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RESEARCH NOTE

Effect of sorbitol concentration on regeneration of embryogenic calli in upland rice varieties (*Oryza sativa* L.)

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Abstract This study describes the impact of sorbitol on plantlets regeneration frequency (PRF) of four rice cultivars (*japonica*, *Oryza sativa* L.) both of which mature and immature embryo-derived calli were investigated. The variance analysis results showed that PRF of the three elite upland rice cultivars, Handao297 (HD297), Handao502 (HD502), Handao65 (HD65) and one lowland rice cultivar Zhongzuo93 (ZZ93) were significantly increased with addition of appropriate amount of sorbitol in culture media. Supplementing appropriate sorbitol in the media of a continous culture from induction and maintenance to regeneration for mature embryo-derived calli could improve PRF dramatically, originally from 27.6% up to a maximum of 71.8%. Especially to low regenerative capacity (LRC) cultivar HD65, the PRF was increased over 7-fold (from 9.7% to 74.0%). The optimum concentrations of sorbitol for calli induction, subculture and differentiation were 5, 20 and 40 g/l, respectively. Adding sorbitol, only in maintenance media at concentration of 20 g/l, also enhanced the PRF greatly in all the cultivars from 27.6% to 43.3%. Similar results were observed when incorporating with maltose in regenerating media both in immature and mature embryo-derived calli. The optimal concentration was 25 g/l sorbitol + 20 g/l maltose and 20 g/l sorbitol + 25 g/l maltose, respectively. HD297 appeared to be the most responsive genotype compared to other cultivars in PRF, 99.2% in immature embryo-derived calli and 76.8% in mature embryo-derived calli. The results and relevant conclusions might be valuable to establish an efficient plant regeneration system from somatic embryogenesis culture in upland rice.

Keywords Sorbitol · Plantlet regeneration frequency (PRF) · Embryogenic callus · Upland rice cultivar

Abbreviations

2, 4-D	Dichlorophenoxyacetic acid
6-BA	6-Benzylaminopurine
MS	Murashige and Skoog (1962)
NAA	α-Naphthalene acetic acid

Introduction

Water scarcity in agricultural production is becoming a serious worldwide issue. It has been widely accepted that

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new crop cultivars with potential of strong drought tolerance can solve or improve this problem. Upland rice is a type of rice that is sown in dry lands and grown in rain-feeding or limited irrigation condition. During its whole life cycle, no coverage of a water layer is required in fields, which saves plenty of water and lessens water pollution (Gupta and O'Toole 1986; Yadav et al. 1997). Recently, a group of elite new upland rice cultivars such as Handao297 (HD297), Handao502 (HD502), Handao65 (HD65), Handao277 etc. have been developed in China Agricultural University and released officially to the rural areas in the North of China successfully. Not only do these cultivars show strong drought tolerance (Yang et al. 2005) but also high yield potential (Bouman et al. 2006). Meanwhile, more and more of these cultivars have been used for researches in tissue culture, transgenic plant technology (La et al. 2003) and other aspects. However, it was found that the plantlet regeneration frequency (PRF) of calli was low normally and very different in cultivars and therefore the application of upland rice cultivars in tissue culture and transgenic plant technology will be restricted seriously.

