Standardization of Culture Medium for Somatic Embryogenesis of Rice Var. MTU 7029

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Abstract

The aim of the study is to establish a routine procedure for high frequency plant regeneration from *in vitro* raised embryogenic callus of *indica* rice var. MTU 7029. In the present piece of work, various concentrations of surfactants [sodium hypochlorite (10 to 30%) and mercuric chloride (0.01 to 1%)] were tested and best concentration of surfactants was chosen according to the parameters like callus induction percentage and its growth as well as status of contamination. On the basis of available data of the said parameters, 0.1% mercuric chloride has shown best performance in all the aspect for surface sterilization of mature rice seeds for 5 minutes and the percentage of the contamination was also very less. The effect of synthetic auxin [2,4-D (0 to 4 mg L^{-1})], cytokinin [kinetin (0 to 1 mg L^{-1})], sucrose (0 to 50 g L^{-1}), agar (6-16 g L^{-1}) and growth medium (MS, N6 and LS) on callus induction were optimized to achieve high frequency plant regeneration from fresh embryogenic callus without further subculture. Results suggests that 2 mg L^{-1} 2, 4-D, 30 g L^{-1} sucrose, 8 g L^{-1} agar and MS basal medium were best for embryogenic callus induction. From the present piece of work an efficient protocol was developed for *in vitro* embryogenic callus formation.

1. Introduction

Rice forms an integral part of human history, tied to us in countless traditions and interwoven in the oldest religious rights. It is a monocot plant belonging to the genus *Oryza* under tribe *Oryzeae* in grass family Poaceae. This genus consists of 26 species (Khush, 1997), of which 24 are wild and two, i.e. *O. sativa* L. and *O. glaberrima* Steud are cultivated. Rice is the major food crop in Asia and the rest of the world, and thus, varieties with improved characteristics are desired. Using rice as a model, monocot system is critical for food safety, hunger eradication and poverty alleviation (Coffman et al., 2004). Plant cell and tissue culture are likely to continue to be of key importance in the application of molecular biology to crop improvement (Chowdhry et al., 1993). Somatic embryogenesis is a unique process in plants and it is of remarkable interest for biotechnological applications such as clonal propagation, artificial seeds and genetic engineering (Quiroz-Figueroa et al., 2006; Namasivayam, 2007). Precisely, when somatic embryogenesis is integrated with conventional breeding programs and molecular and cell biological techniques, it provides a valuable tool to enhanced genetic improvement of crop species (Quiroz-Figueroa et al., 2006). In rice, somatic embryogenesis is the most common regeneration pathway and has been obtained from roots, leaf bases of young seedlings, mature embryos, immature embryos, caryopses, microscopes, cell suspension, protoplast and young inflorescences (Kawata and Ishihara, 1968; Inoue and Maeda, 1980; Wernicke et al., 1981; Heyser et al., 1983; Abe and Futsuhara, 1984; Raghavan Ram and Nabors, 1984; Abe and Futsuhara, 1985; Chen et al., 1985; Abe and Futsuhara, 1986; Kavi Kishor and Reddy, 1986; Raina et al., 1987; Hartke and Lorz, 1989; Koetje et al., 1989; Chowdhry et al., 1993; Valdez et al., 1996a and b; Gairi and Rashid, 2004; Hoque and Mansfield, 2004; Meneses et al., 2005; Ge et al., 2006). Our interest in regeneration of *Oryza sativa* subspecies indica cv. MTU 7029, is a very popular high yielding variety and grown by small and marginal farmers of our country (India). It is very likely that this cultivar can be an important source for preparation of transgenic or genetically engineered rice. So all this aspects kept in mind, in the present piece of work a reliable and reproducible protocol was developed for *in vitro* callus culture from mature seeds of rice var. MTU 7029.

