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# *In vitro* studies to produce double haploid in *Indica* hybrid rice

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**Abstract:** The aim of this investigation was to improve *in vitro* the technique of production of double haploid in *Indica* hybrid rice by combining anther culture, hormone shock and doubling chromosome. It was discussed how to avoid somaclonal variation during culturing and to reduce the time of this process. The anthers of KDML 105 × SPR 1 (*Indica* × *Indica*) were cultured in Linsmaier and Skoog (LS) medium, which contained nutrients, growth regulators [(2,4,-dichlorophenoxy acetic acid (2,4-D) and naphthalene acetic acid (NAA)] and organic compounds, and then subcultured by inducing embryo-like structure (ELS) LS media. During 4 weeks used LS media supplemented with 10 μM $KNO_3$ + 2 mg/L 2,4-D + 2 mg/L NAA + 20% coconut water + 1 mg/L of activated charcoal had induced high embryogenic frequent callus with length of 4–5 mm. The supplementation of 0.2 g/L colchicine and 100 μM 2,4-D was the most efficient in LS media. Over 70% of viable double haploid ELS were produced in 8 weeks and subcultured only twice compared with conventional anther which takes more than 12 weeks. This new technique can therefore be applied to rice in order in shorten time to produce higher number of double haploid plantlets.

**Key words:** hormone shock *in vitro*; caulogenesis; anther culture; double haploid.

**Abbreviations:** 2,4-D, 2,4-dichlorophenoxy acetic acid; ELS, embryo-like structure; KDML, Thai rice variety of donor plants; LS, Linsmaier and Skoog; NAA, naphthalene acetic acid.

## Introduction

It has been indicated that improving of the cooking and eating quality of grain has always been an important consideration in most rice breeding programs (Lapitan et al. 2009). Also this investigation was planned for the same aim. Hu & Zeng (1984) suggested that the doubled haploids technique with homozygous diploid could be induced in fewer generations by doubling chromosomes through inhibiting their anaphase movement. The application of rice anther culture may be one of the alternatives in rice breeding program. Production of doubled haploids through anther culture is a rapid approach to homozygosity. It shortens the period of time required for the development of new rice cultivars; the conventional methods require at least 6–7 generations.

Developments of the *in vitro* techniques offer possibilities of introducing into plants variability that could be utilized for crop improvement. Haploids with their unique genomic constitution have potential for accelerating the production of homozygous new varieties. The production of rice haploids and subsequent homozygous diploid plants by *in vitro* anther culture has dramatically advanced in the last 15 years. The application of this technique for improvement of rice varieties has still been hindered by the difficulty of inducing morphogenesis, either directly from the cultured anthers or indirectly from callus derived from microspores. Also the callus produced frequently loses. The plant regeneration ability with time in culture makes studies on selection of callus mutants difficult when longer periods of the *in vitro* culture are required. Another difficulty arises when during culturing *in vitro* all rice varieties do not respond equally in producing callus and in regenerating plants. This could be due to genetic or environmental characteristics of different varieties (Ozawa et al. 2003).

There are several factors, such as genotype, physiological state of the donor plant, physiological stage of the microspores, culture medium, growth regulators, sucrose and shock pre-treatments that affect the re-

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sponse of anther culture for producing androgenic callus and plant regeneration. Among the external factors the exogenously applied hormones, mainly auxins, such as 2,4,-dichlorophenoxy acetic acid (2,4-D), play a critical role in the reactivation of the cell cycle and in the initiation of the embryo formation. Application of high concentrations of 2,4-D in the culture medium itself is a stress signal since embryogenic induction requires the use of physiological auxin concentrations that inhibit the callus growth (Dudits et al. 1991).

It was shown that anther culture of F1 hybrids leads to fixation of gene combinations. Otherwise, it would be impossible to isolate from a segregating population for developing homozygous lines as well as heterotic F1 hybrids (Hu & Zeng 1984).

There are some reports that use of anther culture technique in rice leads to increase the number of varieties and hybrids in rice where androgenesis is possible. It also increases the efficiency through technique manipulation (Chen & Lin 1976; Tsai & Lin 1977; Chaleff 1978; Miah et al. 1985). Earlier, it was reported that anthers from rice *Japonica* type only were capable of regenerating sufficient number of doubled haploids in anther culture, for which selection can be predicted (Kim et al. 1991). Presently, it is possible to induce high regeneration efficiency also in rice *Indica* type (Narasimman & Rangasamy 1993). However, anther culture technique has some limitations: (a) lacking development of techniques for quick production of large number of doubled haploids; (b) high cost of obtaining haploids and doubled haploids; (c) doubling of chromosome number of the haploids is time-consuming and may not always result in the production of a homozygote; and (d) the risk of somaclonal variation and high frequency of mutation during the tissue culture.

Segui-Simarro & Nuez (2008) suggested that production of doubled haploid plants through androgenesis induction is a promising and convenient alternative to conventional techniques for the generation of pure lines for breeding programs. Also Silva (2010) has pointed out that during the past two decades numerous papers have been published on anther culture of rice. These studies clearly indicate that anther culture is a technique that can be adopted for breeding of rice.

