INFLUENCE OF PHYSIOLOGICAL STATE OF FRESH AND DRY EXPLANTS ON IN VITRO CULTURE RESPONSE IN RICE (*Oryza sativa* L.)

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ABSTRACT

Fresh or dry naked seed-explants of different maturity were cultured in vitro and their responses were investigated on a medium containing 2mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) as growth regulator. It was found that the explants at lower maturity had lower rates of callus induction and lower production of callus masses than those with the more matured ones in the original cultures. However, the quality of the callus induced from the younger explants was much better since the ratio of somatic embryogenic to non somatic embryogenic callus produced by younger explants was higher and grew faster in the subsequent proliferation cultures. In comparison with fresh young seed-explants and dry ones at the same maturity, dry ones generally responded better in terms of the rate of callus induction and the quality of callus. The strategy of using immature naked seed-explants for obtaining more embryogenic callus was highly effective especially for those varieties with relatively poor in vitro culture response.

Key words: *Rice, explant, physiological state, callus, maturity, embryogenic.*

INTRODUCTION

Development of a high efficient *in vitro* plant regeneration system is prerequisite for improvement of the crop by many modern biotechnological means such as breeding using somaclonal variation and genetic transformation. In rice, many factors affecting *in vitro* culture response have been investigated intensively due to its important position in staple food production and the impact of breeding. These factors including medium composition such as basal medium (Chu *et al*. 1975; Alam *et al*. 1994), hormone (Heyser *et al* 1983; Chen *et al*. 1985), additives to the medium (Bajia and Rajam, 1995; Yang *et al* 1999a), carbon source (Alam *et al*. 1994; Jain *et al*. 1997), genotype (Abe and Futsuhara 1986; Khana and Raina 1998), explant material (Kishor and Reddy 1987), culture age (Amarasinghe and Yang 2005), physical factors during the culture period (Yang and Jian 1996) and strategies of culture manipulation (Enoue and Maeda 1981; Zheng *et al*. 2005).In rice *in vitro* cultures, naked seeds were used most frequently as explants for inducing callus because they are available year round, easy to disinfect and have generally higher regeneration potential of the callus than that from other origins such as leaves, shoots and roots. The aim of this study was to investigate the effects of physiological state of the naked seed-explants on rice *in vitro* cultures.

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MATERIALS AND METHODS

Rice varieties and explants

Three popular indica varieties with good cooking quality, Qimiaoxiang, Jingxian 93 and Xiyezhan, and one indica rice variety, Qiuguiai 11, and one japonica variety, Taipei 309, both with good *in vitro* culture response (Yang *et al*. 2005; Yang *et al*. 1999b) were used in this study. These varieties were cultivated in pods in a greenhouse with normal care. The first three varieties were cultivated as early- and late-season in a year but the later two varieties were cultivated only as late-season due to their requirement of short-day for flowering.

Seeds of different maturity were collected and divided into two slots; one slot was used freshly soon after collection while the other slot was dried in a desiccator at least for two months before use. These seeds, fresh or dried, were de-husked and naked seed-explants were obtained.

Callus induction and proliferation cultures

Explants were surface-disinfected by normal procedure and cultured on a medium for inducing callus. The medium used for callus induction consisting of N6 macro elements (Chu et al. 1975), MS micro elements (Murashige and Skoog, 1962), N6 organic elements, 500 mg/l proline, 2 mg/l 2,4-D and 3% sucrose, and was adjusted with 1 N NaOH to a pH of 5.8-6.0 and gelled with 0.65% agar before autoclaving at 121 ^{o}C for 15 min.

Six explants were inoculated in one bottle with 40ml medium and each treatment had 5-8 replicates; treatments with less replicates were generally of those using the youngest fresh naked seed-explants and/or caused by contamination. After one month of culture, all the calli together with the seed-explants in one bottle, were transferred to another bottle with fresh medium of the same composition for proliferation for another month. Callus induction and proliferation cultures were kept in a culture room at 24-26 ^{o}C under darkness.

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After one month culture of the seed-explants, all the calli together with the mother tissues in one bottle were transferred to another bottle with fresh medium of the same composition for proliferation for another month. Callus induction culture and proliferation culture were kept in a culture room at 24-26 ^{o}C under darkness.

Experimental design and Statistical method

Six explants were inoculated in one bottle with 40-ml medium and each treatment had 5-8 replicates; treatments with less replicates were generally of those using the youngest fresh naked seed-explants and/or caused by contamination. Culture bottles with inoculated explants or calli were placed

randomly on shelves in the culture room during the culture periods. Data for callus formation were scored after one month culture of the seed-explants on callus induction medium and expressed as mean percentage ± standard error (SE), and data for callus weight per bottle were obtained by weighing callus in one bottle and expressed as mean weight ± SE.

