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APPENDICES

APPENDIX A

Appendix A Glossary

Abscisic acid : Abbreviated by ABA, plant hormone that plays a role in dormancy and senescence.

Adventitious : Developing from unusual points of origin, such as shoot or root tissues, from callus or embryos, from sources other than zygotes.

Androgenesis : Male parthenogenesis. The development of a haploid individual from a pollen grain.

Aneuploid : A cell in which the number of chromosomes deviates from x (the haploid number) or multiple of x.

Antisepsis : Process or principles using antiseptics.

Aseptic : Free of microorganisms.

Aseptic Technique : Procedures used to prevent the introduction of fungi, bacteria, viruses, mycoplasma or other microorganisms into cultures.

Auxin : A group of plant growth regulators that promotes callus growth, cell division, cell enlargement, adventitious buds, and lateral rooting. Endogenous auxins are auxins that occur naturally. Indole-3-acetic (IAA) is a naturally occurring auxin. Exogenous auxins are auxins that are man-made or synthetic. Examples of exogenous auxins included 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-Butyric acid (IBA), α-Naphthaleneacetic acid (NAA), and 4-Chlorophenoxyacetic acid (CPA).

Callus : An unorganized, proliferate mass of differentiated plant cells, a wound response.

Cell culture : The growing of cell *in vitro*.

Chimera : A plant which contains groups (layers) of cells which are genetically dissimilar.

Clonal Propagation : Asexual reproduction of plants that are considered to be genetically uniform and originated from a single individual or explant.

Contamination : Being infested with unwanted microorganisms such as bacteria or fungi.

Culture : A plant growing *in vitro*.

Cytokinin : A group of plant growth regulators that regulate growth and morphogenesis and stimulate cell division. Endogenous cytokinins, cytokinins that occur naturally, include zeatin and 6-γ,γ-dimethylallylaminopurine (2iP). Exogenous cytokinins, cytokinins that are man-made or synthetic, include 6-furfurylaminopurine (kinetin) and 6-benzylaminopurine (BA or BAP).

Dedifferentiation of cells : Reversion of differentiated to non-differentiated cells (meristematic).

Differentiated : Cells that maintain, in culture, all or much of the specialized structure and function typical of the cell type in vivo. Modifications of new cells to form tissues or organs with a specific function.

Dihaploid : This is an individual (denoted by 2n=2x) which arises from a tetraploid (2n=4x)

Diploid : A nucleus is diploid if it contains twice the base number (x) of chromosomes. The genome formula is 2n=2x.

Embryo abortion : Death of an embryo.

Embryogenesis : Process by which an embryo develops from a fertilized egg cell or asexually from a (group of) cell(s).

Embryoid : Plantlet, embryo-like in structure, produced by somatic cells *in vitro*; also adventitious embryo developing *in vitro* by vegetative means.

Explant : Tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

Epigenetic variation : Non-hereditory variation which is at the same time reversible; often the result of a changed gene expression.

Gibberellins : A plant growth regulator that influences cell enlargement. Endogenous growth forms of gibberellin include Gibberellic Acid (GA_3).

Horizontal laminar flow unit : An enclosed work area that has sterile air moving across it. The air moves with uniform velocity along parallel flow lines. Room air is pulled into the unit and forced through a HEPA (High Energy Particulate Air) filter, which removes particles 0.3 μm and larger.

Hormones : Growth regulators, generally synthetic in occurrence, that strongly affects growth (i.e. cytokinins, auxins, and gibberellins).

Inoculate : Place in or on a nutrient medium.

Internode : The space between two nodes on a stem

In vitro : To be grown in glass (Latin). Propagation of plants in a controlled, artificial environment using plastic or glass culture vessels, aseptic techniques, and a defined growing medium.

In vivo : To be grown naturally (Latin)

Media : Plural of medium

Medium : A nutritive solution, solid or liquid, for culturing cells.

Micropropagation : *In vitro* Clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures.

Node : A part of the plant stem from which a leaf, shoot or flower originates.

Passage : The transfer or transplantation of cells or tissues with or without dilution or division, form one culture vessel to another.

Passage Number : The number of times the cells or tissues in culture have been subcultured or passaged.