2. Materials and Methods

Healthy and disease free seeds of indica rice (*Oryza sativa* L.) cultivar MTU 7029 (Swarna) were collected from Genetics and Plant Breeding, Institute of Agricultural Sciences, BHU, Varanasi, India and the whole work was carried out in the Tissue Culture Laboratory, of the same Institute, in the year 2011-2012. The seeds were manually dehusked and various concentrations of surfactants [sodium hypochlorite (10 to 30%) and mercuric chloride (0.01 to 1%)] were tested and best concentration of surfactants was chosen based on callus induction percentage, growth of the calli and contamination percentage. For the standardization of callus induction medium, dehusked seeds were surface sterilized with 70% (v/v) ethanol for 60 sec followed by 0.1% mercuric chloride for 5 min and thoroughly rinsed five times in sterile distilled water. Surface sterilized seeds were blot dried with sterile Whatman No. 1 filter paper and aseptically cultured on Murashige and Skoog's medium (1962) supplemented with different concentrations of 2,4-D (0, 1, 2, 3 & 4 mg L^{-1}), kinetin (0, 0.25, 0.5 & 1 mg L^{-1}), 30 g L^{-1} sucrose and 8 g L^{-1} agar with a pH value of 5.7 before autoclaving. Again in the same medium, with 2 mg L^{-1} was tested with different concentrations of sucrose (0, 10, 20, 30, 40 & 50 g L^{-1}) and agar as a gelling agent [6, 8, 10 & 16 g L^{-1} (w/v)]. The pH of both media was adjusted to 5.7 before autoclaving. The cultures were incubated at 25±2°C in total darkness upto 45 days. In the another set of experiment, LS (Linsmaier and Skoog, 1965), MS (Murashige and Skoog, 1962) and N6 (Chu et al., 1975) media, were tested which was supplemented with 2 mg L^{-1} of 2,4-D, 30 g L^{-1} sucrose and 8 g L^{-1} agar with a pH value of 5.7 before autoclaving. In all the above mentioned set of experiment the induction of total (embryogenic plus nonembryogenic) and embryogenic calli were represented in percentages as well as the mean and standard errors were also calculated, fresh and dry weights were represented in the same way. All the glasswares and chemicals used in the present investigation were of Borosil Glass Works Limited and HiMedia Laboratories Pvt. Ltd. India respectively.

3. Results and Discussion

Sterilization is the major factor, which affects the tissue culture. Sodium hypochlorite and mercuric chloride used as surface sterilization agent, played an important role in seed germination. However, the data presented in Table 1 reveals the effect of surfactants used for surface sterilization of mature rice seeds var. Swarna for callus induction. Table 1 showed that at 15 and 20% of sodium hypochlorite callus induction frequency was 52 and 72% respectively but above to it at 30% the callus induction frequency was found to zero. The same trend was obtained from the study of callus growth. However, the concentrations above 15% of sodium hypochlorite no contamination was recorded in callus. The study regarding the use of $HgCl_2$ as surfactant suggested that upto 0.1% of $HgCl_2$ the callus induction frequency increased constantly but at 1% concentration of $HgCl_2$ it became zero. The growth rate at all the three used concentration was better, except at 1% but upto 0.1% $HgCl_2$ the contamination was observed in the seed. However, at 1% $HgCl_2$ the contamination percentage was nil. On the basis of available data of the said parameters, 0.1% mercuric chloride has shown best performance in all the aspect for surface sterilization of mature rice seeds for 5 minutes and the percentage of the contamination was also very less. So this concentration was chosen as surface sterilizing agent to proceed further in tissue culture experiments. Similar type of experiment was done by Li et al. (1992) and Noor et al. (2005), where they reported that sterilization of seeds with 45% (v/v) sodium hypochlorite for 30 minutes were effective. The data presented in Table 2 demonstrates the effect of 2,4-D and kinetin on *in vitro* callus induction from mature seed explants of indica rice cultivar Swarna (MTU 7029) on MS medium. 2 to 3 mg L^{-1} 2,4-D were found best and noted to have statistically at par result among the other combinations of 2,4-D and kinetin for total callus induction and percentage of callus induction. However, use of 2 mg L^{-1} 2,4-D showed maximum callus induction frequency (78%), total embryogenic calli (2.8) and also the percentage of embryogenic calli (28%); all these values were statistically significant compared to other used concentration. Further the study regarding fresh and dry weight of the calli (representing average of 3 calli replication^{-1}) case 4 mg L^{-1} 2,4-D (1.2 and 0.2 g) showed statistically significant result as compared to others followed by 3 and 2 mg L^{-1} 2,4-D (1, 1.1 g and 0.16, 0.19 g). Higher doses of kinetin along with 2,4-D was poor performer as compared to 2,4-D alone. Hence, in the present study to proceed further 2 mg L^{-1} 2,4-D was selected for callus induction purpose. The auxin

Table 1: Effects of surfactants for surface sterilization of rice seeds var. MTU 7029 for callus induction

Treatments	Callus induction frequency	Growth of the calli	Contamination (%)
Sodium hypochlorite (%)			
10	0	+++	90-95
15	70	+++	15
20	52	++	No
30	0	-	No
Mercuric chloride (%)			
0.01	66	++++	40
0.05	78	++++	27
0.10	*84*	++++	5
1.00	0	++	No

Table 2: Effect of 2,4-D and Kinetin on *in vitro* callus induction from mature seed explants of indica rice cultivar Swarna (MTU 7029) on MS medium