RESULT AND DISCUSSION

Original cultures for inducing callus

Results of original culture for inducing callus of the varieties cultivated in the early-season are summarized in Table 01. It is obvious that younger seed-explants generally had lower rates of callus formation and yielded less amount of callus.

Table 01: Responses of early-season naked seed-explants of different maturity to callus induction culture[1]

Variety	Maturity[2]	% callus formation[3]		Callus weight per bottle (g)[4]	
		State of seed-explant		State of seed-explant	
		Fresh	Dry	Fresh	Dry
Qimiaoxiang	8	52.38±5.31	68.75±8.32	0.661±0.039	0.707±0.099
	10	57.14±5.07	83.33±8.23	0.696±0.047	1.291±0.102
	12	91.67±6.21	93.75±8.45	1.061±0.036	1.414±0.065
	ck	72.22±8.32	72.22±6.21	1.240±0.194	1.240±0.194
Jingxian 93	8	31.25±5.10	62.50±7.12	0.247±0.041	0.374±0.115
	10	33.33±4.25	88.89±8.36	0.324±0.031	1.014±0.150
	12	41.67±5.63	91.67±8.75	0.670±0.055	1.319±0.119
	ck	88.89±7.14	88.89±7.84	0.946±0.069	0.946±0.069
Xiyezhan	8	26.67±3.65	16.67±2.35	0.074±0.040	0.114±0.056
	10	41.67±4.22	83.33±8.36	0.444±0.044	0.977±0.077
	12	45.83±5.31	91.67±8.56	0.908±0.058	1.268±0.307
	ck	83.33±7.89	83.33±7.68	1.254±0.211	1.254±0.211

1, Data are provided as mean ± SE.

2, Maturity is given as days after the first appearance of flowers.

3, Calculated by: (No. explants yielding callus/ No. explants inoculated) X 100%

4, Weight of the naked seed-explants was included because separating them might cause damages to the calli.

However, in some cases, such as those of Qimiaoxiang, explants obtained from 12 days old or even from 10 days old seeds had higher rates of callus formation. The youngest explants had the lowest rate of callus formation and yielded the least amount of callus for all the varieties and for both fresh and dry explants.

One of the reasons for this might be the higher degree of damages to the explants

during the handling of them both *in vivo* and *in vitro* at the time of preparing and surface-sterilizing these delicate explants and another reason might be due to their physiological state. After being dried and storage, both rates of callus formation and the weight of callus of the young explants were improved for all three varieties.

Results of original culture for inducing callus of the fiv e varieties cultivated in the late-season are summarized in Table 02. In comparison with those of the early-season, degrees of variation differed with varieties; both the rates of callus formation and the weight of callus per bottle dropped considerably in the variety, Qimiaoxiang, and increased considerably in the variety, Xiyezhan, while with less differences in the variety, Jingxian 93. Culture responses of the other two varieties, Qiuguiai 11 and Taipei 309, were generally as those of other three varieties. Furthermore, the rates of callus formation and the weight of callus per bottle were increased when the naked seed-explants became more mature. Dry explants of the same maturity yielded more callus in most cases.

Callus proliferation cultures

After one month of proliferation culture, embryogenic calli were separated from the non-embryogenic ones and weighed because only the embryogenic calli, which were nodular and compact, had the potential to regenerate plants.

Results of the cultures of the early-season materials are summarized in Table 03. The data clearly show that younger seed-explants of the variety, Qimiaoxiang, had evidently more embryogenic calli, no matter that they were fresh or dried when they were inoculated for the original cultures. In the other two varieties, although the differences between treatments of different maturity were little, dried and young explants generally produced more embryogenic calli, especially those in the variety, Xiyezhan, the weights of which was almost doubled comparing that with matured ones.

Table 02: Responses of late-season naked seed-explants of different maturity to callus induction culture[1]