Plant tissue culture and transgenic technique based on calli have proved to be powerful tools to facilitate breeding process. It is well known that successful application of plant tissue culture depends on, to a great extent, establishment of an effective calli regeneration system. In crops, a number of factors had been examined to determine their effects on improving calli regeneration frequency, such as genotypes, hormonal composition, carbohydrate sources, culture methods, and water-deficient treatment, etc. (Asad et al. 2001; Greg and Roberta 1991; Masayoshi and Takayasu 1992; Yoshito et al. 1994; Masayoshi et al. 1996; Kavi 1987; Wang et al. 1999; Jain et al. 1996; She et al. 2002). Some of the results supported that although genotype was a main restricting factor for regeneration frequency, the alteration of hormones, carbohydrate sources, or additional supplementing of some chemicals can increase regeneration frequency dramatically. These measures will overcome genotype constraint to some extent and improve regeneration frequency universally. It was reported that addition of sorbitol into regeneration media promoted regeneration frequency considerably for apple and loquat (Lin and Chen 1997; Ma and Wang 1996). Brad and Roberrt (1993) recommended that sorbitol could work as primary carbon source to improve regeneration frequency of embryogenic calli in maize. In paddy rice, it was also well documented that supplementing of sorbitol in different concentration into calli induction media (Gao et al. 2005; Huang and Huang 1999; Huang et al. 1999; Wu et al. 2002), proliferation media (He and Wang 1991; Jain et al. 1995; Kavi et al. 1987; Liu et al. 2002; Ma et al. 2004; Wang et al. 2001; Zhang et al. 2004) and regeneration media (Liu et al. 2004; Masayoshi and Takayasu 1992; Wu et al. 2002) could improve calli regeneration frequency significantly. In upland rice especially, La et al. (2003) in our lab found that jointly adding sorbitol 25 g/l with maltose 20 g/l in regeneration media increased PRF tremendously of gene *bar* transformed immature embryo-derived calli in upland rice cultivars HD297 and HD502 from 19.5% to 99.3% and 17.2% to 85.1% respectively, which led to the herbicide Basta-resistant transgenic plants to be obtained successfully. Therefore, it is quite necessary to explore further the impacts of sorbitol on PRF of upland rice.

In this paper, we used immature and mature embryos of four rice cultivars including three upland cultivars to test the effect of sorbitol added in the media of induction, subculture and regeneration culture on PRF of calli.

Materials and methods

Explants: immature embryo and mature embryo of four rice cultivars (*japonica*, *Oryza sativa* L.)

Explants: four rice cultivars (*Japonica*, *Oryza sativa* L.) were investigated and the mature and immature embryos of the three upland rice cultivars HD297, HD502 and HD65 and mature embryos of a typical lowland rice Zhongzuo93 (ZZ93) were tested in this study. HD297, HD502 and HD65 were provided and developed in China Agricultural University and officially released to farmers in 2003 and ZZ93 was obtained from China Academy of Agriculture Science and adopted by farmers in 1990s in North of China. HD297 and ZZ93 have been used as control respectively in China National Upland and Lowland Rice Region Trial at present as well.

Preparation of explants

Dehusking and sterilization of seeds: seeds of four *Oryza sativa* varieties were carefully washed with

70% ethanol (v/v) for 3 min then sterilized with 0.1% mercury chloride for 10 min with shaking. Then they were soaked into 10% sodium hypochlorite (v/v) for 20 min with shaking followed by three times rinses in sterile distilled water. Seeds were then placed on solid MB basal media for calli induction with 40 seeds per Petri dish.

Culture media

Immature embryo The basal medium for induction and regeneration used in present study was MB (MS Macro elements, B_5 Micro elements, B_5 Organic elements, MS $FeSO_4$–Na_2EDTA). The induction media is MB supplemented with 2 mg/l 2,4-D, which was modified according to the composition of MS (Murashige and Skoog 1962) and B_5 (Gamborg et al. 1968) medium. The different media is MB adding Myoinositol 0.1 mg/l, Caseinhydrolysate 0.3 mg/l, 6-BA 1 mg/l, KT 2 mg/l and NAA 0.1 mg/l, then we incorporated sorbitol with maltose for immature embryo culture (Table 1), which was named Exp 1.

Mature embryo In order to obtain better culture effects, both induction and proliferating media were MB basal medium containing 2,4-D (2 mg/l). Differentiation medium was basal medium with 6-BA (1 mg/l) and NAA (1 mg/l). Sorbitol was added to the media at various concentrations at different culture period (Table 2). Additionally, in order to investigate the effect of sorbitol on plant regeneration and somatic embryogenesis, various sorbitol and maltose concentrations in combination were used on regeneration (Table 2).

These media were solidified with 0.3% phytagel (Sigma). The pH was adjusted to 5.8 with one Normal HCl or one Normal NaOH before the addition of phytagel prior to autoclaving at 121°C for 20 min.

