollen embryo development [56] and chelated form of iron (Fe-EDTA: Iron-Ethylenediamine tetraacetic acid) is more effective than ferric citrates [57]. Maltose showed better callusing and regeneration response than sucrose. Besides, a higher ratio of green plantlets to albinos in both japonica and indica types was realized due to mannitol as carbon source [13]. Alternatively, ficoll-a synthetic polymer of sucrose could be used to increase the surface density and improve the ratio of green to albino plantlets [58] and to maintain optimum osmotic pressure. Gel rite is another gelling agent found to be the most effective one [59]. Addition of organic additives (yeast extracts at100 mg/l, casein hydrolysate at 500 mg/l, and coconut water 5–10%) to N6 further enhanced androgenic callus induction in indica rice varieties [60]. Besides, the requirement of amino acids, for example, glutamine and alanine is realized in indica rice for callus formation and green plant regeneration [38]. $AgNO_3$ supplementation can induce a callusing response and promote the regeneration of green plants [61].

3.6. Hormonal Requirement

2,4-dichloro-phenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) alone or with kinetin in the culture medium seem to be the major determinants for embryogenic callusing from rice anthers [62]. Neither 2,4-D nor NAA alone in the media can support regeneration, but the use of cytokinins such as kinetin (Kn) and 6 benzyl aminopurine (BAP) with NAA is required for regeneration. A combination of 0.5 ppm indole acetic acid (IAA) and 2.0 ppm facilitates the germination of androgenic embryos. IAA and NAA may induce direct androgenesis, while 2 4-D promotes callus induction and rapid cell proliferation [63]. A lower concentration of BAP enhances microtillering from androgenic plantlets [64], and NAA promotes the formation of roots. Thuan *et al*. [11] reported better callus induction from anthers of F_1 plants derived from four crosses of aromatic and improved rice cultivars cultured in N6 and MS media supplemented with 2,4-D (0.5 mg/L) + NAA (1.0 mg/L) + BAP (0.5 mg/L). Niroula and Bimb [65] reported higher callus induction frequency in N6 medium with 2,4-D (2.5 mg/l) + 0.5 mg/l Kn than N6 + NAA (4 mg/l) + Kn (0.5 mg/l); however, reverse was the case for green plant regeneration. Xa and Lang [66] reported 5.13–9.27% callus induction and 6.17–14% regeneration from four crosses in MS medium with combination 1 mg/L BA + 2 mg/L Kn + 3% sucrose. Besides, MS medium with 10% coconut milk and addition of Kn (0.5 mg/1), BAP (2 mg/1,) and NAA (1 mg/1) at ratio of 1:4:2 gave consistently high frequency of plantlet regeneration from anther derived calli of a wide range of genotypes [67].

Embryo formation (10.8%) was reported to be markedly increased by abscisic acid in the callus induction media or its treatment during the cold pre-treatment period [68]. Ethylene is also produced in plant cell due to the presence of auxin [69], sucrose [70], or calcium [71] in the callus induction medium. Profused callusing may be due to the inhibitory effect of endogenously produced ethylene from excised anthers [72]. Polyamines [73] and $AgNO_3$ [8] also affect anther culture response in rice through inhibition of ethylene synthesis. Putrescine application to the callus medium also increases the frequency of green/albino regeneration frequency both in japonica and indica variety.

3.7. Culture Condition

Anther derived callus was observed in Azucena rice variety (6.66%) while no callusing was found in Budda variety when the cultures were exposed to dark at 23 ± 2°C [74]. Highest callus induction was observed from the anther culture of rice variety Rajalaxmi and Ajay by keeping the culture at 25 ± 1°C for 3–4 weeks [12] and from anthers of six F_1 hybrids within 60 days, and highest regeneration frequency was obtained by keeping at 18 h photoperiod with a light intensity of 27 µmol $m^{-2}s^{-1}$ at 25 ± 1°C [75] which was also observed in japonica variety (Mankeumbyeo, recurrent parent) and a recalcitrant indica variety (Ranta Emas, donor parent) at the same culture condition [76].

Culturing of anthers of PT-1, KDML-105, and HJ rice variety at 25 ± 2°C in darkness for 6 weeks successfully induced embryogenic calli [77]. Highest callus induction frequency was obtained in anther culture of japonica/indica and indica/japonica hybrids [78] at 25 ± 2°C for 8 weeks. Androgenic callus induction as well as green plantlet regeneration was observed by keeping anther culture of five indica rice genotypes (IR 72, Mansarovar, Taraori Basmati, Pusa Basmati, and Karnal local 95) [61] and indica rice genotypes (GR 11 and Gurjari) in the dark at 25 ± 2°C [79]. Callus induction frequency and regeneration efficiency at 30°C/20°C day/night temperature in the dark for 4 weeks were increased 1.4 times and more than double, respectively, than when kept at 25°C [80]. Initial incubation in the dark at 26 ± 2°C for 6 weeks followed by 12 h photoperiod for another 2 weeks resulted highest callus induction and plant regeneration response in six indica (HKR120, HKR 86-3, HKR86-217, PR106, Gobind, and CH 2 double dwarf) and two basmati rice (Basmati 370 and Taraori Basmati) varieties and 14 heterotic indica Basmati F_1 hybrids [23]. Callus induction was also observed by incubating at 27°C, but regeneration ability was lost after 11 days [40]. In contrast, incubation at the above temperature was satisfactory in an interspecific hybrid (*O. sativa* L./*Oryza rufipogon*) for callus induction in the dark and for plantlet regeneration in 16 h photoperiod

(2000 lux) [81]. However, callus induction frequency (0.75–5.15%) was found from anthers of two indica elite lines (MRP5401 and BR29) and one japonica variety (Taipei 309) with exposure in white light for 16 h at 28 ± 20°C until shoots development in about 3–6 weeks [82].

Recovery of a higher frequency of albino plants [4] in regeneration media is a serious problem, and it may be primarily due to breakage of DNA in plastids and nuclei [83]. The frequency of albinism among regenerated plants is controlled by quantitative trait loci on chromosome 9 and 10 [84]. Albinism is reported to be reduced by shortening of culture period [85]. Therefore, fresh calli (3–4 mm diameter) induced in primary culture may be preferred for transfer to regeneration media without allowing subculturing for callus proliferation.

4. CONCLUSION

In rice, several breeding methods are now available to broaden genetic variation and recovery of desirable gene combinations. Each anther of a intervarietal/interspecific hybrid carries thousands of pollen grains with different genotypes, and these can be induced to form stable DH plantlets within short time. Several factors, for example, genotype, physiological status of source plant, pollen stage, media recipes, including hormonal supplementation, pre-treatment of anthers, and culture conditions determine the success of anther culture. A highly reproducible anther culture technique with optimized culture variables can accelerate the breeding process for the isolation of plant types with high yield, disease resistance, and improved quality traits. Besides, the technique once optimized; it can be used to detect and fix (by colchicine) desirable recessive traits induced through mutation or spontaneous gametoclonal variation.

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