COMPARATIVE STUDIES ON *IN-VITRO* RESPONSE OF FRESH AND OLD CALLI OF RICE (*Oryza sativa* L.)

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ABSTRACT

In-vitro responses of fresh and old calli of japonica variety, Taipei 309, and indica variety, Qiuguiai 11, were investigated with different culture procedures to develop an efficient protocol to improve the regeneration efficiency of long-term cultured calli of rice. Pre-culture media were used to restore the declined plant regeneration ability in long-term cultured calli. Both proliferation rates and regeneration efficiencies were compared between 2 varieties with fresh and old calli before and after using pre-culture media. It was clear that in-vitro culture responses were being declined after the second subculture of calli, indicating the loss of abilities to regenerate plants from long-term cultured calli in rice. An efficient protocol developed in this study with a pre-culture medium containing 10 mg/l BA,4 mg/l ABA, 1 mg/l NAA and 3% maltose showed 7-9 folds increase of in-vitro responses in long-term cultured old calli. This protocol not only restored the declined in-vitro ability, but also reduced the somaclonal variation, especially in indica variety, Qiuguiai 11. Furthermore, japonica variety, Taipei 309, performed well in the proliferation cultures before and after using pre-culture media, showing higher proliferation rates with both fresh and old calli, comparing with indica variety, Qiuguiai 11. Higher regeneration efficiencies with less number of albino plants were given by indica variety, Qiuguiai 11 throughout the study. This study clearly indicates that the in-vitro culture responses in rice depend mainly on the varieties of different subspecies, culture conditions and compositions, and the stages of the culture procedures.

Key words: *Rice, Long-term cultured calli, Pre-culture media, Proliferation, Regeneration*

INTRODUCTION

Rice is a monocot and one of the most important cereals in the world. Because of Asia's favorable hot and humid climate, about 90% of the world's rice is grown and consumed in Asia, where it contributes about 50 to 80% of dietary energy (Juliano, 1985). Rice is also a staple food in Latin America, parts of Africa and the Middle East (Bajaj, 1991). Rice belongs to the genus; *Oryza*, which consists of 20 wild species and 2 cultigens. The Asian cultigen is called *Oryza sativa* whereas the African cultigen is known as *Oryza glaberrima*. Both of these have an "A" genome. Most of the species, including these 2 cultigens are diploid (2n = 24) and 7 other species are tetraploids. The wild species represent an important reservoir of genetic diversity and are a source of genes controlling natural resistance to biotic and abiotic stresses, and other characters useful to rice breeders (Bajaj, 1991). Although, uncountable numbers of subspecies are available, mainly 3 subspecies of *Oryza sativa* are more important and more common. Of which, subspecies-indica is tropical and produces longer grain and sticky rice (e.g. basmati and Thai jasmine) compared to japonica, which is short grain and various shades of sticky and grows in temperate climate (e.g. Rice usually grown in Japan, Italy, Egypt, Spain). However, nearly all South East Asian rice is medium to long grain, but they are not

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sticky. They are called javanica, the third subspecies, which apparently originated in Java.

However, there are many constraints to increase the production of rice in achieving increasing demand. Rice is attacked by large number of pests and pathogens, which cause an enormous loss in yield. The major objectives in rice breeding must be to improve the yield both in terms of quality and quantity. New strategies and innovative approaches have to be incorporated into traditional rice breeding to generate new genetic variability in this context. Recently developed *in-vitro* biotechnologies for rice play a major role in these improvements. Out of these, regeneration of complete plants from isolated protoplasts, the synthesis of somatic hybrids and the genetic transformation of plants stand out. Not only that but also, haploid breeding through anther culture, induction of somaclonal variants for disease resistance, selection of cell lines for salt tolerance or for increased amino acids, and the production of male sterile lines are considered as more vital techniques in rice improvements. Such protocols have urged scientists to develop efficient and easily achievable *in-vitro* regeneration techniques.