Table 1 Composition of differentiation media of immature embryo-derived calli culture in four varieties of rice (Exp 1)

Supplement of media	Concentration (g/l)		
	MB1 (CK)	MB2	MB3
Sorbitol	0	15	25
Maltose	30	30	20

Callus induction, subculture and regeneration

Embryogenic calli were induced from scutellum of the immature and mature seeds of four varieties for 15-days culture on induction medium and then proliferated on subculture medium at 20-days intervals twice. After 40-days subculture, calli were placed on regeneration medium with 10 calli each conical flask. Cultures were maintained at ±26°C in darkness for the induction of adventitious buds and somatic embryogenesis and under a 16-h photoperiod from cool white fluorescent lamp (55 μmol/m^2/s) for the rooting of shoots and the embryo development.

Observation and statistical analysis

The appearance and proliferation of calli were observed and recorded every 5 days. After 25–30 days, 10 dishes were randomly chosen for statistical analysis. The rate of callus initiation (RCI) and plantlet regeneration frequency(PRF) were analyzed by SAS ANOVA program, Duncan model, variance analysis and multiple comparisons (Du et al. 1999).

Results

Callus induction from immature embryo

After 6–10 days culture, light yellow, compact calli were induced from immature embryo of each variety. Twenty days later, the diameter of calli was about 0.6–1.2 cm. It was observed that HD297 generated more embryogenic calli. Whereas, calli of HD65 and HD502 were yellow and wet. This might be caused by the difference of genotypes. Although there were no significant differences in induction frequency of calli among various media, it was noticed that HD297 was more responsive to various media than other varieties (data not shown).

Regeneration of rice plantlets from immature embryo-derived calli

Regeneration studies were conducted on 35-day-old calli. From Table 3, we observed that all three varieties gave good response on MB3 medium (99.2%, 88.5%, 18.5% respectively) which contained 25 g/l sorbitol and 20 g/l maltose, and showed poor performance on

Table 2 Concentrations of sorbitol in mature embryo culture in four varieties of rice

Experiment	Treatment		Induction	Subculture	Regeneration
Exp 2	2.1 Induction	I (CK)	0	0	0
		II	5	0	0
		III	10	0	0
		IV	20	0	0
	2.1 Subculture	I (CK)	0	0	0
		II		10	0
		III		20	0
		IV		40	0
	2.3 Regeneration	I (CK)	0	0	0
		II			10
		III			20
		IV			30
		V			40
Exp 3		I (CK)	0	0	0
		II	5	20	40
		III	10	10	20
		IV	10	20	40
					Sorbitol + Maltose
Exp 4		I (CK)	0	0	0 + 45
		II			10 + 35
		III			20 + 25
		IV			30 + 15
		V			40 + 5

sorbitol free medium MB1. Among varieties, HD297 gave better regeneration response on all three media (63.6–99.2%) and HD65 gave poor performance (7.0–19.7%), which showed that genotype affected the PRF significantly. Whereas, for low regenerative capacity (LRC) variety HD65, the highest PRF (19.7%) was obtained on MB2, 4.4-fold higher than that of CK (7.0%), which showed that addition of sorbitol on regeneration media promoted PRF significantly.

Induction of mature embryo (Exp 2.1)

After 15-days culture, calli were induced from all varieties. After 20 days, the size of calli was about 10 mm. It was observed that the HD297 and HD502 showed better callus appearance, which was whiter and more compact (Fig. 1a). Calli of HD65 and ZZ93 were smaller, but their induction frequencies (Table 4) were higher than the other two varieties; HD65 gave the highest induction frequency (59.5%). However, there were no significant differences among induction frequencies on various media. In fact, on sorbitol free medium, average induction frequency was the highest. This signifies that addition of sorbitol had no beneficial effect on callus induction frequency. However, callus growth was improved compared with CK. It is generally recognized that callus status would affect PRF greatly. So the effects of sorbitol supplementing in induction medium were investigated later at regenerating stage.

