Chapter 4

Results and discussion

4.1 Pre-Treatment :

Application of a variety of stresses, such as temperature pre-treatment, osmotic shock and sugar starvation, during the labile developmental period of pollen grains is known to be promontory or essential for the induction of androgenesis in several plants, including cereals (Bhojwani and Razdan, 1996; Bhojwani *et al*., 2001). However, the type, duration and the time of application of these pre-treatments may vary with the species or even variety (Datta, 2005). The most widely used pre-treatment for androgenesis is the low temperature shock. In first experiment the cold treatment is used to determine suitable stage of anther for caulogenesis.

The table 4.1 and 4.2 shown effect of low temperature treatment on development stage of F1 and H1 pollen. The results shown that the anthers were pre - treatment with low temperature (5^{o}C) have effect to microspores division more than other temperature (10, 15^{o}C and control). The pollen in mid-late uninucleate stage (Fig. 4.1) most response at distance between flag leaf and penultimate leaf over 5 cm. when treated with 5 ^{o}C at 7 and 14 days (over 50%) both anthers of F1 and H1 were determine from caulogenesis percentage. In culture experiments the best results were obtained from pretreatment at 5 ๐C for 14 days (the distance between flag leaf and penultimate leaf 9 cm). The response of anthers from different panicle positions on androgenesis are shown in Table 4.3. It can induce caulogenesis in high number

72.33% (F1) and 65.67% (H1). The induction frequency of callus was over 65 % more than in the control. It was observed that F1 and H1 hybrids required nearby temperatures and pretreatment durations. Zhao (1983) reported that cold treatment at 6-8 ºC for 6 days was optimal for keng rice. Three days was optimal for hsein rice. However, when the pretreatment duration exceeded a certain limit, the induction frequency in keng rice and in hsien rice decreased markedly. The callus aged and lost its ability to regenerate into plantlets. Based on the study by Mercy and Zapata (1986), the distance between the flag leaf and the subtending leaf as well as the late uninucleate and early binucleate pollen stage has been used as markers for callus induction although with inconsistent success. Shahjahan *et al*. (1992) observed that the best microspore stage for highest callus induction in four indica rice varieties was the mid-uninucleate stage and this corresponded with spikelets of yellowish green colour and anthers reaching the middle of the spikelet. Yin *et al*. (1976) using a japonica cultivar observed that the best microspore stage for callusing was the late-uninucleate stage when spikelets were yellowish green in colour and the length of stamen was 1:3 to 1:2 of the glume.

The part cold treatment plays in improving anther culture has been discussed in many reports (Nitsch (1977); Sunderland and Roberts (1977) and Takahashi *et al*. (1975)). Zhao (1983) found that during cold pretreatment the intensity of breath and the consumption of nutrients in anthers incubated under different temperature levels. As a result the lives of the anther wall cells were prolonged the degeneration of microspores was delayed and the proportion of cultures that survived was enhanced. We found that microspores not only initiated androgenesis, but also divided several times during the course of the cold pretreatment of panicles (Chen *et al*., 1991). In

most of the published works on androgenesis in rice, panicles were given a cold pre-treatment but the temperature and duration varied. Matsushima *et al.* (1988) had reported that a pre-treatment at 10 ^{o}C for 10-30 days was necessary to induce sporophytic divisions in microspores of the *japonica cv Nipponbare*. This was subsequently confirmed by several workers for japonica and indica cultivars (Ogawa *et al*., 1992; Datta *et al*., 1990; Raina and Irfan, 1998). Ogawa *et al*. (1995) observed that 28 days of pre-treatment at 10 ^{o}C was optimum for the *indica cv IR 24.* Gupta and Borthakur (1987) had selected pre-treatment at 10 ^{o}C for 11 days for anther culture of the *indica cv khonorullo*. Although the frequency of anthers showing pollen callusing after cold-treatment for 25 days was fairly high, most of the plants regenerated from the calli formed after such a long cold pre-treatment were albinos. Similarly, Pande (1997) observed that cold pre-treatment was essential for androgenesis in anther cultures of the *indica cv* IR43, and 10 ^{o}C for 10 days was most suitable. Pre-treatments longer than 11 days resulted in albino production. Reddy *et al*. (1985) reported that a brief (10 min) exposure to high temperature (35 ^{o}C) before cold-treatment was better for pollen callusing but it adversely affected green plant production.