Concentration of PGR (mg L^{-1})		Total callus induction (Mean±SE)	Percentage of callus induction	Total embryogenic calli (Mean±SE)	Percentage of embryogenic calli	Fresh weight of callus (g) (Mean±SE)	Dry weight of callus (g) (Mean±SE)
2,4-D	Kinetin						
0	0	0±0	0	0±0	0	0±0	0±0
1	0	1.2±0.84	12	0±0	0	0.34±0.01	0.045±0.0033
2	0	*7.8±0.45*	78	*2.8±0.84*	28	1.0±0.014	0.160±0.007
3	0	*7.0±0.71*	70	1.6±0.55	16	1.1±0.010	0.19±0.0025
4	0	6.5±1.10	65	1.2±0.84	12	*1.2±0.008*	*0.2±0.007*
0	0.25	0±0	0	0±0	0	0±0	0±0
1	0.25	0.4±0.55	4	0±0	0	0.11±0.145	0.008±0.011
2	0.25	2.8±0.84	28	0.6±0.55	6	0.27±0.009	0.025±0.003
3	0.25	3.6±0.89	36	1.0±0.71	10	0.49±0.010	0.055±0.003
4	0.25	3.8±0.84	38	0.8±0.84	8	0.77±0.014	0.11±0.006
0	0.5	0±0	0	0±0	0	0±0	0±0
1	0.5	0±0	0	0±0	0	0±0	0±0
2	0.5	1.4±0.55	14	0±0	0	0.45±0.012	0.05±0.002
3	0.5	1.6±1.14	16	0.4±0.55	4	0.56±0.012	0.074±0.004
4	0.5	2.2±0.84	22	0.4±0.55	4	0.71±0.013	0.09±0.004
0	1	0±0	0	0±0	0	0±0	0±0
1	1	0±0	0	0±0	0	0±0	0±0
2	1	1.6±0.55	16	0±0	0	0.54±0.01	0.07±0.003
3	1	2.4±0.55	24	0.4±0.55	4	0.78±0.01	0.12±0.004
4	1	2.6±0.55	26	0.6±0.55	6	0.90±0.001	0.13±0.003
SEm±		0.41		0.30		0.02	0.002
CD (p=0.05)		0.81		0.59		0.04	0.005
CD (p=0.01)		1.08		0.78		0.06	0.007

(2,4-D or 2,4,5-T) in caryopses cultures of rice selectively stimulates epithelial cells of the scutellum to form somatic embryo as reported by Jones and Rost, (1989). Morita et al. (1999) reported that addition of adequate levels of synthetic auxins, such as 2,4-D into basal medium resulted in prolific callus formation from a variety of rice explants. The results of the present study also showed that the presence of 2,4-D in culture medium is critical for rice callus induction from mature seeds. Again Lutts (1996) observed that MS medium or callus induction medium if supplemented with 0.5 mg L^{-1} 2,4-D+1 mg L^{-1} NAA+1 mg L^{-1} BA showed maximum callus induction in the two japonica (I Kong Pao and Aiwu) and two indica cultivar (IR 2153 and Nona Bokra). In contrary with the present study, Karthikeyan et al. (2009) reported that among the used 8 concentrations of 2,4-D, 2.5 mg L^{-1} 2,4-D showed maximum callus induction and mean fresh weight as compared to the other concentrations. The observations of present study also found to be consistent with the earlier observation of Tyagi et al. (2007) as they have used 2 mg L^{-1} 2,4-D for the better callus induction of rice. Necrosis of callus was visible with increasing concentration of 2,4-D (≥ 4 mg L^{-1}, which also depends on the varietal character). In the present study, any callus that turned brown during subculture was found to be unsuitable for regeneration. With low concentration (≤1 mg L^{-1}) callus induction was poor. Growth regulator 2,4-D at the rate of 2 mg L^{-1} was suggested and proved to be best for callus induction in both the varieties i.e. Basmati-370 and Basmati-385 as demonstrated by Ullah et al. (2007).

Table 3 demonstrates the effect of culture media, sucrose and agar on *in vitro* callus induction from mature seed explants of indica rice cultivar Swarna. Among the three callus induction medium MS (8.2 and 82% respectively) and N6 (7.8 and 78% respectively) showed statistically at par results and significant with LS (7.6 and 76%) medium for the parameters like total callus induction and percentage of callus induction, but N6 medium showed more values for standard error as compared to other which represented the unstableness of the data. However in case of total embryogenic calli and their percentage in the entire three used medium were found to represent statistically at par results. Calli obtained from MS medium has maximum

Table 3: Effect of culture media, sucrose and agar on *in vitro* callus induction from mature seed explants of indica rice cultivar Swarna (MTU 7029)