Regeneration of mature embryo

Effect of sorbitol concentrations on PRF in induction medium (Exp 2.1)

PRF studies were also conducted on 35-day-old calli. While there was no substantial distinction in medium treatment, there were significant differences among varieties as shown in Table 5. With supplement of various sorbitol concentrations in induction medium, HD297 gave the highest PRF 49.8%, and HD65 gave

Table 3 Effect of incorporation of sorbitol and maltose on PRF of calli derived from immature embryos in three varieties of rice (Exp 1)

Media	Concentration (g/l) Sorbitol + Maltose	Plantlet regeneration frequency (%)			Average (%)	*P* value of treatment effects
		HD297	HD502	HD65		
MB1	0 + 30 (CK)	63.6 ± 22.5 b	47.6 ± 15.9 b	7.0 ± 6.8 b	39.4 B	0.0001**
MB2	15 + 30	71.6 ± 20.2 b	80.6 ± 13.9 a***	19.7 ± 12.2 a	57.3 A	
MB3	25 + 20	99.2 ± 2.4 a	85.5 ± 14.9 a	18.5 ± 9.2 a	67.7 A	
Average (%)		78.1 A	71.2 A	15.1 B		
P value of varieties effects		0.0001**				
P value of varieties × Medium treatments effects		0.0001**				
P value of model testing		0.0001**				

*Comparison significant at the 0.05 level

**Comparison significant at the 0.01 level; *, if there is, at 0.05 level

***Duncan's multiple comparisons for variables

The footnotes and the meanings above also be used and same in Tables 4–9 below

a, b, c refer to differences of differentiation frequency on different media for each varieties; A, B, C refer to differences of differentiation frequency among varieties and differences of differentiation frequency on different media. Means with the same letter are not significantly different. Means with the same letter are not significantly different

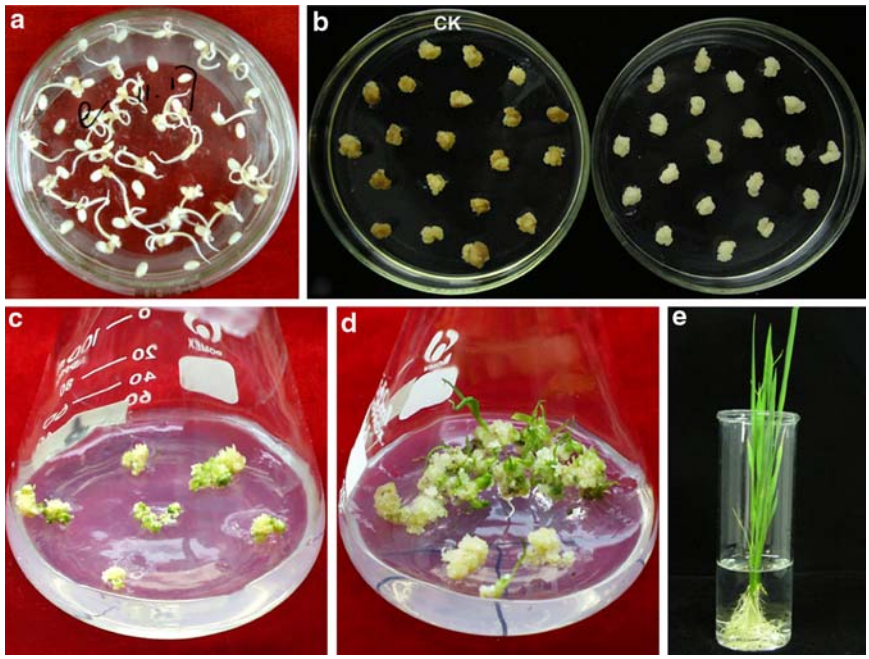

Fig. 1 Plant regeneration from mature seed-derived embryogenic calli of upland rice cv. HD297. (**a**) Callus induction from mature seeds after a 20-days culture on callus induction medium; (**b**) embryogenic calli with somatic embryos from mature seeds after twice subculture (40d) on proliferating medium, sorbitol-free (CK) and 20 g/l sorbitol (Right); (**c**) embryogenic callus showing green spots 25–30 days after culture on regeneration medium; (**d**) shoot regeneration from embryogenic calli approximately 35–40 days after culture on regeneration medium; (**e**) plantlets with healthy root 7 days after transfer onto 1/2 MS basal medium without plant growth regulators