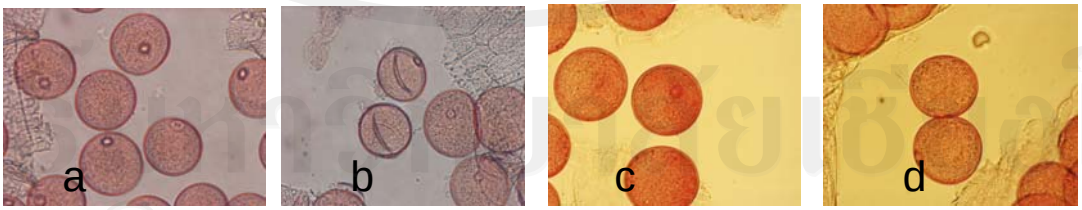


Figure 4.1 Stage of microspore development of hybrid rice (a) early uninucleate (b) mid- uninucleate (c) late- uninucleate (d) binucleate.

Table 4.1 Effect of low temperature treatment on development stage of F1 pollen. [a]

Distance between flag leaf and penultimate leaf (cm)	Storage time (days)	Temperature (°C)	Stage of pollen (%)			
			Early uninucleate	Mid uninucleate	Late uninucleate	Binucleate
5	7	5	79.11	20.89	-	-
		10	86.78	13.22	-	-
		15	87.89	12.11	-	-
	14	5	68.56	31.44	-	-
		10	79.67	20.33	-	-
		15	85.44	14.56	-	-
		Control	84.33	15.67	-	-
7	7	5	40.89	40.67	14.11	4.33
		10	51.22	11.00	13.00	4.78
		15	65.11	23.26	8.63	3.00
	14	5	30.44	52.56	11.67	5.33
		10	52.67	34.11	9.11	4.11
		15	63.89	31.00	4.00	1.11
		Control	67.00	27.67	5.33	
9	7	5	6.00	53.67	37.33	3.00
		10	5.67	25.33	40.67	28.33
		15	7.22	27.00	30.78	35.00
	14	**5**	**3.67**	**37.33**	**54.00**	**5.00**
		10	9.11	35.67	31.22	26.00
		15	5.00	27.67	31.00	36.33
		Control	3.33	8.67	27.00	61.00

[a] This experiment observed from 3 replicates with 300 cells per treatment.

Table 4.2 Effect of low temperature treatment on development stage of H1 pollen. [a]

Distance between flag leaf and penultimate leaf (cm)	Storage time (days)	Temperature (°C)	Stage of pollen (%)			
			Early uninucleate	Mid uninucleate	Late uninucleate	Binucleate
5	7	5	82.33	17.67	-	-
		10	88.44	11.56	-	-
		15	86.11	13.89	-	-
	14	5	75.22	24.78	-	-
		10	84.56	15.44	-	-
		15	88.56	11.44	-	-
		Control	79.67	20.33	-	-
7	7	5	34.33	51.00	7.67	7.00
		10	42.67	36.33	15.33	5.67
		15	47.00	31.67	17.00	4.33
	14	5	23.33	56.33	12.34	8.00
		10	44.11	43.67	7.00	5.22
		15	53.67	34.33	7.33	4.67
		Control	28.00	39.67	18.33	14.00
9	7	5	6.67	26.00	61.00	6.33
		10	2.33	21.67	31.33	44.67
		15	5.33	17.67	26.67	50.33
	14	**5**	**3.00**	**19.00**	**62.33**	**15.67**
		10	1.00	16.67	31.00	51.33
		15	3.67	11.67	23.33	61.33
		Control	0.00	14.67	17.00	68.33

[a] This experiment observed from 3 replicates with 300 cells per treatment.

Table 4.3 Effect of low temperature treatment on F1 and H1 androgenesis in LS Media. [a]

Distance between flag leaf and penultimate leaf (cm)	Storage time (days)	Temperature (°C)	Androgenesis			
			Calli regeneration		Green plant regeneration	
			F1	H1	F1	H1
5	7	5	17.33	15.00	-	-
		10	12.00	11.67	2.67	-
		15	11.33	12.67	1.00	1.67
	14	5	21.33	18.33	2.00	-
		10	16.67	9.67	3.67	2.33
		15	13.33	9.33	2.50	1.00
		Control	10.67	7.67	3.00	1.67
7	7	5	28.67	34.00	4.00	2.67
		10	11.67	16.22	7.33	5.00
		15	9.33	13.67	7.33	4.67
	14	5	31.33	38.67	4.33	6.67
		10	14.67	29.11	12.00	14.00
		15	20.00	27.33	14.67	19.67
		Control	18.67	23.67	21.67	27.33
9	7	5	63.44	68.33	7.00	12.33
		10	46.67	48.33	15.67	20.67
		15	34.67	41.00	21.00	17.67
	14	5	**72.33**	**65.67**	5.33	6.00
		10	41.67	38.00	23.00	27.67
		15	37.33	35.67	26.22	23.33
		Control	32.00	25.67	**34.67**	**37.33**