Pathogen : A disease-causing organism.

Pathogenic : Capable of causing a disease.

Plant Tissue Culture : The growth or maintenance of plant cells, tissues, organs or whole plants *in vitro*.

Regeneration : In plant cultures, a morphogenetic response to a stimulus that results in the products of organs, embryos, or whole plants.

Shoot Apical Meristem : Undifferentiated tissue, located within the shoot tip, generally appearing as a shiny dome-like structure, distal to the youngest leaf primordium and measuring less that 0.1 mm in length when excised.

Somaclonal Variation : Phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones.

Somaclones : Plants derived from any form of cell culture involving the use of somatic plant cells.

Stage I : A step in *in vitro* propagation characterized by the establishment of an aseptic tissue culture of a plant.

Stage II : A step in *in vitro* propagation characterized by the rapid numerical increase of organs or other structures.

Stage III : A step in *in vitro* propagation characterized by preparation of propagules for successful transfer to soil, a process involving rooting of shoot cuttings, hardening of plants, and initiating the change from the heterotrophic to the autotropic state.

Stage IV : A step in *in vitro* plant propagation characterized by the establishment in soil of a tissue culture derived plant, either after undergoing a Stage III pretransplant treatment, or in certain species, after the direct transfer of plants from Stage II into soil.

Sterile : (A) Without life. (B) Inability of an organism to produce functional gametes. (C) A culture that is free of viable microorganisms.

Sterile Techniques : The practice of working with cultures in an environment free from microorganisms.

Subculture : See "Passage". With plant cultures, this is the process by which the tissue or explant is first subdivide, then transferred into fresh culture medium.

Subculture number : The number of times cells, etc. have been subcultured i.e. transplanted from one culture vessel to another.

Tissue Culture : The maintenance or growth of tissue, in vitro, in a way that may allow differentiation and preservation of their function.

Totipotency : A cell characteristic in which the potential for forming all the cell types in the adult organism are retained.

Undifferentiated : With plant cells, existing in a state of cell development characterized by isodiametric cell shape, very little or no vacuole, a large nucleus, and exemplified by cells comprising an apical meristem or embryo.

Appendix B **Composition of nutrient media after Murashige and Skoog (MS), and Linsmaier and Skoog (LS).**

Components	Concentration (mg/L)	
	MS	LS
Macronutrients		
KNO_3	1900	1900
$CaCl_2.2H_2O$	440	440
$MgSO_4.7H_2O$	370	370
$(NH_4)_2SO_4$	-	-
$NaH_2PO_4.H_2O$	-	-
NH_4NO_3	1650	1650
KH_2PO_4	170	170
K_2SO_4	-	-
$Ca(NO_3)_2.4H_2O$	-	-
Micronutrients		
KI	0.83	0.83
H_3BO_3	6.2	6.2
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
$FeSO_4.7H_2O$	27.85	27.8
Na_2EDTA	37.25	37.3
Amino acid/Vitamins/sugar		
Glycine	2.0	2.0
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	0.4
Inositol	100	100
Sucrose	30000	30000
pH	5.8	5.8

Appendix C Reagents and solutions

Acetic Orcein Stain

- Add 1g of Orcein to 45 ml of glacial acetic acid
- Heat solution from step 1 to dissolve
- Slowly add solution from step 2 into 55ml of distilled water
- Allow to cool and store

Fixation

- EtOH 95% 75 ml
- acetic acid 25 ml

HCl Stock 1 N

- HCl 3.65 g
- Distilled water 100 ml

KOH Stock 1N

- KOH 5.611 g
- Distilled water 100 ml

70 % Ethanol

- EtOH 95% 715 ml
- Distilled water 285 ml

Appendix D Publication I

Proceedings the 2nd International Rice for the Future

PP-RICE-113

Influence of Some Components in Tissue Culture Media on Caulogenesis Inducement in Local Thai Rice Genotypes