Anyhow, the answers for all limit actions are still for further investigations. Therefore, in this *in vitro* study we tried to solve some of these limitations by combining the anther culture technique and hormone shock for doubled haploids production. The terminology of hormone shock was used because calli were cultured in various high concentrations of 2,4-D (50, 100 150 and 200 μM) Linsmaier and Skoog (LS) media for 6 h and then subcultured to LS media without supplemented 2,4-D. Thus, the objectives of this study were as follows: (a) to investigate *Indica* rice responses to anther culture process for callus and plantlets production; and (b) to improve *Indica* rice doubled haploid production technique for higher survival rate as well as reducing both the time and the cost of production.

## Materials and methods

*Plant material*

Donor plants (KDML 105 × SPR 1 seeds) were grown in field to produce F1 anther. Plants are usually ready for anther culture from 60 to 90 days after planting. The KDML 105 × SPR 1 (*Indica* × *Indica*) anthers were used for this study. Stems containing panicles with pollen at this stage were identified in rice by the relative positions of the flag leaf and penultimate leaf collars. Anthers from the distance 4–9 cm between the base of the flag and auricle of the last leaf which were in the middle to late uninucleate stage of development before pollen mitosis were collected. The anthers were stored in the dark for 14 days at 4℃ in a cold room before being cultured, wrapped in aluminium foil and placed in plastic boxes. F1 hybrid panicles from KDML 105 × SPR 1 were brought in the laminar air flow and surface sterilized for 8–10 min by soaking them in 10% (v/v) sodium hypochloride solution and then by rinsing 3–4 times with sterile distilled water.

*Caulogenesis inducement*

10 formulas of LS medium were used: (1) 2 mg/L of 2,4-D (control); (2) 2 mg/L of 2,4-D + 2 mg/L of naphthalene acetic acid (NAA); (3) 2 mg/L of 2,4-D + 2 mg/L of kinetin; (4) 2 mg/L of 2,4-D + 2 mg/L of kinetin + 2 mg/L of NAA; (5) 10 μM $KNO_3$ + 2 mg/L of 2,4-D + 2 mg/L of NAA; (6) 10 μM $NH_4NO_3$ + 2 mg/L of 2,4-D + 2 mg/L of NAA; (7) 2 mg/L of 2,4-D + 2 mg/L of NAA + 15% coconut water; (8) 2 mg/L of 2,4-D + 2 mg/L of NAA + 20% coconut water; (9) 10 μM $KNO_3$ + 2 mg/L of 2,4-D + 2 mg/L of NAA + 20% coconut water; and (10) 10 μM $KNO_3$ + 2 mg/L of 2,4-D + 2 mg/L of NAA + 20% coconut water + 1 mg/L of activated charcoal). Macronutrient concentrations ($KNO_3$, $NH_4NO_3$), growth regulators (2,4-D, NAA) and other organic compounds (coconut water and activated charcoal) were modified and applied in order for embryogenic callus to be transformed to the embryo-like structure (ELS). The pH of each composition was adjusted to 5.8 before autoclaving at 115℃ for 15 min. The anthers were cultured in a 5×8 cm sterile glass bottle, 10 anthers were inoculated and 3 replicas (100 anthers per replica) were cultured in each treatment which contained 25 mL callus-inducing medium. The cultures were kept at 25 ± 2℃ under continual illumination from white fluorescent lamps (3,000 Lux under 16 h photoperiods). Callus was formed during 4–6 weeks. After producing doubled haploid plantlet from embryogenesis in next experiment, the same procedure was used again with doubled haploid anther (H1 anther) to compare the responses to anther culture process for callus and plantlets.

*Hormone shock and embryogenesis inducement*

LS media supplemented with various concentrations of colchicines and 2,4-D (Tables 1 and 2) were used for embryoids inducement. The 2 mg calli was transferred to LS liquid media in which 2 mg/L NAA, 1 mg/L kinetin, 1 g/L sodium salt of 2-(N-morpholino)-ethanesulfonic acid, 1 g/L casein hydrolyzate, 30 g/L sucrose, 30 g/L sorbitol and 0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/L colchicines were added. The comparison was done with hormone shock by culturing in various high concentrations of 2,4-D (50, 100 150 and 200 μM) LS media. The treatments took 6 hours and then those calli were subcultured to LS media without supplemented 2,4-D in a 250 mL Erlenmayer flask and placed on a rotary shaker at 100 rpm at 25 ± ℃ under continual illumination from white fluorescent lamps (3,000 Lux under 16 h photoperiods). Subculture of cell suspension into induced

Table 1. Influence of various LS media formulas on the anther culture response of F1 hybrid rice.[a]

| Formula | Organogenesis | | | | | | Caulogenesis | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Shoot | | Root | | Shoot+Root | | Friable | | Compact | |
| | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. |
| 1 | neg | | 7.33 | 2.08 | neg | | 22.33 | 2.52 | neg | |
| 2 | neg | | 45.00 | 7.00 | neg | | neg | | 22.67 | 3.79 |
| 3 | 18.00 | 3.61 | 24.00 | 6.56 | neg | | neg | | 25.67 | 4.16 |
| 4 | 50.67 | 2.52 | 16.67 | 3.51 | neg | | 17.00 | 3.00 | neg | |
| 5 | neg | | neg | | 45.33 | 6.66 | 43.33 | 9.07 | neg | |
| 6 | neg | | neg | | 24.33 | 4.04 | neg | | 27.00 | 4.00 |
| 7 | neg | | neg | | 21.67 | 4.73 | 54.00 | 8.54 | 13.33 | 3.51 |
| 8 | neg | | neg | | 17.00 | 6.00 | 75.67 | 6.66 | neg | |
| 9 | neg | | neg | | neg | | 53.33 | 5.03 | 42.67 | 6.51* |
| 10 | neg | | neg | | neg | | 91.33 | 2.52* | neg | |

[a]The asterisk signifies the significantly highest value (p-value <0.01); mean difference was tested by Kruskal Wallis test. The "neg" means that the formation of organogenesis or caulogenesis was not found.