Culture medium	Total callus induction (Mean±SE)	Percentage of callus induction	Total embryo-genic calli (Mean±SE)	Percentage of embryogenic callus induction	Fresh weight of callus (g) (Mean±SE)	Dry weight of callus (g) (Mean±SE)
LS	7.6±0.55	76	2.6±0.55	26	0.96±0.009	0.15±0.004
MS	*8.2±0.45*	82	*3.0±0.71*	30	*1.12±0.018*	*0.17±0.003*
N6	7.8±0.84	78	2.8±0.84	28	1.10±0.011	0.16±0.004
SEm±	0.18		0.2		0.004	0.0011
CD (p=0.05)	0.32		0.36		0.007	0.002
CD (p=0.01)	0.48		0.54		0.01	0.003
			Sucrose concentration (g L^{-1})			
0	0±0	0	0±0	0	0±0	0±0
10	3.0±0.71	30	0±0	0	0.64±0.006	0.09±0.005
20	5.8±0.84	58	1.2±0.45	12	0.87±0.010	0.13±0.006
30	*8.0±0.71*	80	*3.2±0.84*	32	*1.17±0.015*	*0.18±0.005*
40	6.8±0.45	68	2.6±0.55	26	0.91±0.010	0.13±0.008
50	6.6±0.55	66	2.2±0.84	22	0.52±0.012	0.07±0.004
SEm±	0.17		0.16		0.0032	0.0015
CD (p=0.05)	0.29		0.27		0.0054	0.0025
CD (p=0.01)	0.43		0.40		0.0079	0.0037
			Agar concentration (g L^{-1})			
6	1.8±1.10	18	0.4±0.55	4	0.93±0.02	0.15±0.004
8	*7.8±0.84*	78	*3.2±0.84*	32	*1.19±0.01*	*0.2±0.0045*
10	7.4±0.55	74	2.8±0.84	28	1.00±0.02	0.145±0.004
16	0.4±0.55	4	0±0	0	0±0	0.006±0.009
SEm±	0.22		0.18		0.004	0.0017
CD (p=0.05)	0.39		0.32		0.007	0.0029
CD (p=0.01)	0.58		0.48		0.01	0.0044

fresh (1.12 g) and dry weights (0.17 g) as compared to the other two medium and represented statistically significant values. On the basis of results among the three medium MS medium was selected as a standard medium for conducting the next experiment. The data regarding different concentrations of sucrose (0 to 50 g L^{-1}) used in the MS medium supplemented with the 2 mg L^{-1} 2,4-D presented in the Table 3.30 g L^{-1} sucrose has shown statistically significant result in all the studied parameters. Hence this concentration of sucrose was screened out for further experimentation. However, in the presence of high sucrose concentration (above 40 g L^{-1}), inhibition of callus induction was observed in mature rice seeds, possibly due to the osmotic stress resulting from high concentration of sucrose in the medium. This result is in agreement with the reports of Kishor and Reddy (1986); Garin et al. (2000) and Lee et al. (2002), who have shown the effects of osmotic potential affects the growth and morphogenesis during somatic embryogenesis. Besides all these aspects, the concentration of agar in the medium was another critical factor for callus induction. So while using different concentrations of agar (6 to 8 g L^{-1}) in the medium (MS) supplemented with 2 mg L^{-1} 2,4-D and 30 g L^{-1} it was observed that 8 and 10 g L^{-1} agar in the medium were found optimum for total callus induction and embryogenic callus induction and the values were statistically significant also. But while measuring fresh and dry weights of the calli, 8 g L^{-1} agar in the medium showed maximum value and those were statistically significant. High humid condition was formed while used agar concentration was less in the medium which repressed the callus growth and development as well as repressed the shoot regeneration frequency from embryogenic calli as reported by Tsugawa and Suzuki (2000). Zhao et al. (1999) reported that rice callus induction is depending on the type of callus medium used; he used three basal medium, LS, MS and N6, supplemented with 3.0 mg L^{-1} of 2,4-D and 30 g L^{-1} of sucrose for comparison. Khaleda and Al-Forkan (2006) demonstrated that dehusked seeds from five deepwater rice when placed in two basal media MS and LS, supplemented with 2 mg L^{-1} 2,4-D, 0.1% CH, 0.5 mg L^{-1} BAP and 0.5 mg

L^{-1} kinetin on the basis of the requirement and also 30 g L^{-1} sucrose added and media were solidified with 0.8% (w/v) agar, the growth of the callus is very good.

4. Conclusion

In short, frequencies of the embryogenic callus induction derived from matured rice seeds were tested in this study depended on the concentrations of 2,4-D, sucrose, agar and growth medium. From the present piece of work an efficient protocol was developed for *in vitro* embryogenic callus formation of rice cv. MTU 7029. The present protocol provides a rapid regeneration system, which could be conveniently used for producing genetically modified plants of rice.

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