Regeneration of plants from callus is an indispensable step for breeding when the strategies such as protoplast fusion (somatic hybridization), somaclonal variation, gene transformation using callus as the acceptor tissue, most of the anther and spore culture (haploid breeding) are used. Also, production of callus and its subsequent regeneration are the prime steps in crop plant to be manipulated by biotechnological means (Saharan *et al*. 2004). Many researchers in most of the laboratories have reported that *in-vitro* culture responses in indica varieties are poorer comparing with those in japonica rice varieties (Abe and Futsuhara, 1986; Khanna and Raina, 1998). Nevertheless, it has been reported that there are large variations among varieties of the same subspecies in terms of *in-vitro* culture response. Furthermore, rapid decline of the regeneration efficiency of rice callus cultures can frequently been observed with several subcultures. The aim of this study was to compare *in-vitro* culture responses between fresh and old calli of both indica and japonica rice varieties under different culture conditions so that a protocol to improve the *in-vitro* responses with old calli would be established.

MATERIALS AND METHODS

Varieties and method for preparation of explants

An indica variety, Qiuguiai 11, which had been used for many *in vitro* culture experiments (Xiong and Yang, 2004; Yang et al. 2000; Yang et al. 1999a; Yang et al. 1999b; Yang et al. 1999c; Jian and Jintaananankul, 1998; Yang and Jian, 1996) and a japonica variety, Taipei 309, a model rice variety used in *in-vitro* studies, were used for these experiments. Mature dry seeds of these varieties were de-husked and then surface-sterilized with 70% (v/v) ethanol for 30 s and with 1% (v/v) sodium hypochlorite for 15 min. They were rinsed three times with autoclaved distilled water.

Callus induction and proliferation cultures

The disinfected naked seeds were placed on medium composed of N6 macro elements (Chu *et al*.1975), MS micro elements (Murashige and Skoog,

1962), B5 vitamins (Gamborg *et al*., 1968), 1000 mg/l proline, 2 mg/l 2,4-D, 3% (w/v) sucrose. The medium was gelled with 0.75% (w/v) agar. One month after culture initiation under dark at 24-26^{0}C, compact and nodular calli were collected and subcultured on fresh medium of the same composition but with varied concentrations of 2,4-D (1, 2 and 4 mg/l 2,4-D) for maintenance and proliferation. Calli of both varieties were subcultured in every three weeks intervals. At the second and the fourth subcultures, they were considered as fresh and old calli respectively and used as material in plant regeneration experiments.

Plant regeneration cultures

Calli for experiments on plant regeneration were first dehydrated (Tsukahara and Hirosawa, 1992) to lose about 25% of the fresh weight within 24 h and incubated at this state for three days in the culture room for obtaining a better dehydration effect of enhancing plant regeneration. These dehydrated calli were then cultured on regeneration media composed of N6 macro elements, MS micro elements, B5 vitamins, 3% sucrose, 1000 mg/l proline, 1 mg/l NAA and various other ingredients (BA and/ or $CuSO_4.5H_2O$) for the comparison and evaluation of their effects on plant regeneration. About 150 mg calli, dehydrated from 200 mg calli, was cultured on 40 ml medium in one flask and kept under 10/14 h light/dark photoperiod of about 20 μEM^{-2} s^{-1} provided by daylight fluorescent lamps at 24-26 ^{0}C. Results of the experiments were evaluated 30-days after culture initiation.

Culture for enhancing plant regeneration ability of old callus

Old calli of both varieties were cultured on pre-culture media for 30 days before transferring them onto regeneration media. Although, the composition of the pre-culture media is basically similar to that in plant regeneration cultures, they have been supplemented with a higher level of BA (10 mg/l), ABA (4 mg/l) and various kinds and combinations of other ingredients such as copper sulfate, sucrose, sorbitol, maltose and glucose. Pre-cultures were placed under the same conditions as the plant regeneration culture. After about one month of culture, the callus masses, with or without shoot-buds were collected separately from each treatment and used respectively for the subsequent plant regeneration culture. The media for plant regeneration were composed of the same basal elements and the same amounts of sucrose as in the regeneration media used for fresh calli, but were gelled with 0.18 % (w/v) Phytagel ^{TM}. Two kinds of regeneration media were used with and without 1 mg/l BA.

All the media were adjusted to pH 5.8 and autoclaved at 121^{0}C and 1.1 kg/m^2 for 20 minutes.