Table 2. Influence of various LS media formulas on the anther culture response of H1 hybrid rice.[a]

| Formula | Organogenesis | | | | | | Caulogenesis | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Shoot | | Root | | Shoot+Root | | Friable | | Compact | |
| | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. |
| 1 | neg | | 43.33 | 4.73 | neg | | neg | | 25.00 | 1.73 |
| 2 | neg | | 52.33 | 4.16 | neg | | 20.33 | 3.21 | 18.33 | 3.21 |
| 3 | 42.67 | 8.50 | 18.00 | 2.65 | neg | | neg | | 21.33 | 4.93 |
| 4 | 18.33 | 1.53 | 40.00 | 7.55 | neg | | 19.00 | 3.00 | neg | |
| 5 | neg | | 16.00 | 4.58 | 42.33 | 11.67 | 19.00 | 4.00 | neg | |
| 6 | neg | | 17.00 | 3.00 | 23.33 | 6.11 | 24.67 | 6.03 | neg | |
| 7 | neg | | 18.00 | 2.65 | 22.00 | 5.00 | 47.67 | 3.06 | neg | |
| 8 | neg | | neg | | neg | | 86.67 | 4.51* | neg | |
| 9 | neg | | neg | | neg | | 60.67 | 6.51 | 20.00 | 3.61 |
| 10 | neg | | neg | | neg | | 50.33 | 6.66 | 40.00 | 2.00* |

[a] The asterisk signifies the significantly highest value (p-value <0.001); mean difference was tested by Kruskal Wallis test. The "neg" means that the formation of organogenesis or caulogenesis was not found.

ELS media formula was carried out for every 3 to 6 weeks in order to induce ELSs. Analyzed metaphase chromosome of ELSs after treating with colchicines and 2,4-D by aceto-orcein squash method was done according to Giri & Giri (2007). Anthers were fixed from 4 to 24 h in ethanol: acetic acid (3:1). After removing the embryoid from the fixative, it was hydrolyzed with 1 N HCl for 4 min at 60 ℃. Then HCl was removed and replaced with aceto-orcein at 90 ℃ for 1–2 min. ELSs were squashed and observed ploidy by microscope. This experiment was done in 3 replicates with 100 cells per treatment.

*Statistical analysis*

The caulogenesis and organogenesis percentages were shown according to LS media formulas. Kruskal Wallis test was used to determine caulogenesis and organogenesis frequency differentiation among various LS media formulas.

## Results and discussion

*Caulogenesis inducement*

The embryogenic calli induction was the most efficient in LS media supplemented with 10 μM $KNO_3$ + 2 mg/L of 2,4-D + 2 mg/L of NAA + 20% coconut water + 1 mg/L of activated charcoal. It showed a high-quality calli formation with high regeneration capacity (Fig. 1a,b) after 4 weeks of the cultured period both in F1 and H1 anther. This result supports findings of Visarada et al. (2002), who showed that the calli regeneration response was also determined by the induction medium. On the other hand, LS media supplemented with 2 mg/L of 2,4-D + 2 mg/L of kinetin + 2 mg/L of NAA promotes organogenesis in H1 anther culture (Fig. 1c). The LS media supplemented with 2 mg/L of 2,4-D + 2 mg/L of kinetin + 2 mg/L of NAA promoted organogenesis in F1 anther culture (Fig. 1d). Embryogenic callus formation and plant regeneration from anther are shown in Figure 1e,f. Moreover, H1 anthers response on modified LS media was better than that of F1 anthers since their forming calli resulted in a shorter period. The optimal concentration of media component for the highest embryogenic calli frequency was significantly different among the anther response tested (Tables 1 and 2).

Khatum & Nenita (2005) reported that variation between callus induction media and genotype was non-significant. Similarly, the callus forming abilities from rice anther culture and time required for callus induction depend on genotype (Reddy et al. 1985; Abe 1992). Kim et al. (1991) reported that the best type response