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Abstract

In rice tissue culture, many different tissue medium have been applied for different varieties because each variety needed a specific for some components in media. Therefore in studying many varieties, different preparation many tissue media should be evaluated to induce callus. This research demonstrates the influence of some components in tissue culture media which affects caulogenesis inducement by using indirect somatic embryogenesis method of mature zygotic embryo explants. The 4 varieties of rice seeds: RD 6, KDML 105, SPR 1 and CNT 1 which replesent of indica rice (have different tissue media need) were cultured in developed MS and LS medium whose macro nutrient concentration (KNO_3, NH_4NO_3), growth regulators (2,4-D, NAA) and other organic compounds (coconut milk and activated charcoal) were modified in order for high embryogenic frequence which transformed to best embryo-like structure. The effects of different component concentrations were found necessary for the development of callus. From the research results, it was found that the use of LS media supplemented with 10 μM KNO_3 + 9.05 μM 2,4-D + 4.42 μM NAA+ 15% Coconut milk + 0.5 mg/l activated charcoal could induce high embryogenic frequent callus which was 1- 2.5 cm long in 4-6 weeks but not having plantlets regeneration before caulogenesis. Unlikely, using standard MS and LS media took 8-10 weeks to induce callus because of having plantlets regeneration before caulogenesis. The results revealed the influence of some components in tissue culture media which had effect on callus form, type, size and caulogenesis time. Some components could promote caulogenesis and inhibit plantlet regeneration which was advantageous to reduce time to produce cell suspension and synthetic seed production. Moreover, can be applied this knowledge in other monocotyl plants in order to increase chance to produce more plantlets within shorter period of time.

Keywords : callus, embryogenesis, caulogenesis, tissue culture media, rice seeds

Materials and methods:

Plant material:

Four rice varieties: RD6, CNT1, SPR1 and KDML105 were used in this study. Mature rice seeds were manually dehusked and washed with sterilized water and then the seeds were transferred to the laminar airflow cabinet. The surface of mature dehulled seeds of these genotypes were sterilized by soaking in 70 %(v/v) ethanol solution for 3 min and rinsing with distilled water before sterilizing with 15%(v/v) Clorox for 20 min by continual shaking. Treated seeds were rinsed three times with sterile distilled water.

Caulogenesis inducement.

For further study, the surface sterilized seeds were cultured in a 5x8 cm sterile glass bottle, 5 seeds were inoculated and 60 seeds were cultured in each treatment which contained 25 ml callus inducing MS and LS medium whose macro nutrient concentration (KNO_3, NH_4NO_3) ,growth regulators (2,4-D, NAA) and other organic compounds (coconut milk and activated charcoal) were modified in order for high embryogenic frequence which transformed to the best embryo-like structure. In MS and LS media ten formulars: 1) 2 mg l^{-1} of 2,4-D (control), 2) 2 mg l-1 of 2,4-D + 2 mg l^{-1} of NAA, 3) 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of KI, 4) 2 mg l^{-1} of 2,4-D + 2 mg l^{-1}

of KI + 2 mg l^{-1} of NAA, 5) 10 µM KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA, 6) 10 µM NH_4NO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA, 7) 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk, 8) 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk, 9) 10µM KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk and 10) 10 µM KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk + 3 mg l^{-1} of Activated Charcoal) were applied. The pH of every formular was adjusted to 5.8 before autoclaving at 115◦C for 15 min. The cultures were kept at 25±2 ◦C under continual illumination from white fluorescent lamps (3,000 Lux under 16 hour's photoperiods).

Results and discussion:

The embryogenic calli induction was most efficient in LS media supplemented with 10 µM KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk + 3 mg l^{-1} of activated charcoal because it could produce high-quality calli formation with high regeneration capacity (Fig.1(a)) after the culture period of 4 weeks. This result supports Visarada et al. (2002)'s findings which showed that the calli regeneration response was also determined by the induction medium. On the other hand, MS media supplemented with 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of KI + 2 mg l^{-1} of NAA promoted organogenesis (Fig. 2(b)). Embryogenic callus formation and plant regeneration from mature seeds are shown in Fig.1. The optimal concentration of component for the highest embryogenic calli frequency was different among the varieties tested (Table 1 and Table 2). Khatun and Nenita (2005) reported that variation between callus induction media and genotype x callus induction media was non-significant. Optimization of the condition for an efficient induction of embryogenic calli and regeneration of plants from mature seeds of indica rice varieties was attempted. The number, colour, size, shape and appearance time of the induced embryogenic calli depends on the type of basal medium (MS, LS). In this study, the high-quality calli was green or dark green in colour, recalcitrant and friable. Moreover, it took short inducement period. The media have several differences in composition, one important factor is the ratio of NO_3 to NH_4, which affects somatic embryogenesis greatly in monocots (Visarada et al. 2002). Growth regulator concentrations in culture medium are critical to control the growth and morphogenesis. Generally, high concentration of auxins and low cytokinins in the medium promotes abundant cell proliferation with the formation of callus. Shoot regeneration is better on hormone- free medium or that containing 2,4-D at low concentration than on medium supplemented with NAA and KI. Al-Khayri et al. (1992) also reported that the addition of 15% coconut milk improved callus culture and shoot regeneration of Spinacia oleracea L. (spinach), relevant to the result in Table 1 and Table 2. Addition of activated charcoal can promote callus forming and growth because it promotes pH balance, adsorption of the inhibitors and growth preventers (Wang et Hong,1976; Anagostakis,1974).

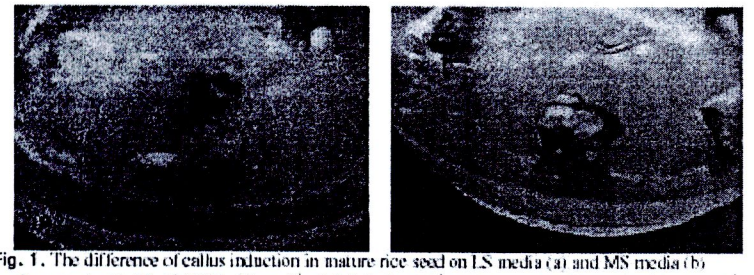

Fig. 1. The difference of callus induction in mature rice seed on LS media (a) and MS media (b) supplemented with 10 μM KNO_3 +2 mg l^{-1} of 2,4-D+2 mg l^{-1} of NAA + 15% Coconut milk+3 mg l^{-1} of activated charcoal after 4 weeks of culture.

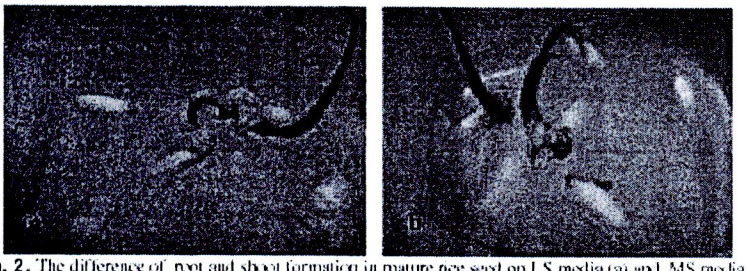

Fig. 2. The difference of root and shoot formation in mature rice seed on LS media (a) and MS media (b) supplemented 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of KI + 2 mg l^{-1} of NAA after 4 weeks of culture.

Table 1. Callus induction in rice mature seed explants cultured on MS supplemented with different Components.