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

4.2 Caulogenesis inducement

The caulogenesis inducement 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. 4.2 a,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 10 μM KNO_3 + 2 mg/L of

2,4-D + 2 mg/L of NAA promotes organogenesis in H1 anther culture (Fig. 4.2 c) and F1 anther culture (Fig. 4.2d). Embryogenic callus formation and plant regeneration from anther are shown in Figure 4.2 e, 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 4.4 and 4.5). Khatum and Nenita (2005) reported that variation between callus induction media and genotype was nonsignificant. 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 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 and Lin, 1976; Tsai and 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 and 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 4.6). 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 and Raina, 1998; Lee *et al*., 2002; Ozawa *et al*., 2003; Lin and 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 4.4). 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 and Raina (1998). Somatic embryogenesis was a successive developmental process that involves multiple phases (Arnold *et al*., 2002).

In china considerable work has been done to develop media that would favour the formation of green haploid and diploid plants in anther cultures of cereals at a high frequency. Low inorganic nitrogen, particularly ammonium, in the medium is reported to promote androgenesis (Chu *et al*., 1975) and the yield of green plants (Olesen *et al*., 1988) in some cereals. Ten times reduction in NH_4NO_3 concentration in LS medium substantially enhanced the overall androgenic response in *Lolium perenne* and *L. multiflorum* (Bante *et.al*., 1990). Even KNO_3 in the medium was inhibitory for embryogenesis. Halving the concentration of KNO_3 in MS basal medium remarkably enhanced pollen embryogenesis in *Hevea brasiliensis* (Chen, 1990). Generally, high concentration of auxins and low cytokinins in the medium promoted abundant cell proliferation with the formation of callus. Shoot regeneration was better on hormone-free medium or that containing 2,4-D at low concentration than on medium supplemented with NAA and KI (Table 4.4). In most cases, 2,4-D as a strong synthetic auxin was sufficient to initiate and sustain embryogenic callus grown in rice and has been used as the only growth regulator in callus induction media (Khanna and Raina, 1998; Lee *et al*., 2002; Ozawa *et al*., 2003; Lin and Zhang , 2005). There were also a few reports that used of 2,4-D alone only produced a non-embryogenic one (Wu *et al*., 2002; Fan *et al*., 2002; Wang *et al*., 2004). Al-Khayri *et al.* (1992) also reported that the addition coconut water improved callus culture and shoot regeneration of *Spinacia oleracea* L. (spinach), relevant to the result in Table 1. Addition of activated charcoal could promote callus forming and growth because it promotes pH balance, adsorption of the inhibitors and growth preventers (Wang and Hong, 1976; Anagostakis, 1974). Similar results were found by Khanna and Raina (1998). Somatic embryogenesis was a successive developmental process that involves

multiple phases (Arnlod *et al*., 2002). Casein hydrolysate which is a source of calcium, several micronutrients, vitamins and amino acids, was added in modified LS media. Improvement in callus induction and growth by the addition of casein hydrolysate was also reported by Khaleda and Al-Forkan (2006) in deepwater rice. They also started that callus formation and plant regeneration was influenced by interaction of media components.