Data Analysis

Proliferation rates and regeneration efficiencies of fresh and old calli of both varieties were recorded at each step of the experimental procedure. Experiments were arranged in Randomized Complete Block Design. Data were analyzed by analysis of variance (ANOVA) using the SAS system for Microsoft Windows, release 8.01. Significant differences among means were determined by Duncan's multiple range tests at $P \leq 0.05$.

RESULTS AND DISCUSSION

Comparison of proliferation rate with fresh and old calli

Formation of calli in both varieties could be observed from two weeks after the initiation of the cultures. Extreme variation existed among explants and 2 varieties in the formation of callus, both quantitatively and qualitatively. This extreme variation in callus formation seemed to be mainly due to the difference in the physiological state of the explants, some of the physical factors such as the position of the explants on the medium and the varietal differences.

Compact and nodular calli were selected and cultured on fresh medium for proliferation. Calli proliferated quite fast on the fresh medium with a proliferation rate of 8-10 folds within a culture period of 21 days. In the proliferation culture, some calli with undesirable features, such as with hairy roots or brown color, could be observed and removed before being subcultured them on the subsequent fresh medium. At the end of the second subculture, a maximum proliferation rate could be seen in both varieties although the japonica variety, Taipei 309, showed comparatively higher responses in producing more nodular compact calli. After the second subculture, further subcultures gradually reduced the proliferation both in quantitatively and in qualitatively in both varieties. The proliferation rate was significantly lower ($P \leq 0.05$) at the end of the fourth subculture comparing with that at the second subculture. However, japonica variety, Taipei 309, still showed higher responses comparing with indica variety, Qiuguiai 11, at the end of the fourth subculture (Table 01). Furthermore, the production of watery and soft calli rather than producing nodular and compact calli was obvious at the end of the fourth subculture.

Out of the 3 concentrations of 2,4-D used, significantly higher responses ($P \leq 0.05$) could be seen in the media containing 1 or 2 mg/l 2,4-D, without no significant difference ($P \leq 0.05$) between these 2 concentrations, comparing with the medium containing 4 mg/l 2,4-D in both varieties, Qiuguiai 11 and Taipei 309, and in both types of calli, fresh and old, used in this study (Table 01)

Table 01: Comparison of the proliferation rate at the end of the second and fourth subculture

Type of calli[1]	2,4-D conc. (mg/l)	Variety	
		Qiuguiai 11	Taipei 309
Fresh calli	1	496.74 aA[2]	629.66 aA
	2	448.91 aB	626.81 aA
	4	306.19 bB	512.12 bA
Old calli	1	151.85 cB	232.77 cA
	2	151.60 cA	204.73 cdA
	4	70.09 dA	137.28 dA

1. Proliferation rate was calculated at the 2nd and 4th sub culture for fresh and old calli respectively.
2. Means followed by the same lower case letters in the same columns and by the same upper case letters in the same rows in the same category of calli are not significantly different at 5% level in Duncan's Multiple Range Test.

These results confirms that repeated subcultures gradually decline the proliferation rate and the ability to produce nodular calli in both indica and japonica varieties. Changes in various metabolic and catabolic reactions in the cells, which are becoming old, are the reasons for losing the ability of proliferation with nodular calli under more passages. Rapid decline of such abilities could be observed within several subcultures in rice. These results agree with previous reports published by Kishor and Reddy (1987) and Yang *et al*. (1999c).