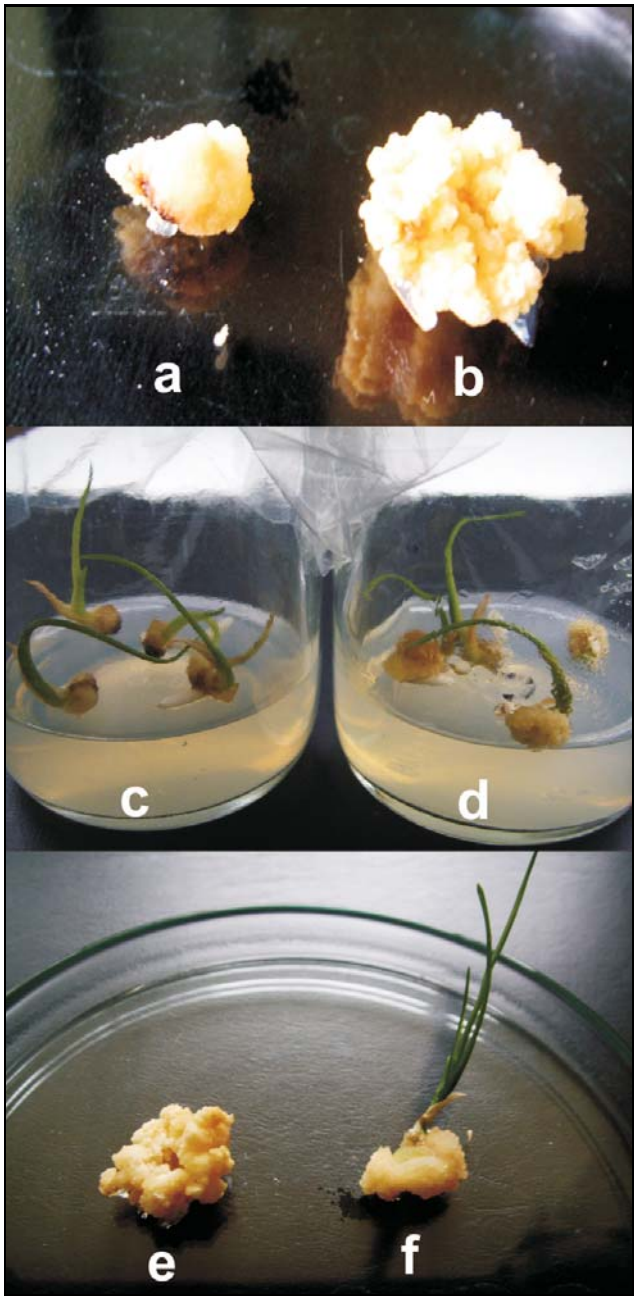


Fig. 1. The difference of callus induction from anther on LS media formula No. 10 after 4 weeks of culture in F1 hybrid anther (a) and H1 anther (b). The difference of organogenesis formation in anther culture on LS media formula No. 4 promoted organogenesis in F1 anther culture (c) and in H1 anther culture (d) after 4 weeks of culture. Embryogenic callus formation (e) and plant regeneration (f) from anther culture after 4 weeks of culture.

was from *Japonica* × *Japonica* hybrids followed by *Indica* × *Japonica* and then by *Indica* × *Indica* crosses. Several other researchers have also noticed a decline in androgenesis in the following order: *Japonica* > *Japonica* × *Indica* > *Indica* (Chen & Lin 1976; Tsai & Lin 1977; Chaleff 1978; Miah et al. 1985). Both callus induction and green plant regeneration have varied considerably depending on the specific cultivars used to construct the hybrids (Narasimman & Rangasamy 1993).

Optimization of the condition for an efficient induction of embryogenic calli and regeneration of plants from anther of *Indica* rice varieties has been improved. The characteristic and appearance time of the induced embryogenic calli depend on the type of basal medium. Production of embryogenic calli with high regeneration capacity was a prerequisite for highly efficient transformation of rice.

In this study the high-quality calli were green or light green colour, recalcitrant, friable and it took short inducement period (Table 3). The media had several differences in composition. One important factor is the ratio of $NO_3^-$ : $NH_4^+$ which greatly affects somatic embryogenesis in monocots (Visarada et al. 2002). Decreasing of $NO_3^-$ (formulas 5, 9 and 10) could induce friable callus in F1 and H1 anther, but decreasing of $NH_4^+$ (formula 6) did not show an effect on caulogenesis. Growth regulator concentrations in culture medium were critical to control the growth and morphogenesis. Generally, high concentration of auxins and low concentration of cytokinins in the medium promoted abundant cell proliferation with the formation of callus. Root regeneration was better on hormone-free medium or on that containing 2,4-D at low concentration (formulas 1 and 2) than on medium supplemented with NAA and kinetin (formulas 3 and 4) which induced both shoot and root regenerations (Tables 1 and 2). In most cases, 2,4-D as a strong synthetic auxin was sufficient to initiate and to sustain embryogenic callus grown in rice and has been used as the only growth regulator in callus induction media (Khanna et al. 1998; Lee et al. 2002; Ozawa et al. 2003; Lin & Zhang 2005). There were also a few reports that the use of 2,4-D alone only produced a non-embryogenic one (Fan et al. 2002; Wu et al. 2002; Wang et al. 2004). Al-Khayri et al. (1992) also reported that the addition of coconut water (formulas 8, 9 and 10) improved caulogenesis and shoot regeneration of *Spinacia oleracea* (spinach). The fact that auxin and cytokinin are essential for callus induction was fully appreciated after the discovery of the presence of cytokinin in coconut water. This is relevant and corresponds with the obtained data (Table 1). Addition of activated charcoal (formula 10) could promote callus forming and growth because of pH balance, adsorption of the inhibitors and growth preventers (Anagostakis 1974). Similar results were found by Khanna & Raina (1998). Somatic embryogenesis was a successive developmental process that involves multiple phases (Arnlod et al. 2002).