4.3 Embryogenesis inducement and doubling chromosome

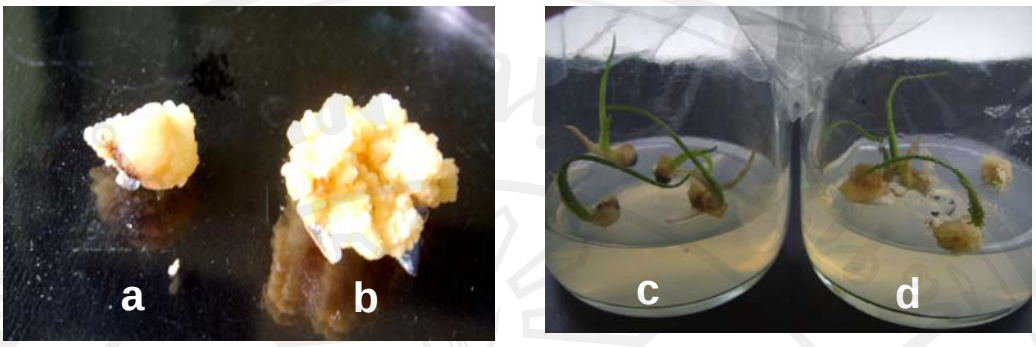
Combination *in vitro* techniques between hormone shock for induced embryogenic development and doubling chromosome to produce double haploid were the most efficient in LS media supplemented with 0.2 g/L colchicine and 100 μM 2,4-D (Fig. 4.4 a). It could induce high rate of viable double haploid embryoid over 70% in 6 weeks (Table 4.7) and subcultured only twice (Fig. 4.3a),in comparison with the conventional anther cultured method, which takes more than 12 weeks and subcultured more than 4 times to produced double haploid embryoid (Fig. 4.3b). 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 and 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 (Bögre *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 inorganized 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 and Barnabas, 1995), Oryza sativa (Alemanno and 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 and Dickinson, 1990). Similar observation for *Triticum* was reported by Szakacs and Barnabas (1995). Both, the concentration and the duration of colchicine treatments are important for promoting androgenesis and doubling chromosome. According to Zaki and 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 and Dickinson, 1995). For colchicine treatment in high concentration (more than 0.3 g/L), somaclonal variation in high rate (Table 4.8) and denaturation of chromosome (Fig. 4.4b) would occur, which caused the death of cells. Redha *et al*. (1998) reported that the use of colchicines in concentration over 0.2 g/L caused the reduction of embryogenesis and lead to chimera of polyploids cells in wheat. The results from this experiment supported Redha *et al*. (1998) 'result, single cells could not develop to embryoid because chimera of polyploids cells (Fig. 4.5 a and b). 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.

The use of hormones in the culture medium at a specific time might allow the creation of worthy mutants originating from callus-regenerating buds. The technique, when properly used, can be reliable for the production of normal isogenic lines. That is most important for rice breeding. We do not think a universal method should exist for plant breeding. A choice between different techniques has to be made in terms of the cultivars. It is possible to adapt the androgenetic technique to some extent. It also is possible to introduce favorable characters into a hybrid using the F1 for pollen culture. Several ways are open. The *in vitro* method is one. It shortens the time necessary for selection, produces original information on a given genotype rapidly and creates new genotypes *in vitro*.



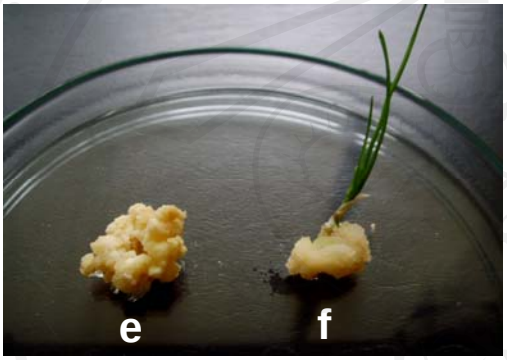


Figure 4.2 The difference of callus induction from anther on LS media formula no.10 after 4 weeks of culture (a) F1 hybrid anther and (b) H1 anther.The difference of organogenesis formation in anther culture on LS media formula no.5 promoted organogenesis in F1 anther culture (c) and in H1 anther culture (d) after 4 weeks of culture. Embryogenic callus formation (e) and plant regeneration (f) from anther culture after 4 weeks of culture.

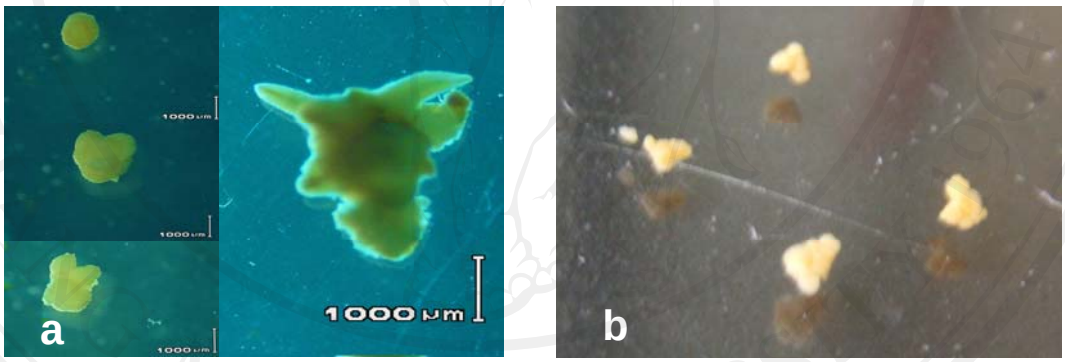


Figure 4.3 Differentiation of embryoids after culture calli in LS media supplemented 0.2 g /L colchicine and 100 µM 2,4-D (a). compare with conventional anther culture method (b) after 8 weeks.