Comparison of regeneration efficiencies with fresh and old calli

At the end of the second and the fourth subcultures, the calli were dehydrated as explained in the section of materials and methods, and transferred them as fresh and old calli respectively onto 3 regeneration media containing 2 mg/l BA and 1 mg/l NAA or 1 mg/l NAA or 2 mg/l BA, 1 mg/l NAA and 5 mg/l copper sulfate. According to the results shown in Table 02, significantly higher regeneration efficiency ($P \leq 0.05$) could be seen with indica variety, Quiguiai 11, in the media containing BA than that with japonica variety, Taipei 309 in the same media although Taipei 309 responded well in the proliferation media than Quiguiai 11. Regeneration from the calli in the media containing only NAA was negligible. Use of copper sulfate with BA and NAA in the regeneration medium significantly improves the regeneration efficiency and the early growth of plants producing more number of elongated plants within few days, especially with fresh calli (obtained at the end of the second subculture) of Quiguiai 11 ($P \leq 0.05$). This treatment has also reduced the frequency of mutations so that the production of albino plants could not be obvious (Table 02 and Figure 01). This agrees with the studies carried out by Yang *et al*. (1999c). According to them, this can be explained by the regulatory effects of copper in plant growth and development. Copper is an essential element in plant growth and development as it acts as a component or activator of some enzymes, which are critical in most of the metabolic pathways. High concentrations of copper in the medium have stimulated the plant regeneration in *Cucumis melo* (Carcia-Sogo *et al*., 1991), barley (Dahleen, 1995), wheat, triticale, and tobacco (Purnhauser and Gyulai, 1993), rice (Yang *et al*., 1999a). In the popular tissue culture media, copper is not enough for the efficient high quality shoot bud formation. Therefore, enrichment of copper is more important in these media. It was reported that 5 µmol/l copper, which is 50 times higher than that in the MS medium, was approximately the minimum amount of copper for maximum stimulatory effects on plant regeneration (Yang *et al*., 1999b).

It is also very clear that the ability to regenerate shoots from the calli of both varieties is generally declined with the repeated subcultures so that old calli responded poorly in plant regeneration media (Table 02). However, plantlet regeneration from long-term cultures is a pre-requisite for any genetic manipulation at cellular level in crop improvement. Therefore, retention or restoring of cellular totipotency with rice calli, which are becoming old, is more important.

Table 02: Comparison of regeneration efficiencies of fresh and old calli in different regeneration media

Type of calli [1]	Reg. media [2]	Qiuguiai 11					Taipei 309				
		No. of plants in different heights (cm) [3]				Reg. efficiency [4]	No. of plants in different heights (cm)				Reg. efficiency
		<1	1-3	3-6	>6		<1	1-3	3-6	>6	
Fresh calli	R11	52(2)	15(2)	6	2	31.63 bA[5]	3	1(1)	1(1)	0	2.78 aB
	R12	3	0	0	0	1.44 cA	0	0	0	0	0.00 bA
	R13	81	43	16	8	61.31 aA	2(1)	2(2)	0(1)	0	2.81 aB
Old calli	R11	5(1)	1	0	0	2.60 cA		0	0	0	0.00 bB
	R12	1	0	0	0	0.10 cA	0	0	0	0	0.00 bA
	R13	8	2	1	1	4.51 cA	0 1	1	0	0	0.78 bB

1. Fresh calli were transferred to regeneration media at the end of the 2nd subculture whereas old calli were transferred to regeneration media at the end of the 4th sub culture.
2. Three regeneration media were used for both types of calli,
   R11 – 2 mg/l BA + 1 mg/l NAA;
   R12 – 1 mg/l NAA;
   R13 – 2 mg/l BA + 1 mg/l NAA + 5 mg/l Copper sulfate
3. No. of plants in different heights (cm) produced per 250 mg calli cultured. Number of albino plants produced in each treatment is indicated in parenthesis.
4. Regeneration efficiency = $\frac{\text{Number of regenerated plants}}{\text{Weight of calli cultured (mg)}} \times 100 \text{ (mg)}$

   (Regeneration efficiency is expressed as number of regenerated plants per 100 mg of calli cultured)
5. Means followed by the same lower case letters in the same columns and by the same upper case letters in the same rows in the same category of calli are not significantly different at 5% level in Duncan's Multiple Range Test.