Components	Varieties	Organogenesis		Callus types		Callus forming period (days)	Callus size after 3 weeks (mm)	Colour
		Shoot	Root	Friable	Compact			
1) 2 mg l⁻¹ of 2,4-D (control)	RD6		+	+		31	[illegible]	Light Yellow
	KDML 105		++	+	++	42	<1	Gray
	SPR1		+	+		37	2	Yellow
	CNT1		+	+		41	[illegible]	Yellow
2) 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of NAA	RD6		+	++		30	1	Light Green
	KDML 105		+	++		40	[illegible]	Dark Green
	SPR1			++		35	2	Light Green
	CNT1			++		35	2	Green
3) 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of KI	RD6	++	++	+	+	40	2	Light Yellow
	KDML 105	++			++	44	[illegible]	Yellow
	SPR1	+	+		++	30	3	Yellow
	CNT1	+	++		+	30	1	Dark Yellow
4) 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of KI + 2 mg l⁻¹ of NAA	RD6	++	+++	+	+	42	<1	Gray
	KDML 105	+		+++	+	43	<1	Gray
	SPR1	+	+	+	+	35	1	Dark Yellow
	CNT1	+	+	+	+	43	1	Dark Yellow
5) 10μM KNO_3 + 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of NAA	RD6		+	++		26	[illegible]	Green
	KDML 105		+	++		33	1	Green
	SPR1			++		31	1	Green
	CNT1			+++		33	1	Dark Green
6) 10μM NH_4NO_3 + 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of NAA	RD6		+++	+	+	35	1	Dark Yellow
	KDML 105		+		+	47	2	Yellow
	SPR1		+	+		35	[illegible]	Yellow
	CNT1		++	+		43	[illegible]	Yellow
7) 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of NAA + 15% Coconut milk	RD6			+++		27	[illegible]	Dark Green
	KDML 105		+	++		33	[illegible]	Green
	SPR1			+++		27	[illegible]	Dark Green
	CNT1		+	+++		20	[illegible]	Green
8) 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of NAA + 15% Coconut milk	RD6		++	+	+	38	1	Gray
	KDML 105		+	+	++	41	[illegible]	Green
	SPR1		+	+++	++	31	[illegible]	Green
	CNT1		++	+		31	1	Green
9) 10μM KNO_3 + 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of NAA + 15% Coconut milk	RD6		+	+		22	1	Light Green
	KDML 105		++	+		26	1	Light Green
	SPR1			++		25	[illegible]	Green
	CNT1			++		23	[illegible]	Green
10) 10μM KNO_3 + 2 mg l⁻¹ of 2,4-D + 2 mg l⁻¹ of NAA + 15% Coconut milk + 3 mg l⁻¹ of activated charcoal	RD6			++		20	[illegible]	Green
	KDML 105		+	++		24	[illegible]	Green
	SPR1			+++		24	[illegible]	Dark Green
	CNT1			+++		21	2	Dark Green

Legends: + = Low, ++ = Optimal/good, +++ = Excellent/very good

Table 2. Callus induction in rice mature seed explants cultured on L6 supplemented with different Components.

Components	Varieties	Organogenesis		Callusgenesis				
		Shoot	Root	Callus types		Days forming period (days)	Callus size after 6 weeks (mm)	Colour
				Friable	Compact			
1: 2 mg l^{-1} of 2,4-D (control)	RD6		+	+	-	35	2	Yellow
	KDML105		+	-	-	41	2	Dark Yellow
	SPR1		+	+	-	34	3	Yellow
	CNT1		++	+	-	35	4	Yellow
2: 2 mg l^{-1} of 2,4-D + 3 mg l^{-1} of NAA	RD6			++		32	4	Green
	KDML105			-		33	5	Green
	SPR1			+++		40	4	Green
	CNT1			+++		28	5	Dark Green
3: 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of KI	RD6	++	++	+	+	30	1	Gray
	KDML105	+	+		+	39	2	Dark Yellow
	SPR1	+	+		++	39	1	Light Yellow
	CNT1	+	+++			39	1	Yellow
4: 1 mg l^{-1} of 2,4-D + 2 mg l^{-1} of KI + 3 mg l^{-1} of NAA	RD6	++	+++	++	+	37	1	Gray
	KDML105	+	+	++	++	43	1	Dark Yellow
	SPR1		+	+	+	35	2	Dark Yellow
	CNT1	++	+	+	+	33	2	Dark Yellow
5: 10 gN KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA	RD6			++		26	6	Dark Green
	KDML105			+		35	5	Green
	SPR1		++	++		37	4	Green
	CNT1			+++		42	4	Dark Green
6: 10 gN NH_4NO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA	RD6	++	+	-	+	36	3	Light Yellow
	KDML105		+	+		42	2	Yellow
	SPR1					34	3	Light Yellow
	CNT1		+	+		33	3	Yellow
7: 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk	RD6			++		35	6	Dark Green
	KDML105			++		28	6	Dark Green
	SPR1			+++		33	6	Dark Green
	CNT1			+++		32	5	Dark Green
8: 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk	RD6		++	++	++	33	1	Yellow
	KDML105		+	-	-	33	5	Light Green
	SPR1		+			30	3	Light Green
	CNT1		-	-		34	3	Green
9: 10 gN KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk	RD6		+	++	++	26	5	Light Green
	KDML105		+	+++	++	34	5	Light Green
	SPR1			+++		33	5	Light Green
	CNT1			+++		34	5	Dark Green
10: 10 gM KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk + 3 mg l^{-1} of activated charcoal	RD6			+++		19	8	Dark Green
	KDML105			++		19	8	Dark Green
	SPR1			+++		19	7	Dark Green
	CNT1			+++		17	7	Dark Green