Table 4 Effect of sorbitol concentrations on induction frequency of mature embryo-derived callus in four varieties of rice (Exp 2.1)

Sorbitol concentration (g/l)	Callus induction frequency (%)				Average (%)	*P* value of treatment of effects
	HD297	HD502	HD65	ZZ93		
0 (CK)	50.3 ± 7.4 a	52.5 ± 7.3 a	63.0 ± 7.5 a	59.1 ± 19 a	56.2 A	0.1257
5	33.9 ± 14.4 b	22.8 ± 6.1 b	61.3 ± 12.9 a	38.4 ± 0.013 a	42.0 A	
10	30.9 ± 18.3 b	28.0 ± 10.0 b	68.8 ± 12.8 a	40.5 ± 0.012 a	42.1 A	
20	21.2 ± 13.7 b	43.9 ± 6.0 b	45.0 ± 25.1 a	48.2 ± 0.012 a	40.3 A	
Average (%)	34.1 C	36.8 C	59.5 A	50.2 B		
P value of varieties effects	0.0046**					
P value of varieties × Medium treatments effects	0.5661					
P value of model testing	0.0510					

Table 5 Effect of Sorbitol concentrations on PRF of mature embryo-derived calli in induction media in four varieties of rice (Exp 2.1)

Sorbitol concentration (g/l)	Plantlet regeneration frequency* (%)				Average (%)	*P* value of treatment of effects
	HD297	HD502	HD65	ZZ93		
0 (CK)	43.7 ± 31.1 a	25.5 ± 6.1 a	2.5 ± 2.5 a	37.3 ± 13.0 a	27.3 ± 22.0 A	0.8320
5	55.6* ± 29.0 a	35.1** ± 3.7 a	0.5 ± 0.4 a	39.4 ± 5.1 a	32.7 ± 24.5 A	
10	54.8 ± 34.3 a	36.7 ± 7.6 a	2.4 ± 3.2 a	30.4 ± 16.5 a	31.1 ± 25.7 A	
20	45.0 ± 37.4 a	27.2 ± 10.8 a	3.1 ± 4.8 a	29.2 ± 34.7 a	26.1 ± 27.2 A	
Average (%)	49.8 ± 28.9 A	31.1 ± 8.2 B	2.1 ± 2.9 C	34.1 ± 18.0 B		
P value of varieties effects	<0.0001**					
P value of varieties × Medium treatments effects	0.9974					
P value of model testing	0.0130**					

the lowest response 2.1%. Sorbitol, at 5 g/l, enhanced the PRF in three varieties except HD65.

Effect of sorbitol concentrations on PRF in subculture medium (Exp 2.2)

Marked differences were found in both varieties and media effects (Table 6). The optimal sorbitol concentration was 20 g/l, at this concentration the PRF reached 43.4%, which was 56.9% higher than CK. Similar to addition in induction medium, among the varieties tested HD297 gave the best regeneration response of 44.6%, HD65 the poorest at 19.0%. Significant differences were also observed in interaction between varieties and medium treatments. Compared with HD502 and ZZ93, HD297 and HD65 were more responsive to sorbitol concentrations in

Table 6 Effect of sorbitol concentrations on PRF of mature embryo-derived callus in subculture media in four varieties of rice (Exp 2.2)

Sorbitol concentration (g/l)	Plantlet regeneration frequency (%)				Average (%)	*P* value of treatment of effects
	HD297	HD502	HD65	ZZ93		
0 (CK)	33.8 ± 14.8 b	26.7 ± 10.1 b	17.5 ± 2.5 b	32.5 ± 2.5 b	27.6 B	0.0001**
5	27.7 ± 19.6 b	20.0 ± 10.0 b	20.0 ± 0.0 b	40.0 ± 5.0 b	26.9 B	
10	47.0 ± 3.3 b	30.0 ± 0.0 b	10.0 ± 0.0 c	42.5 ± 5.0 a	32.4 B	
20	70.0**± 0.00 a	35.0 ± 0.020 a	42.5 ± 5.0 a	40.0 ± 5.0 b	43.3 A	
Average (%)	44.6 A	27.9 B	19.0 C	38.8 A		
P value of varieties effects	<0.0001**					
P value of varieties × Medium treatments effects	0.0014**					
P value of model testing	<0.0001**					