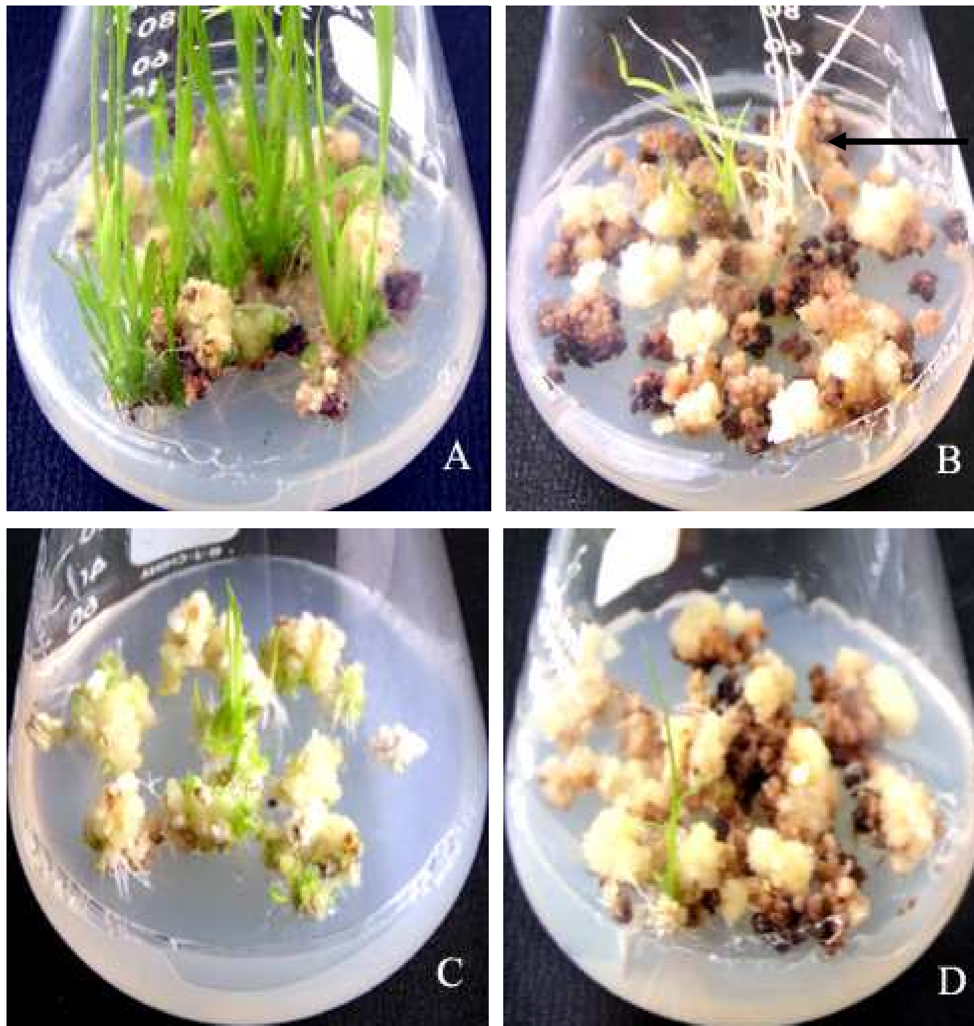

Figure 01: A-D Effects of copper sulfate in media on regeneration of shoots from fresh and untreated old calli. Photos were taken after 30 days of regeneration culture initiation. A. Fresh calli of indica variety, Quiguiai 11, produced more elongated plants. B. Fresh calli of japonica variety, Taipei 309, did not respond for copper sulfate well and produced some albino plants (arrow points the albino plants). C. Old calli of Quiguiai 11 were also responded for copper sulfate in the media, but produced more tiny shoots. D. Old calli of Taipei 309 showed very poor responses even in the media containing copper sulfate.

Effect of pre-culture media on proliferation and regeneration from old calli

It is clear that long-term cultured calli of rice are poor in *in-vitro* responses. Therefore, it is important to develop efficient protocols to restore their totipotency. Basal medium supplemented with 10 mg/l BA and 4 mg/l ABA was used to prepare six kinds of pre-culture media containing various compounds with various combinations such as 3% sucrose or 30 mg/l copper sulfate and 3% sucrose or 2% sorbitol and 1% sucrose or 3% maltose or 3% glucose or 1.5% sorbitol and 1.5% glucose. These media were tested with dehydrated old calli obtained at the end of the forth subculture.

It is obvious that the proliferation rate of old calli could be increased by 7-9 folds with the use of pre-culture media even though they produced soft calli with some media combinations. Japonica variety, Taipei 309, showed higher proliferation rates with all the pre-culture media comparing with indica variety, Quiguiai 11. The highest response could be seen with the calli on the proliferation medium containing 3% maltose and they produced shoot buds even in the pre-culture medium about 2 weeks after culture initiation (Table 03).