*Embryogenesis inducement and doubling chromosome*

Combination *in vitro* techniques between hormone shock for induced embryogenic development and doubling chromosome to produce double haploid were the most efficient in LS media supplemented with 0.2 g/L colchicine and 100 μM 2,4-D (Fig. 3a). It could induce high rate of viable double haploid embryoid over 70% in 6 weeks (Table 4) and subcultured only twice (Fig. 2a), in comparison with the conventional anther cultured method, which takes more than 12 weeks and subcultured more than 4 times to produced double haploid

Table 3. Influence of various LS media formulas on the caulogenesis of F1 and H1 anther hybrid rice.[a]

| Formula | Anther type | Caulogenesis | | | | |
|---|---|---|---|---|---|---|
| | | Callus type | | Callus forming period (days) | Callus size after 4 weeks (mm) | Callus colour |
| | | Friable | Compact | | | |
| 1 | F1 | + | | 29 | 2 | Light yellow |
| | H1 | | + | 20 | 2.5 | Dark yellow |
| 2 | F1 | | + | 32 | 1 | Dark yellow |
| | H1 | + | + | 21 | 2 | Dark yellow |
| 3 | F1 | | + | 35 | 1.5 | Gray |
| | H1 | | + | 18 | 2 | Light yellow |
| 4 | F1 | + | | 31 | 1.5 | Gray |
| | H1 | + | | 24 | 2 | Dark yellow |
| 5 | F1 | ++ | | 27 | 3 | Light green |
| | H1 | + | | 24 | 4 | Light green |
| 6 | F1 | | + | 38 | 3 | Light yellow |
| | H1 | + | | 19 | 4 | Light green |
| 7 | F1 | ++ | + | 25 | 4 | Light green |
| | H1 | ++ | | 15 | 4.5 | Light green |
| 8 | F1 | +++ | | 24 | 3 | Light green |
| | H1 | +++ | | 17 | 4.5 | Green |
| 9 | F1 | ++ | ++ | 22 | 3 | Light green |
| | H1 | ++ | + | 19 | 5 | Green |
| 10 | F1 | +++ | | 16 | 4 | Light green |
| | H1 | ++ | ++ | 15 | 5 | Green |

[a] Abbreviations: +, low; ++, optimal; +++, excellent; F1, anther from F1 hybrid; H1, anther from F1 anther culture.

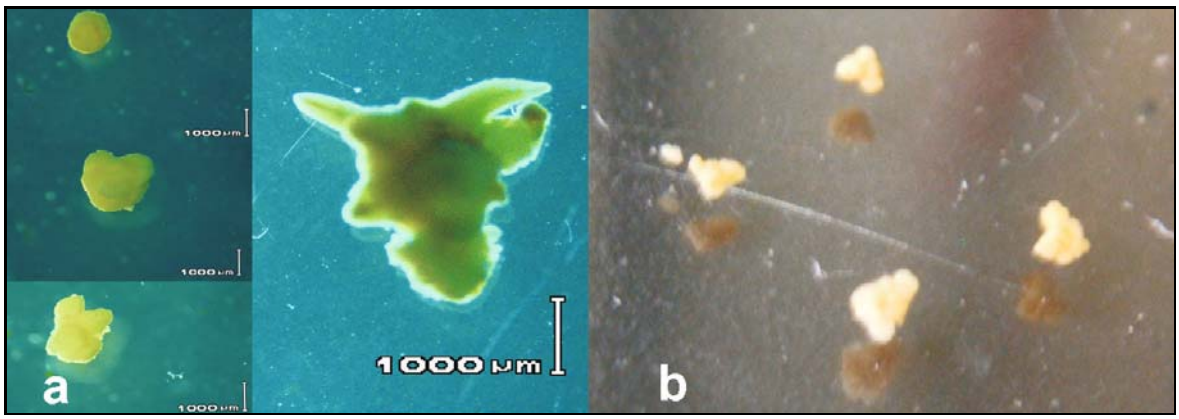


Fig. 2. Differentiation of embryoids after culture calli in LS media supplemented with 0.2 g/L colchicine and 100 µM 2,4-D (a) in comparison with conventional anther culture method (b) after 8 weeks.

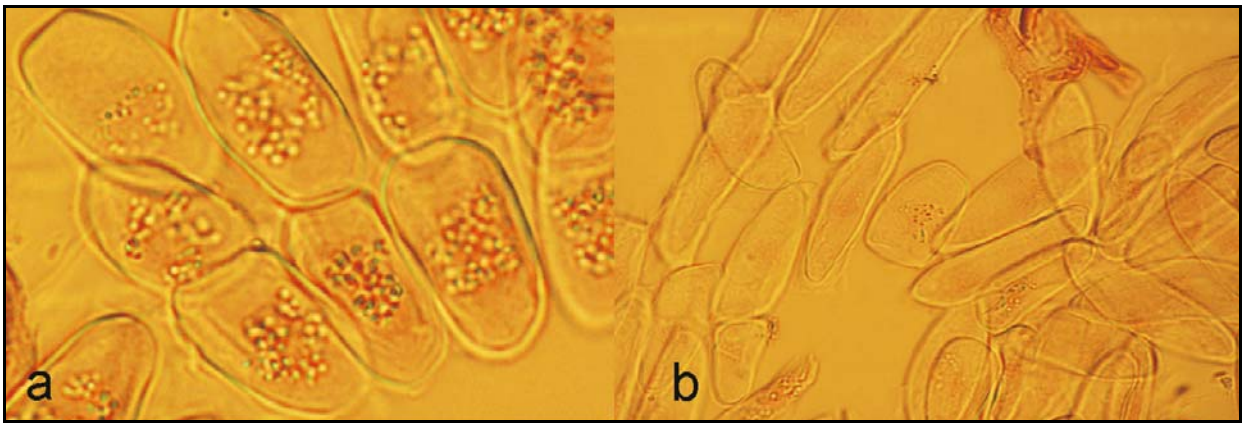


Fig. 3. Analyzed metaphase chromosome of ELSs after treatment with 0.2 g/L colchicine and 100 µM 2,4-D (a) and treatment with over 0.3% colchicines (denatured chromosomes) (b).