Legends: + – Low; ++ – Optimal/good; +++ – Excellent/very good

Conclusions

Generally, standard MS and LS media took 8-10 weeks for callus inducement because of plantlets regeneration before caulogenesis. Therefore, this study provided more advantageous results. LS medium adding 10 mM KNO_3 + 2 mg l^{-1} of 2,4-D + 2 mg l^{-1} of NAA + 15% Coconut milk + 3 mg l^{-1} of Activated Charcoal is considered as a medium suitable for caulogenesis inducement in all rice varieties. Since it could induce high embryogenic frequent callus which was 1-2.5 cm long in 4-6 weeks without plantlets regeneration during caulogenesis. This was beneficial to embryogenesis inducing because it could shorten the culture period and increase the chance for transformed to best embryo-like structure after culturing in suspension media. The potential of the embryogenic callus to produce somatic embryos and their conversion into plants provides the best and accessible efficiency.

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Appendix E Publication II

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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 × SPR1 (*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 1.5 mm. The supplementation of 0.2 g/L colchicine and 100 nM 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 to shorten time to produce higher number of double haploid plantlets.

Key words: hormone shock; *in vitro*; embryogenesis; 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 (1981) 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; Tzai & 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 rices.

Anyhow, the answers for all limitations 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 productions; 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.

Material 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°C 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.

Callogenesis induction

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°C 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°C 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 anthers) to compare the responses to anther culture process for callus and plantlets.

Hormone shock and embryogenesis induction

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 hydrolysate, 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 these 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 ± 2°C 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						Embryogenesis			
	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	21.00	6.56	neg.		neg.		25.67	4.16
4	30.67	2.52	16.67	3.51	neg.		17.00	3.00	neg.	
5	neg.		neg.		45.33	6.66	45.33	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	18.33	3.51
8	neg.		neg.		17.00	6.00	75.67	6.66	neg.	
9	neg.		neg.		neg.		58.33	7.63	42.67	6.51*
10	neg.		neg.		neg.		91.33	2.52*	neg.	

[a]The asterisk signifies the significantly highest value (p-value < 0.05); mean difference was tested by Kruskal Wallis test. The "neg." means that the formation of organogenesis or embryogenesis was not found.

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

Formula	Organogenesis						Embryogenesis			
	Shoot		Root		Shoot + Root		Friable		Compact	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
1	neg.		45.33	4.73	neg.		neg.		25.00	4.73
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	1.53
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	3.00	neg.	
6	neg.		17.00	3.00	23.33	6.11	24.67	6.03	neg.	
7	neg.		18.00	2.65	22.00	3.00	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.		70.33	6.66	30.00	2.00*

[a] The asterisk signifies the significantly highest value (p-value < 0.0001); mean difference was tested by Kruskal Wallis test. The "neg." means that the formation of organogenesis or embryogenesis 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 Ciri & Ciri (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 °C. Then HCl was removed and replaced with aceto-orcein at 90 °C for 1-2 min. ELSs were squashed and observed ploidy by microscope. This experiment was done in 5 replicates with 100 cells per treatment.

Statistical analysis

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

Results and discussion

Embryogenesis induction

The embryogenic calli induction was the most efficient in LS media supplemented with 10 uM 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).