Variety	Maturity [2]	% callus formation[3]		Callus weight per bottle (g)[4]	
		State of seed-explant		State of seed-explant	
		Fresh	Dry	Fresh	Dry
Qimiaoxiang	8	20.00±3.58	38.89±4.54	0.144±0.026	0.283±0.113
	10	33.33±4.10	69.44±7.11	0.278±0.051	1.026±0.189
	12	61.11±7.23	100.00±0.00	0.444±0.048	1.279±0.043
	ck	77.78±6.89	77.78±9.21	0.766±0.026	0.766±0.026
Jingxian 93	8	37.50±4.52	50.00±5.02	0.333±0.066	0.200±0.021
	10	42.86±3.95	80.56±7.26	0.522±0.125	0.415±0.013
	12	38.10±3.56	77.78±7.58	0.457±0.062	0.492±0.051
	ck	66.67±5.24	66.67±7.68	1.001±0.077	1.001±0.077
Xiyezhan	8	41.67±5.14	75.00±7.35	0.154±0.028	0.805±0.065
	10	58.33±6.63	92.86±7.88	0.549±0.077	0.791±0.083
	12	80.56±6.35	80.00±7.36	1.074±0.090	1.693±0.191
	ck	77.78±8.45	77.78±8.52	0.957±0.053	0.957±0.053
Qiuguiai 11	8	41.67±5.48	83.33±8.66	0.236±0.017	0.292±0.031
	10	58.33±4.12	68.75±7.32	0.265±0.015	0.602±0.107
	12	85.71±6.23	87.50±5.69	0.418±0.058	1.058±0.119
	ck	100.0±00.00	100.00±0.00	1.591±0.158	1.591±0.158
Taipei 309	8	54.17±6.12	91.67±8.34	0.247±0.028	0.450±0.042
	10	58.33±4.68	91.67±8.65	0.364±0.042	0.815±0.220
	12	66.67±7.59	80.00±8.35	0.745±0.050	1.514±0.390
	ck	73.33±8.10	73.33±7.36	1.316±0.142	1.316±0.142

1. Data are provided as mean±SE.

2, Maturity is given as days after the first appearance of flowers.

3, Calculated by: (No. explants yielding callus/No. explants inoculated)X100%

4, Weight of the naked seed-explants was included because separating might cause damage to the calli.

Table 03: Responses in the first proliferation-subculture of the callus derived from early-season naked seed-explants at different maturity[1]

Variety	Maturity[2]	Weight of embryogenic callus per bottle (g)	
		State of seed-explant	
		Fresh	Dry
Qimiaoxiang	8	1.679±0.211	2.199±0.244
	10	1.079±0.219	2.030±0.091
	12	0.875±0.148	1.727±0.038
	ck	0.900±0.054	0.900±0.054
Jingxian 93	8	1.205±0.050	1.356±0.102
	10	1.194±0.016	1.779±0.003
	12	1.264±0.057	1.914±0.162
	ck	1.549±0.073	1.549±0.073
Xiyezhan	8	1.165±0.079	2.395±0.045
	10	1.316±0.016	2.441±0.041
	12	1.155±0.046	2.318±0.042
	ck	1.378±0.123	1.378±0.123

1, Data are provided as mean ± SE.

2, Maturity is given as days after the first appearance of flowers.

Results of the cultures of the late-season materials are summarized in Table 04. In comparison with those of the early-season, the data for the late season materials seem to be more fluctuated. However, there were always some treatments of the young explants giving obviously much better results than that of the mature ones, such as 8-days old dried explants in Qimiaoxiang, 8-days old fresh explants in Jingxian 93, and both the dried and fresh 8-days old explants in Xiyezhan produced more embryogenic calli. The immature naked seed-explants of the varieties, Qiuguiai 11 and Taipei 309, which were not cultivated in the early-season, did not show any enhancement of the proliferation of embryogenic calli. However, Yang *et al*.2005 and Yang *et al*. 1999b have showed the high responses of mature naked seed-explants of these two varieties to their established protocols and it could not be further improved by the present strategy.

Table 04: Responses of the first proliferation-subculture of the callus derived from early season naked seed-explants at different maturity

Variety	Maturity	Weight of embryogenic callus per bottle (g)	
		State of seed-explant	
		Fresh	Dry
Qimiaoxiang	8	0.999±0.016	1.866±0.027
	10	1.562±0.016	1.686±0.018
	12	1.080±0.029	1.511±0.023
	ck	1.417±0.056	1.417±0.056
Jingxian 93	8	1.841±0.037	1.021±0.023
	10	1.593±0.081	1.512±0.012
	12	1.334±0.022	1.519±0.089
	ck	1.082±0.048	1.082±0.048
Xiyezhan	8	1.454±0.018	1.517±0.067
	10	1.086±0.100	1.494±0.074
	12	1.411±0.053	0.986±0.026
	ck	1.282±0.016	1.282±0.016
Qiuguiai 11	8	1.372±0.024	1.524±0.026
	10	1.770±0.019	0.991±0.041
	12	1.628±0.013	0.999±0.083
	ck	1.809±0.013	1.809±0.013
Taipei 309	8	3.194±0.062	3.044±0.067
	10	3.569±0.128	2.976±0.095
	12	3.046±0.026	2.880±0.100
	ck	3.111±0.099	3.111±0.099

CONCLUSIONS

Many factors influence the *in vitro* culture response of rice, but the factor of physiological state of the starting material of explants had not yet been carefully investigated so far. Results of the present experiments indicate clearly that by selection of suitable immature seeds as explant source, culture response of some varieties, especially for those with poor *in vitro* culture response, in terms of the rates of callus production, weight of callus and the quality of callus such as embryogenic callus could be largely improved.

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