Table 4. Influence of LS liquid media supplemented with different concentration of colchicine and 2,4-D on the embryogenesis of F1 hybrid rice.[a]

| Colchicine (g/L) | 2,4-D (μM) | Percentage of embryogenesis after 6 weeks | | |
|---|---|---|---|---|
| | | Globular | Heart | Torpedo |
| 0 | 25 | 85 | 15 | – |
| | 50 | 18 | 49 | 33 |
| | 100 | 22 | 35 | 43 |
| | 150 | 15 | 60 | 25 |
| | 200 | 37 | 54 | 9 |
| 0.1 | 25 | 71 | 21 | 8 |
| | 50 | 11 | 62 | 27 |
| | 100 | 4 | 24 | 72 |
| | 150 | 27 | 37 | 36 |
| | 200 | 28 | 49 | 23 |
| 0.2 | 25 | 78 | 12 | 10 |
| | 50 | 17 | 50 | 33 |
| | 100 | 16 | 13 | 71 |
| | 150 | 35 | 21 | 44 |
| | 200 | 31 | 43 | 26 |
| 0.3 | 25 | 73 | 16 | 11 |
| | 50 | 25 | 24 | 51 |
| | 100 | 8 | 17 | 75 |
| | 150 | 16 | 19 | 65 |
| | 200 | 20 | 37 | 43 |

[a] This experiment observed from 3 replicates with 100 cells per treatment.

Table 5. Effect of different colchicine and 2,4-D concentrations on chromosome doubling in F1 hybrid rice anther culture.[a]

| Colchicine (g/L) | 2,4-D (μM) | Percentage of cells showing chromosomal complement | | | |
|---|---|---|---|---|---|
| | | $n$ | $2n$ | $3n$ | $4n$ |
| 0 | 25 | 100 | – | – | – |
| | 50 | 97 | 3 | – | – |
| | 100 | 98 | 2 | – | – |
| | 150 | 100 | – | – | – |
| | 200 | 97 | 3 | – | – |
| 0.1 | 25 | 42 | 58 | – | – |
| | 50 | 36 | 64 | – | – |
| | 100 | 37 | 63 | – | – |
| | 150 | 44 | 56 | – | – |
| | 200 | 31 | 69 | – | – |
| 0.2 | 25 | 19 | 81 | – | – |
| | 50 | 14 | 81 | 5 | – |
| | 100 | 11 | 85 | 4 | – |
| | 150 | 5 | 87 | 8 | – |
| | 200 | 19 | 73 | 8 | – |
| 0.3 | 25 | 3 | 82 | 11 | 4 |
| | 50 | 7 | 71 | 15 | 7 |
| | 100 | 11 | 66 | 18 | 5 |
| | 150 | 6 | 69 | 24 | 1 |
| | 200 | 18 | 74 | 6 | 2 |

[a] This experiment observed from 3 replicates with 100 cells per treatment.

embryoid (Fig. 2b). Applied 2,4-D could be identified as one of the key inducers of embryogenic development in somatic plant cells cultured *in vitro*. Only a fraction of the cells appears to be capable of an embryogenic response. Differences in auxin sensitivity of the cells could be suggested as a limiting factor in the complex interaction between cells and synthetic hormones. Among the external factors, the exogenously applied hormones, mainly auxins such as 2,4-D, play a critical role in the reactivation of the cell cycle and the initiation of the embryo formation. Application of high concentrations of 2,4-D in the culture medium itself is a stress signal, since embryogenic induction requires the use of a physiological auxin concentration that inhibited the callus growth (Smith 1990; Bidhan & Asit 2004).

Indeed, the detailed comparison between embryogenic and non-embryogenic clones from the same genotype of alfalfa *(Medicago varia* cv. Rambler) has revealed considerably increased sensitivity to 2,4-D in protoplast-derived cells or root explants of the embryogenic genotype (Bogre et al. 1990). The inductive effect of a short auxin shock can clearly be demonstrated with the help of microcallus suspentions from alfalfa (*Medicago sativa*). Treatment of dedifferentiated cells grown in the presence of weak auxin NAA with 100 μM 2,4-D for a few minutes up to a few hours is sufficient to induce embryo formation of embryogenic somatic cells. In addition, the use of this culture allows the exact timing of the inductive phase. In contrast, the proembryogenic nature of carrot suspension cultures makes it difficult to determine the time of commitment of somatic cell towards embryogenesis. Differences between carrot and alfalfa embryogenic culture systems are summarized by Dudits et al. (1991). Embryogenesis occurs in tissues or colonies grown in the presence of 2,4-D at concentrations that already inhibit the growth of callus tissues. The minimum concentration or the duration of 2,4-D treatment required for inductive effect differed in various genotypes and species. Induction of cell division as a 2,4-D response could result in unorganized callus growth or well-coordinated pattern-forming polarized growth of embryo development. Some of the factors are extremely critical for the success of anther culture. The factors, such as the genotype of the plant as a source of anthers, developmental stage of the pollen, and composition of the nutrient media and pre-treatment of the anthers prior to *in vitro* culture, are important.