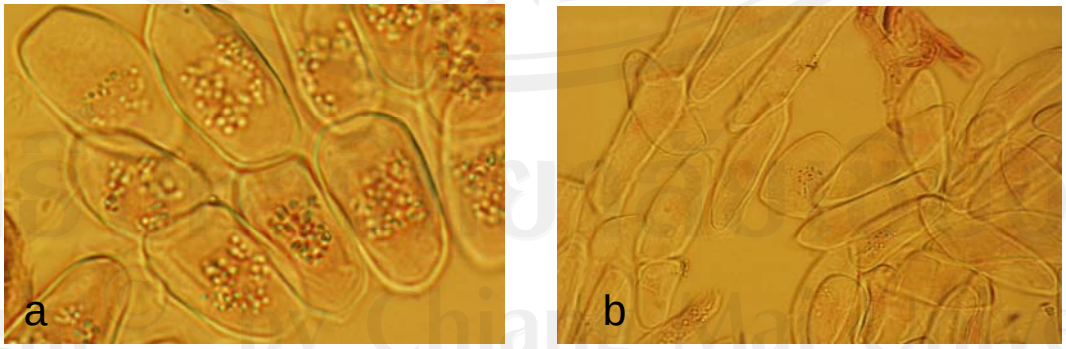


Figure 4.4 Analyzed metaphase chromosome of ELSs after (a). treated with 0.2 g /L colchicine and 100 µM 2,4-D and (b) treated with over 0.3 g/L colchicines (denature chromosome).

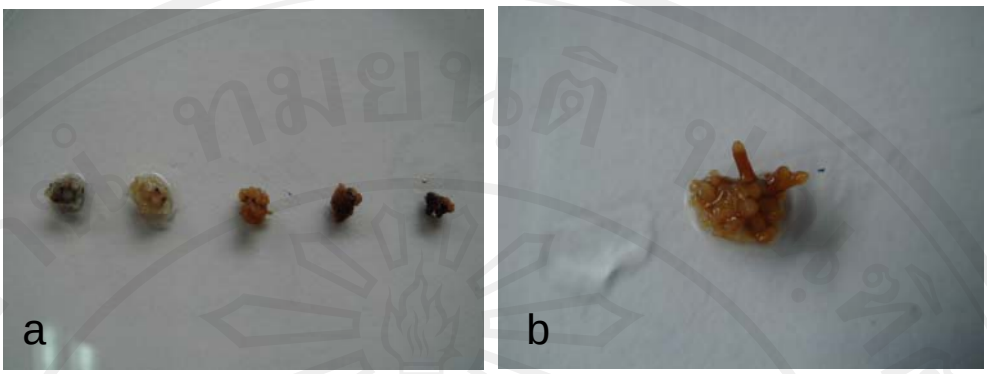


Figure 4.5 Single cells after (a). treated with 0.1, 0.2, 0.3, 0.4 and 0.5 g/L colchicine and 100 µM 2,4-D and (b) treated with over 0.3 g/L colchicines (denature chromosome).

Table 4.4 Influence of the 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 4.5 Influence of the 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.

Table 4.6 Influence of the various LS media formulas on the caulogenesis of F1 and H1 anther hybrid Rice.

Formula	Anther Type	Caulogenesis				
		Callus type		Callus forming period (days)	Callus size after 4 weeks (mm)	Color of callus
		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**

Abbreviations: + = Low; ++ = optimal; +++ = Excellent
F1 = Anther from F1 hybrid; H1= Anther from F1 anther culture

Table 4.7 Influence of LS liquid media supplemented with different concentration of colchicine and 2,4-D on the embryogenesis of F1 hybrid rice. [a]

Colchicine concentration (g/L)	2,4-D concentration (μM)	% 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 4.8 Effect of different colchicine and 2,4-D concentrations on chromosome doubling in F1 hybrid rice anther culture. [a]

Colchicine concentration (g/L)	2,4-D concentration (μM)	% of cells showing chromosomal complement of			
		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.