Khatun & Nemita (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

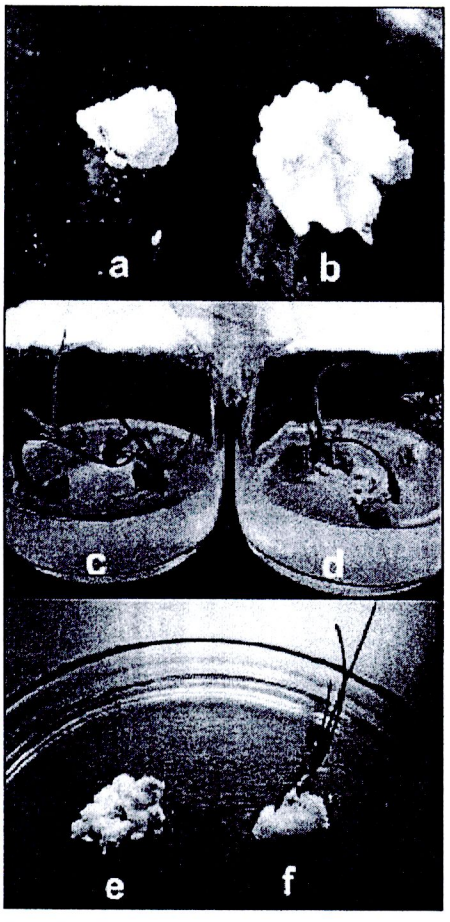

Fig. 1 The difference of callus induction from anther on LS media formula No. 10 after 4 weeks of culture in F1 hybrid anther (a) and H1 anther (b). The difference of organogenesis formation in anther culture on LS media formula (c) 4 promoted organogenesis in F1 anther culture (c) and in H1 anther culture (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 embryogenesis. 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 (Han 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 embryogenesis 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 (Anagnostakis 1974). Similar results were found by Khanna & Raina (1998). Somatic embryogenesis was a successive developmental process that involves multiple phases (Arnold 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 formulations on the embryogenesis of F1 and H1 anther hybrid rice[a]

Formula	Anther type	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	1.5	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.

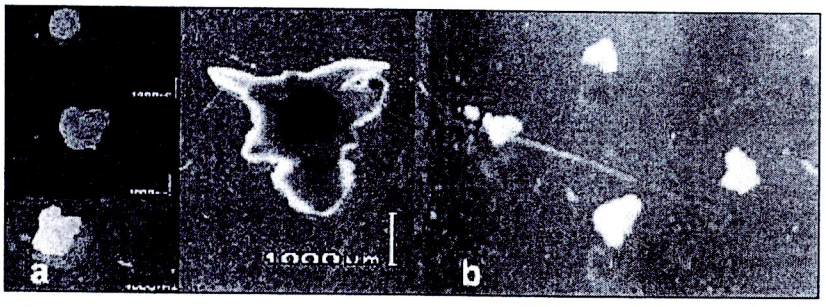

Fig. 2. Differentiation of embryoids after culture calli in LS media supplemented with 0.2 g/L colchicine and 100 µM 2,4-D (a) in comparison with conventional anther culture method (b) after 8 weeks.

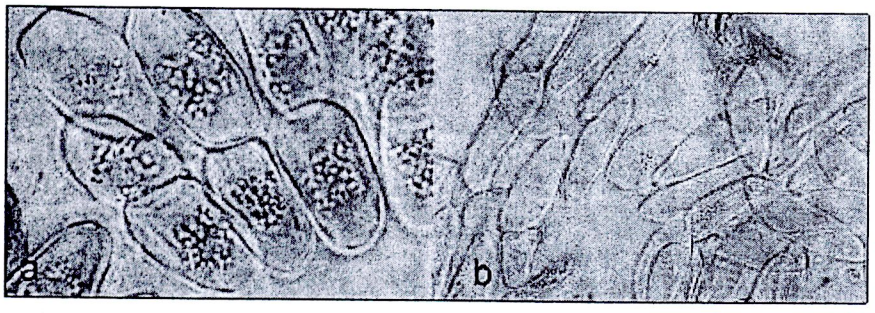

Fig. 3. Analyzed metaphase chromosome of H1Rs after treatment with 0.2 g/L colchicine and 100 µM 2,4-D (a) and treatment with over 0.3% colchicine (denatured chromosome) (b).

Table 4. Influence of 1/8 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	13	85	1	–
	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	71	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 2001).

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 suspensions 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 pretreatment 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), *Origo 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. 1995). 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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