Subsequently, promotional effect of colchicine on androgenesis has also been observed in *Triticum aestivum* (Szakacs & Barnabas 1995), *Oryza sativa* (Alemanno & Guiderdoni 1994) and *Zea mays* (Barnabas et al. 1991). Colchicine probably disrupts the microtubular cytoskeleton, which is responsible for positioning the nucleus on one side to maintain asymmetric division. Consequently, the nucleus moves to a central position followed by equal division of the microspore (Zaki & Dickinson 1990). Similar observation for *Triticum* was reported by Szakacs & Barnabas (1995). Both, the concentration and the duration of colchicine treatments are important for promoting androgenesis and doubling chromosome. According to Zaki & Dickinson (1991), treatment with 25 mg/L colchicine for 12 h was optimal for two cultivars of *Brassica napus*. It caused 3–4 fold increase in androgenic response. For the same species, Iqbal et al. (1994) found that 100 mg/L colchicine treatment for 24 h gives the best results. Although colchicine

significantly promotes androgenesis, very few of the embryos attain full development (Zaki & Dickinson 1995). For colchicine treatment in high concentration (more than 0.3%), somaclonal variation in high rate (Table 5) and denaturation of chromosome (Fig. 3b) would occur, which caused the death of cells. Redha et al. (1998) reported that the use of colchicines in concentration over 0.2% caused the reduction of embryogenesis and lead to chimera of polyploids cells in wheat.

Doubling haploid techniques could also be used together with other biotechnological tools. The application of mutagenic agents to single haploid cells offers the possibility of screening recessive mutants in the first generation, avoiding chimerism and rapid fixing the selected genotype (Maluszynski et al. 1996). In barley, a protocol has been reported for efficient production of mutants from anthers and isolated microspores cultured *in vitro* (Castillo et al. 2001). When a selective agent is available, the probability of identifying the beneficial mutants from a large microspore population increases.

In conclusion, our presented *in vitro* technique applied for doubled haploid production by combining an anther culture, doubling chromosome and hormone shock is very effective since it improves viability rate, reduces ploidy chimera, time and cost production.

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## References

Abe K. 1992. Genealogical study on callus formation ability in anther culture of rice variety, Koshohikara. Jap. J. Breed. **42:** 403–413.

Alemanno L. & Guiderdoni E. 1994. Increased doubled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. Plant Cell Rep. **13:** 432–436.

Al-Khayri J.M., Huang F.H., Morelock T.E. & Busharar T.A. 1992. Spinach tissue culture improved with coconut water. Hort. Sci. **27:** 357–358.

Anagnostakis S.L. 1974. Haploid plants from anthers of tobacco enhancement with charcoal. Planta **115:** 281–283.

Arnlod S.V., Sabala I., Bozhkov P., Dyachok J. & Filonova L. 2002. Developmental pathways of somatic embryogenesis. Plant Cell Tiss. Org. Cult. **69:** 233–249.

Barnabas B., Pfahler P.L. & Kovacs G. 1991 Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum aestivum* L.). Theor. Appl. Genet. **81:** 675–678.

Bidhan R. & Asit B.M. 2005. Anther culture response in *indica* rice and variations in major agronomic characters among the androclones of a scented cultivar, Karnal local. Afr. J. Biotechnol. **4:** 235–240.

Bogre L., Stefanov I., Abraham M., Somogyi I. & Dudits D. 1990. Differences in responses to 2,4-D-dichlorophenoxy acetic acid (2,4-D) treatment between embryogenic and non-embryogenic lines of alfalfa, pp. 427–436. In: Progress in Plant Cellular and Molecular Biology, Nijkamp H.J.J., Van der Plas L.H.W. & Van Aartrijk J. (eds), Kluwer Academic Publishers, Dordrecht.

Castillo A.M., Cistue L., Valles M.P., Sanz J.M., Romagosa I. & Molina-Cano J.L. 2001. Efficient production of androgenic doubled-haploid mutants in barley by the application of sodium azide to anther and microspore cultures. Plant Cell Rep. **20:** 105–111.

Chaleff R.S. 1978. Anther culture as a rice breeding technique. Intl. Rice Res. Newsl. **3:** 2–3.

Chen C.C. & Lin M.H. 1976. Induction of rice plantlets from anther culture. Bot. Bull. Acad. Sin. (Taipei) **17:** 18–23.

Dudits D. Bogre L. & Gyorgyey J. 1991. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells in vitro. J. Cell. Sci. **99:** 475–484.

Fan Q., Xu X.P., Huang X.L. & Li B.J. 2002. Callus formation and plant regeneration of *indica* rice variety Pei'ai 64S. Acta Bot. Boreal Occident Sin. **22:** 1469–1473.

Giri C.C. & Giri A. 2007. Plant biotechnology: Practical Manual. I K International Publishing House Pvt., Ltd., New Delhi, 156 pp.