4.4 Synthetic seed production

Encapsulating somatic embryo with sodium alginate and benomyl in different concentration brought about synthetic seeds. Then, dehydration were persued until the seeds lost 80% of their moisture contents (Fig 4.6 a and b). The experiment suggested that germination percentage would decrease every week and so as the speed of germination. The result was; every concentration of benomyl not affected survival percentage, speed of germination due to the contamination appeared less number during storage. The synthetic seeds took 4-6 days for germinating. However, the germination percentage decreased significantly in the 6 week of storage. The germination percentage decreased to 46% whereas 9% were abnormal seedling. Considering the result in Table 4.9, the most suitable synthetic seed storage period at 25 ºC was 2 weeks with 74 % germination and 3 % abnormal seedling. This was considered to be the satisfied percentage. Nevertheless, Nieves *et al.* (2001) suggested that the viability would increase more if applying 3.8 µM ABA before encapsulation with sodium alginate and dehydration. Their results leaded to 57 % synthetic seed germination. The storage by inducing desiccation tolerance with 0.5 mg/l ABA was done for 1, 2 and 3 weeks whose results were 48 %, 47 % and 32 % of germination, respectively. Conforming with Takahata *et al*. (1993) who could produce the most inducing desiccation tolerance when applying 100 µM ABA, developing into 27-48 % plantlets. In alfalfa (*M. sativa*) desiccation-tolerance of somatic embryos was induced by exogenous application of abscisic acid (ABA) by Senaratna *et al*. (1990). Subsequently, the embryos were dried to 10–15% moisture and stored for at least 3 weeks in the dry state. Anyhow, from this experiment the result demonstrates the possibility of inducing desiccation tolerance without ABA into somatic embryos.

Under appropriate treatment conditions, 65% of these somatic embryos survived and germinated in a manner analogous to a true seed. Desiccation-tolerance has also been induced in alfalfa somatic embryos by exposure to sub-lethal levels of low temperature, water, nutrient or heat stress. The bead quality was also modified by impregnating them with 3% sucrose, by coating the microcapsules with a fungicidal mixture comprising 8% Elvax 4260 and beeswax, and 0.1% Topsin M (Onishi *et al*., 1994). To facilitate the emergence of shoot and root meristems during embryo germination, However, further research is needed to optimize protocols for production of viable synthetic seeds that could be stored for longer periods and could be commercially viable.

Attree and Fowke (1993) have reported that somatic embryos of spruce matured in the presence of PEG and ABA were very tolerant to low moisture levels. According to them, such somatic embryos had less than 50% moisture content which was further reduced to less than 10% following desiccation. These embryos were stored at –20°C for a year and thereafter successfully germinated following imbibition with no loss in viability. The coating material may also limit success of the synthetic seed technology, and at present none of the embryo encapsulation methods described earlier is completely satisfactory. The hydrated capsules are more difficult to store because of the requirement of embryo respiration (Redenbaugh, 1990). A second problem is that capsules dry out quickly unless kept in a humid environment or coated with a hydrophobic membrane(Redenbaugh *et al*., 1987). Calcium alginate capsules are also difficult to handle because they are very wet and tend to stick together slightly. In addition, calcium alginate capsules lose water rapidly and dry down to a hard pellet within a few hours when exposed to the ambient atmosphere. These

problems can be offset by coating the capsules with Elvax 4260 (ethylene vinyl acetate acrylic acid terpolymer, Du Pont, USA) (Redenbaugh *et al*., 1990). Redenbaugh, Fuji and Slade (1993) have reported that the limitations caused by coating materials can be overcome by selecting appropriate coating material for encapsulation. According to them, the coating material should be non-damaging to the embryo, mild enough to protect the embryo and allow germination and be sufficiently durable for rough handling during manufacture, storage, transportation and planting. The concentration of the coating material is also an important limiting factor for the synthetic seed technology.

The coat must contain nutrients, growth regulator(s) and other components necessary for germination and conversion and it should be transplantable using the existing farm machinery. Though many coating materials have been tried for encapsulation of somatic embryos, sodium alginate obtained from brown algae is considered the best and is being popularly used at present. Alginate has been chosen for ease of capsule formation as well as for its low toxicity to the embryo. The rigidity of the gel beads protects the fragile embryo during handling. According to Redenbaugh *et al.* (1987), the capsule gel can potentially serve as a reservoir for nutrients (like an artificial endosperm) that may aid the survival and speed up the growth of the embryo. A key factor for synthetic seed technology is the assessment of the effects of various concentrations of sodium alginate and calcium chloride on the texture, shape and size of the bead. The beads differed morphologically with respect to texture, shape, diameter and transparency with different combinations of sodium alginate and calcium chloride. An optimal ion exchange between Na^{+} and Ca^{2+} producing firm clear, isodiametric beads was achieved by complexation of a 3%

solution of sodium alginate (Sigma) with 100 mM calcium chloride for 30 min thereby forming an insoluble gel matrix of calcium alginate. This is in agreement with many other reports on tree species such as Camellia sp. (Janeiro *et al*., 1997), *Malus sp*., *Pyrus sp*. and *Morus bombysis* (Niino and Sakai, 1992). At lower concentrations (1–2%), sodium alginate became unsuitable for encapsulation perhaps because of a reduction in its gelling ability following exposure to a high temperature during autoclaving (Larkin *et al*., 1988). At a higher concentration of sodium alginate (5–6%) beads were isodiametric but hard enough to cause considerable delay in sprouting.