subculture medium, the regeneration frequency varying from 27.7% to 70.0% and 10.0% to 42.5% respectively. Whereas, compared with lowland rice variety ZZ93, the three upland rice varieties gained highest PRF at 20 g/l sorbitol concentration. Notably, to LRC variety HD65, the PRF was increased sharply from 10.0% to 42.5%. What's more, obvious enhancement of the appearance of calli generated on media containing sorbitol was observed, light yellow and more vigorous than that of CK (sorbitol free medium), especially after twice subculture (Fig. 1b).

Effect of sorbitol concentrations on PRF in regenerated medium (Exp 2.3)

As shown in Table 7, HD297 still gave the highest regeneration response and HD65 the poorest. The difference in the PRF in sorbitol concentrations was not significant. However, PRF of four varieties were enhanced with the increase of sorbitol concentration, varying from 24.4% to 36.3%, especially for HD297 and HD65 which increased from 49.0% and 1.1% to 76.8% and 20.1% at 30 and 40 g/l sorbitol concentration respectively. There were significant differences among varieties, HD297 gave the highest regeneration response 66.7%, while HD65 gave the lowest at 9.4%.

Effect of sorbitol concentrations on PRF when supplementing at entire culture period (Exp 3)

According to the results from Exp 2, sorbitol concentrations 5, 20 and 40 g/l were the optimum in induction, subculture and regeneration media respectively. The treatments of Exp 3 were designed based on Exp 2 to investigate the optimal incorporation of sobritol supplementing at each culture stage (Table 8). There were significant differences of PRF in both varieties and media as well interaction between variety and medium. Particularly, the variation of PRF among media was more considerable compared with that of varieties, which was promoted greatly in succession from 27.6% (CK) to the highest 71.8% averagely. To all the four varieties, the highest PRF with over 70% was all gained not at 5–20–40 g/l but 10–20–40 g/l sorbitol concentration level without exception, which indicated that the optimal concentration for the whole culture period was not a simple combination of three optimum concentrations of each culture stage, implying a possible interaction among the three culture processes. Furthermore, we found in Exp 3 that to LRC HD65, the PRF was increased tremendously from 9.7% (CK) to 74.0% at the level 10–20–40 g/l which almost reached to the highest 76.8% of HD297 in Exp 2.3.

Table 7 Effect of sorbitol concentrations on PRF of mature embryo-derived callus in regenerated media in four varieties of rice (Exp 2.3)

Sorbitol concentration (g/l)	Plantlet regeneration frequency (%)				Average (%)	*P* value of medium treatment effects
	HD297	HD502	HD65	ZZ93		
0	49.0 ± 7.4 b	17.2 ± 12.5 a	1.1 ± 1.9 b	30.4 ± 16.5 a	24.4 A	0.6915
10	62.0 ± 13.5 a	20.6 ± 5.9 a	2.5 ± 2.5 b	37.3 ± 13.0 a	30.6 A	
20	55.7 ± 2.4 a	22.2 ± 11.1 a	12.2 ± 6.9 a	29.2 ± 34.7 a	29.8 A	
30	76.8**± 4.2 a	20.4 ± 8.1 a	12.2 ± 1.9 a	37.3 ± 13.0 a	34.7 A	
40	60.0 ± 25.0 a	25.5 ± 6.1 a	20.1**± 6.8 a	39.4 ± 5.1 a	36.3 A	
Average (%)	60.7 A	20.4 C	9.4 D	34.7 B		
P value of varieties effects	<0.0001**					
P value of varieties × Medium treatments effects	0.4414					
P value of model testing	<0.0001**					

Table 8 Effect of sorbitol concentrations on PRF of mature embryo-derived callus at entire culture period in four varieties of rice (Exp 3)