Hu H. & Zeng J.Z. 1984. Development of new varieties via anther culture, pp. 65–90. In: Ammirato P.V., Evans D.A., Sharp W.R. (eds), Handbook of Plant Cell Culture, Vol. 3, Macmillan, New York.

Iqbal M.C.M., Mollers C. & Robbelen G. 1994. Increased embryogenesis after colchicine treatment of microspore culture of *B. napus*. J. Plant Physiol. **143:** 222–226.

Khanna H.K. & Raina S.K. 1998. Genotype culture media induction effects on regeneration response of three *indica* rice cultivars. Plant Cell Tiss. Org. Cult. **52:** 145–153.

Khatun M.M. & Desamero N.V. 2005. Callus induction and plant regeneration from rice epicotyl. Plant Tiss. Cult. **15:** 51–56.

Kim H.S., Lee Y.T., Lee S.Y. & Kim T.S. 1991. Anther culture efficiency in different rice genotypes under different cold treatment durations and culture temperatures. Research Reports of the Rural Development Administration. Bio/Technol. **33:** 5–13.

Lapitan V.C., Redona E.D., Abe T. & Brar D.S. 2009. Mapping of quantitative trait loci using a double-haploid population from the cross of Indica and Japonica cultivars of rice. Crop. Sci. **49:** 1620–1628.

Lee K.S., Jeon H.S. & Kim M.Y. 2002. Optimization of a mature embryobased in vitro culture system for high-frequency somatic embryogenic callus induction and plant regeneration from *japonica* rice cultivars. Plant Cell Tiss. Org. Cult. **71:** 9–13.

Lin Y.J. & Zhang Q. 2005. Optimizing the tissue culture conditions for high efficiency transformation of *indica* rice. Plant Cell Rep. **23:** 540–547.

Maluszynski M., Szarejko I. & Sigurbjörnsson B. 1996. Haploidy and mutation techniques, pp. 67–93. In: Mohan Jain S., Sopory S.K. & Veilleux R.E. (eds), In Vitro Haploid Production in Higher Plants, Vol. 1: Fundamental Aspects and Methods, Kluwer Academic Publisher, Dordrecht, The Netherlands.

Miah M.A.A., Earle E.D. & Khush G.S. 1985. Inheritance of callus formation ability in anther cultures of rice, *Oryza sativa* L. Theor. Appl. Genet. **70:** 113–116.

Narasimman R. & Rangasamy S.R.S. 1993. Comparison of fertility between the F1, F2 and anther derived lines in the crosses of Indica Japonica and Japonica Indica in rice (*Oryza sativa* L.). Euphytica **66:** 19–25.

Ozawa K, Kawahigashi H, Kayano T. & Ohkawa Y. 2003. Enhancement of regeneration of rice (*Oryza sativa* L.) calli by integration of the gene involved in regeneration ability of the callus. Plant Sci. **165:** 395–402.

Reddy Y.S., Leelavathi S. & Sen S.K. 1985. Influence of genotype and culture medium on microspore callus, induction and green plant regeneration in anthers in *Oryza sativa*. Physiol. Plant. **63:** 309–314.

Redha A., Attia T., Bütter B., Saisingtong S., Stamp P. & Schmid J.E. 1998. The improvement in regenerated doubled haploids

from anther culture of wheat by anther transfer. Plant Cell Tiss. Org. Cult. **105:** 167–172.

Segui-Simarro J.M. & Nuez F. 2008. Pathways to doubled haploidy: chromosome doubling during androgenesis. Cytogenet. Genome Res. **120:** 358–369.

Silva T.D. 2010. Indica rice anther culture: can the impasse be surpassed? Plant Cell Tiss. Org. Cult. **100:** 1–11.

Smith H. 1990. Signal perception, differential expression within multigene families and the molecular basis of phenotypic plasticity. Plant Cell Environ. **13:** 585–594.

Szakacs E. & Barnabas B. 1995. The effect of colchicine treatment on microspore division and microspore-derived embryo differentiation in wheat (*Triticum aestivum* L.). Euphytica **83:** 209–213.

Tsai S.C. & Lin M.H. 1977. Production of rice plantlets by anther culture. J. Agric. Res. (China) **26:** 100–112.

Visarada K.B.R.S., Aailaja M. & Sarma N.P. 2002. Effect of callus induction media on morphology of embryogenic calli in rice genotypes. Biol. Plant. **45:** 495–502.

Wang Y.Q., Duan Z.G., Huang J.K. & Liang C.Y. 2004. Efficient regeneration from in vitro culture of young panicles of rice (*Oryza sativa* L.). Chinese Bull. Bot. **21:** 52–60.

Wu J.G., Chen S.Y., Shi C.H. & Fan L.J. 2002. Study on culture system in gene transformation of *indica* rice. Chin. Agric. Sci. Bull. **18:** 36–40.

Zaki M.A. & Dickinson H.G. 1990. Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma **156:** 149–162.

Zaki M.A. & Dickinson H.G. 1991. Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex. Plant Reprod. **4:** 48–55.

Zaki M.A. & Dickinson H.G. 1995. Modification of cell development *in vitro*: the effect of colchicine on anther and isolated microspore culture in *Brassica napus*. Plant Cell Tiss. Org. Cult. **40:** 225–270.

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