Table 4.9 Result of storage time on inducing desiccation tolerance synthetic seed germination percentage and speed of germination after dehydration was persued until seeds lost 80% of their moisture contents. [a]

storage time (week)	Germination (%)		abnormal seedling(%)		speed of germination (day)	
	5 ºC	25 ºC	5 ºC	25 ºC	5 ºC	25 ºC
0	91	76	-	2	3	4
2	89	**74**	-	3	3	4
4	86	61	1	5	3	4
6	**84**	46	4	9	4	6
8	81	28	7	16	6	6

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

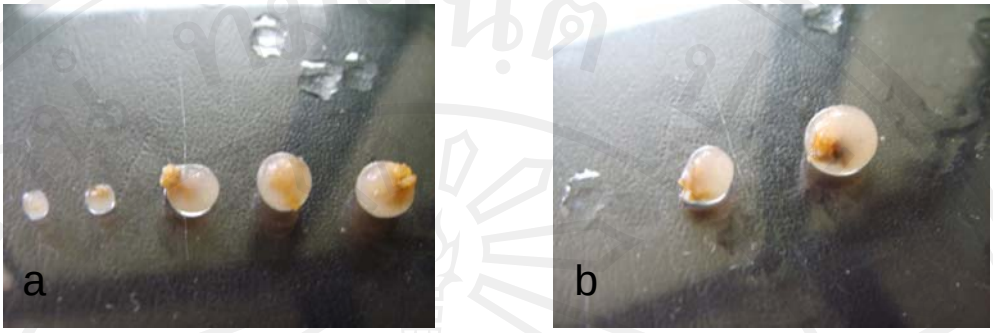


Figure 4.6 Synthetic seed (a). during dehydration persued until the seeds lost 80% and (b) compare size of Synthetic seed before and after dehydration.

The survival of F1 seeds and H1 synthetic seed at 14 days after sowing in soil (Figure 4.7 b) were higher by 80 and 75%, respectively, in all the treatments (0, 0.2, 0.4 and 0.6 mg/L benomyl) applied (Table 4.10). As well, there were no significant differences in the different concentrate of benomyl used and complexing time in $CaCl_2$ among the aforementioned variables. However, a response was presented similar to what was obtained by authors such as Castillo *et al.* (1998), who observed a higher percentage of germination of encapsulated somatic embryos with increased fungicide, sodium alginate content and reduced exposure time to $CaCl_2$ (Prewein and Wilhelm, 2002; Malabadi and Van Staden, 2005). This is because high concentrations or excessive exposure of the embryos to the complexing agent (benomyl) results in more absorption and penetration of benomyl to the embryo, which can generate growth inhibition that is reflected in decrease in the germinative response and subsequent development in the field (Redenbaugh *et al.*, 1986; Malabadi and Van Staden, 2005). The importance of the high percentage of survival and germination

obtained in the encapsulated zygotic embryos should be noted (Table 3). From these results it is clear that if the embryo reaches necessary vigor during the maturation phase, can break the mechanical resistance and lack of oxygen with increased sodium alginate content in the matrix, and thus the germinative response is not affected (Jiménez and Quiala, 1998). This can be extrapolated to somatic embryos, refining the maturation protocols to obtain better percentages of artificial seed germination (Nieves *et al*., 2001).

It is important to point out that the zygotic embryos germinated at 4 days of cultivation (Figure 4.7 a), achieving the conversion to plants (Figure 4.7 b), while the somatic embryos germinated at approximately 2 weeks and in an irregular manner, manifesting a lack of maturity and asynchrony in the development of these embryos (Castellanos *et al.*, 2004). However, refining the encapsulation process for somatic embryos, with the goal of improving the percentages of germination and subsequent conversion to plants, can mean the generation of a cloning product with the potential to outperform seeds of sexual origin (Tapia *et al*., 1999; Nieves *et al*., 2001).