Concentration of sorbitol (g/l) Induction–subculture–regeneration	Plantlet regeneration frequency (%)				Average (%)	*P* value of treatment effects
	HD297	HD502	HD65	ZZ93		
0–0–0 (CK)	43.3 ± 17.3 c	21.5 ± 7.8 c	9.7 ± 7.5 c	35.8 ± 5.1 c	27.6 D	<0.0001**
5–20–40	49.5 ± 3.7 b	23.3 ± 3.8a b	33.4 ± 5.2 b	59.9 ± 3.4 b	41.6 C	
10–10–20	61.1 ± 7.8 a	61.7 ± 6.3 a	26.7 ± 1.2 b	62.3 ± 2.5 b	53.0 B	
10–20–40	70.5 ± 2.4 a	72.5** ± 13.9 a	74.0** ± 4.0 a	70.0 ± 1.7 a	71.8 A	
Average (%)	56.1 A	44.8 B	36.0 C	57.0 A		
P value of varieties effects	<0.0001**					
P value of varieties × Medium treatments effects	<0.0001**					
P value of model testing	<0.0001**					

Effect of incorporation of sorbitol and maltose on PRF in regenerating medium (Exp 4)

The effects of incorporating appropriate concentrations of sorbitol and maltose on PRF was investigated in Exp 4. As shown in Table 9, there were significant differences among media, varieties and interaction between media and variety. The difference was especially remarkable on media; the highest PRF was 64.8%, CK the lowest at only 19.1%. Among varieties, the highest was ZZ93, which gave the best response (59.2%) followed by HD297 (41.3%), HD65 (36.8%) and HD502 the lowest at 22.4%. All varieties reached highest PRF,

Table 9 Effect of incorporation of sorbitol and maltose on PRF of mature embryo-derived callus in regenerated media in four varieties of rice (Exp 4)

Maltose concentration (g/l)	Sorbitol concentration (g/l)	Plantlet regeneration frequency (%)				Average (%)	*P* value of treatment effects
		HD297	HD502	HD65	ZZ93		
45	0	26.7 ± 7.6 c	7.3 ± 4.8 c	9.2 ± 5.2 c	33.3 ± 6.3 b	19.1 ± 12.7 E	<0.0001**
35	10	23.3 ± 1.4 d	25.5 ± 1.6 d	26.7 ± 8.0 b	60.0 ± 6.6 a	27.5 ± 22.8 D	
25	20	70.0** ± 5.0 a	45.0** ± 5.2 a	74.2** ± 10.9 a	70.0** ± 5.0 a	64.8 ± 13.2 A	
15	30	53.3 ± 5.2 b	39.8 ± 2.3 a	40.0 ± 7.5 b	70.0** ± 7.5 a	50.8 ± 13.9 B	
5	40	33.3 ± 5.2 c	20.0 ± 0.0 b	33.8 ± 1.3 b	62.5 ± 2.5 a	37.4 ± 16.4 C	
Average (%)		41.3 ± 18.8 C	22.4 ± 18.4 B	36.8 ± 22.9 A	59.2 ± 14.8 B		
P value of varieties effects		<0.0001**					
P value of varieties × Medium treatments effects		<0.0001**					
P value of model testing		<0.0001**					

average 64.8% at 25 g/l maltose + 20 g/l sorbitol, which was the optimal incorporation concentration to callus maintenance and differentiation. This result also was congruent with the optimal concentration in immature embryo culture (25 g/l sorbitol + 20 g/l maltose). Furthermore, we found that the regenerative response was obviously different among tested varieties. Compared with higher regenerative capacity varieties HD297 and ZZ93, to LRC varieties HD65 and HD502, the PRF were promoted sharply from 9.2% to 74.2% with over 7-fold increase and 7.3% to 45.0% with over 5-fold increase respectively.

The results presented here demonstrate that PRF was enhanced greatly in the presence of sorbitol in media of rice somatic callus culture. Especially when supplementing sobitol at each culture stage and in combination with maltose in differentiation media, the PRF of mature embryo culture were improved from 27.6% and 19.1% to71.8% and 64.8% respectively. In immature embryo culture, the PRF increased from 39.4% to 67.7% with incorporation of sorbitol and maltose. The optimum concentration of sorbitol at each culture stage was: 10–20–40 g/l; in differentiation culture it was 20 g/l sorbitol + 25 g/l maltose in mature embryo culture, 25 g/l sorbitol + 20 g/l maltose in immature embryo culture respectively; in subculture the best sorbitol concentration was 20 g/l in mature embryo culture.

Observation of regenerated plantlets

After 25–30 days culture on regenerated media, green spots was observed of all tested varieties. The calli of HD297 had better appearance than other varieties, and showed more green spots (Fig. 1c). Generally the test results concluded more in numbers and stronger in appearance of shoot differentiating from Exp 3 to Exp 4 (Fig. 1d). Regenerated plantlets of HD297 and HD502 were dark green, faster growing, more vigorous and strong, while those of HD65 and ZZ93 were weak, yellow and grew slowly (Fig. 1e). Plantlets regenerated were grown on hormone free half-salt MS media for rooting and subsequently transferred to soil.

Discussion

To our knowledge this is the first report that systematically investigated the effect of sorbitol concentrations on PRF of embryogenic callus in rice (*Oryza sativa* L.). The results presented here indicate

that supplementing sorbitol in culture media could enhance morphogenesis and increase PRF greatly. It signifies that sorbitol as an addition matter is a very important and positive factor to enhance PRF of somatic-derived calli in rice cultivars, specifically to LRC cultivars. Therefore the restriction of genotypes to PRF can possibly be altered effectively by adding sorbitol in continual culture period or cooperating with other sugars such as maltose in LRC cultivars tissue culture. However, further evidence from LRC cultivars is necessary to support this discovery.

High PRF is an essential requirement for plant transformation technology. This means this research may be useful for tissue culture of other rice genotypes to obtain higher PRF, particularly to multiple subculture periods. Additionally, a number of studies showed that immature embryo has better regenerative capacity than mature embryo, while mature embryo is widely used in plant transformation because of easy handling and availability. In current research, with addition of sorbitol in regenerating medium, the PRF of mature embryo was stimulated sharply and reached comparable frequency to that of immature embryo.

Sorbitol, a six-carbon alcohol, with mannitol is commonly regarded as an osmotic regulator. Some papers were of the view that sorbitol can only play an osmotic agent role, it can not be metabolized by calli (Wang et al. 1999), while others regarded that sorbitol can also act as energy or carbohydrate (Brad and Roberrt 1993; Kavi et al. 1987; Masayoshi and Takayasu 1992). Kumria et al. (2001) suggested that GUS activity was enhanced greatly by using appropriate incorporation of sorbitol and maltose in co-cultivation medium. In addition, the accumulation of sugars in response to applied stress conditions is also quite well documented (Swarup et al. 1991; Wang et al. 1999; Yoshito et al. 1994).

In this study, sorbitol concentrations must be increased to an optimal range with the growing of calli thus reflecting the test results (5, 20, 40, 10–20–40 g/l). Consequently the optimal sorbitol concentration changes according to the variation of culture conditions. We suggest that there is a complex internal mechanism of sorbitol responsibility that caused remarkable interactions in different culture stages, during the entire culture period sorbitol might act as an osmotic agent at an early stage, while tend to be a carbon source later. Yoshito et al. (1994) also suggested that loss of plant regeneration in long-term cultures is caused, at least in part, by specific cultural conditions and not by genetic changes. Furthermore, in Exp 4, adding further amount of sorbitol (above 20 g/l) in regenerating medium therefore led to the decline of PRF as indicated in test results. This possibly signifies that interaction occurred between sorbitol and maltose, and that sorbitol plays a similar role to maltose, regarding the dual role of sugar as carbon and osmotic source in shoot regeneration. As to the LRC cultivars, the PRF was increased dramatically at optimal sorbitol concentration, which possibly implies that the LRC cultivars are more fastidious to osmotic pressure. However, all the published papers and comments, up to now, cannot explain our results in this report. The mechanism of how sorbitol works in regeneration system in rice remains to be clarified necessarily in our next studies.

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