Table 4.10 Effect of different benomyl concentration of encapsulating applied to embryoid of KDML 105 x SPR 1 (H1)

Benomyl concentration (mg/l)	Survival (%)	Germination in LS media (%)		Germination in soil (%)	
		7 days	14 days	7 days	14 days
Control (F1)	100a	93.0a	92.5a	95.0a	80.4b
0	97a	95.8a	94.5a	93.7a	86.4a
0.2	**100a**	**100.0a**	**98.0a**	**94.5a**	**85.8a**
0.4	100a	97.5a	97.5a	91.0a	75.4c
0.6	98a	95.0a	92.8a	82.0b	74.8c

Means following by the same letter in the columns do not differ significantly among themselves according Minimum Significant Difference test (P>0.1)

4.5 Germination and conversion of encapsulated embryoids

Table 4.10 shows the germination and conversion response of encapsulated embryoids obtained from androgenesis. More than 90% of encapsulated embryoid germinated in 1 weeks, both on the aseptic and non-aseptic substrata. There were no significant differences between aseptic and nonaseptic conditions in first week. Under aseptic conditions, the conversion rates of encapsulated embryoids were more than the control (F1), and the conversion rates of embryoids encapsulated in 3% sodium alginate and 0.2 mg/l benomyl were highest among all treatment (Table 4.10). Under non-aseptic conditions, however, the germinated capsules needed to be transferred to fertile soil and vermiculite (2:1) (Fig. 4.7a) since substrata with 8% agar alone could not support further growth of the germinated shoot buds. The treatment which have concentration over 0.2 mg/l benomyl turned yellow and died 2 weeks after transplanting. The synthetic seed which encapsulated in 3% sodium alginate and 0.2 mg/l benomyl could growth from embryo to normal plant and produced normal seeds after 60 days (Fig. 4.7d).

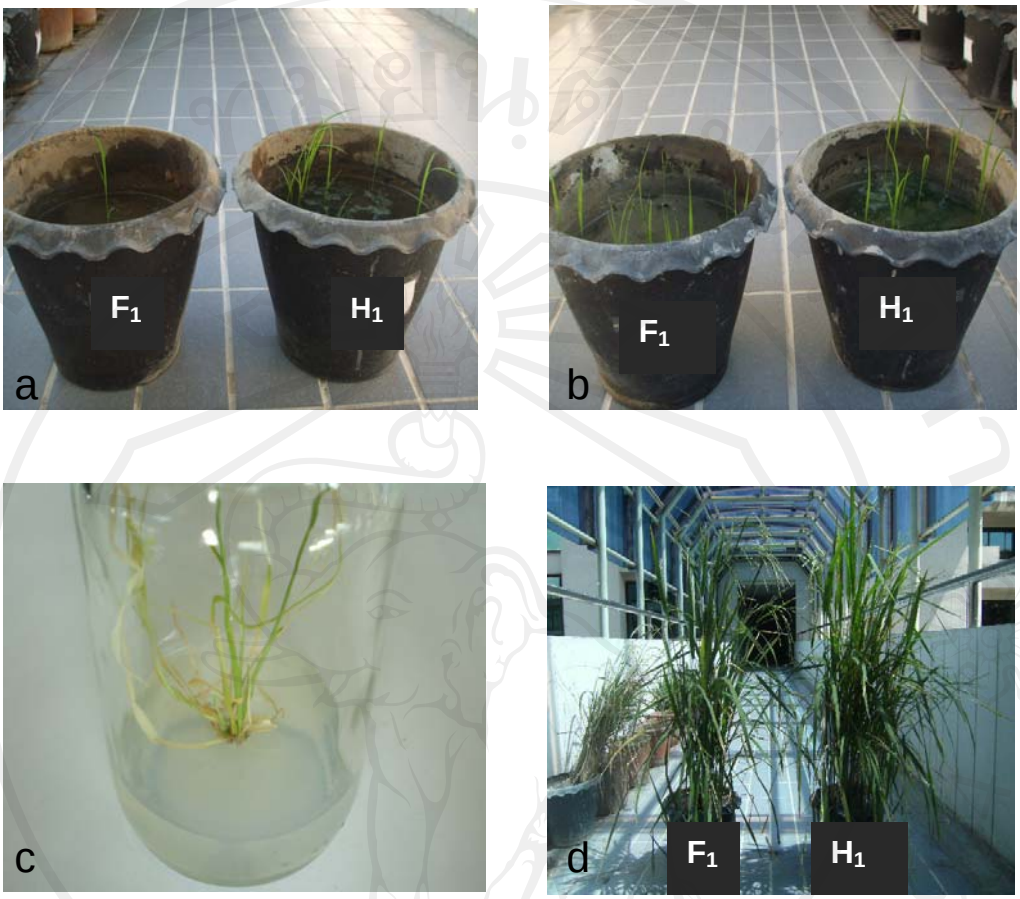


Figure 4.7 F1 hybrid seed germination compare with H1 synthetic seed (a). 4 days, (b) 14 days and synthetic seeds after culturing on LS media 14 days (c). and (d) after 60 days in soil.