Table 03: Comparison of the proliferation rate of old calli in different pre-culture media

Pre-culture media [1]	Qiuguiai 11	Taipei 309
PC 1	881.0 aA[2]	1247.7 aA
PC 2	358.4 cB	858.4 aA
PC 3	515.3 bC	1133.0 aA
PC 4	1007.7 aA	1340.2 aA
PC 5	439.1 bcB	1058.2 aA
PC 6	771.8 abB	1251.0 aA

1. Proliferation rate was calculated after 1 month culture of calli (obtained from the 4th sub culture) in pre-culture media. Pre-culture media contained;
PC1 – 3% sucrose;
PC2 – 30 mg/l Copper sulfate + 3% sucrose;
PC3 – 2% sorbitol + 1% sucrose;
PC4 – 3% maltose;
PC5 – 3% glucose;
PC6 – 1.5% sorbitol + 1.5% glucose
2. Means followed by the same lower case letters in the same columns and by the same upper case letters in the same rows are not significantly different at 5% level in Duncan's Multiple Range Test.

Table 04: Comparison of regeneration efficiency of the calli obtained from different pre-culture media.

Pre-Culture media[1]	Reg. Media (mg /l BA)[2]	Qiuguiai 11					Taipei 309				
		No. of plants in different heights (cm)[3]				Reg. efficiency[4]	*No. of plants in different* heights (cm)				Reg. efficiency
		<1	**1-3**	**3-6**	**>6**		**<1**	**1-3**	**3-6**	**>6**	
PC 1	0	35	5	2	1	17.31 bA[5]	2(2)	1(1)	1	1(1)	3.32 bB
	1	36(1)	4	1	1	17.87 aA	1(2)	2(2)	0(1)	0	3.30 abcB
PC 2	0	16	1	1	0	7.95 bA	0(1)	0	0	0	0.22 cB
	1	13	1	0	0	5.95 cAB	0(1)	0	0	0	0.68 cB
PC 3	0	23	4	2	1	12.08 bA	1(2)	0(2)	0	0	2.35 bcB
	1	24	4	2	1	12.63 abcA	0(4)	1(5)	0(1)	0(1)	4.75 abB
PC 4	0	52(3)	8(3)	5(1)	4(1)	30.05 aA	4(7)	1(2)	1(3)	2(2)	8.90 aB
	1	27	2	1	1	15.41 abB	1(4)	1(3)	0(1)	1(3)	6.28 aB
PC 5	0	19(2)	3(1)	1	1	10.65 bA	0(3)	1(4)	0(1)	0(1)	3.73 bA
	1	17(1)	3	0	0	8.18 bcA	0(2)	0(2)	0(1)	0	2.38 abcA
PC 6	0	23	3	1	0	10.63 bA	0(1)	0(1)	0	0	0.91 bcB
	1	19	2	1	0	8.46 bcA	0(3)	0(1)	0	0	1.60 bcB

1. Calli in each pre-culture medium were transferred to 2 kinds of regeneration media.
   PC1 – 3% sucrose;
   PC2 – 30 mg/l Copper sulfate + 3% sucrose;
   PC3 – 2% sorbitol + 1% sucrose;
   PC4 – 3% maltose;
   PC5 – 3% glucose;
   PC6 – 1.5% sorbitol + 1.5% glucose
2. Each regeneration medium contained 0 and 1 mg/l BA.
3. Number of plants in different heights (cm) produced per 250 mg calli cultured
4. Regeneration efficiency = $\frac{\text{Number of regenerated plants}}{\text{Weight of calli cultured (mg)}} \times 100\text{ (mg)}$

   (Regeneration efficiency is expressed as number of regenerated plants per 100 mg of calli cultured)
5. Means followed by the same lower case letters in the same regeneration medium used for the calli obtained from different pre-culture media and by the same upper case letters in different regeneration media used for the calli obtained from the same pre-culture media are not significantly different at 5% level in Duncan's Multiple Range Test.

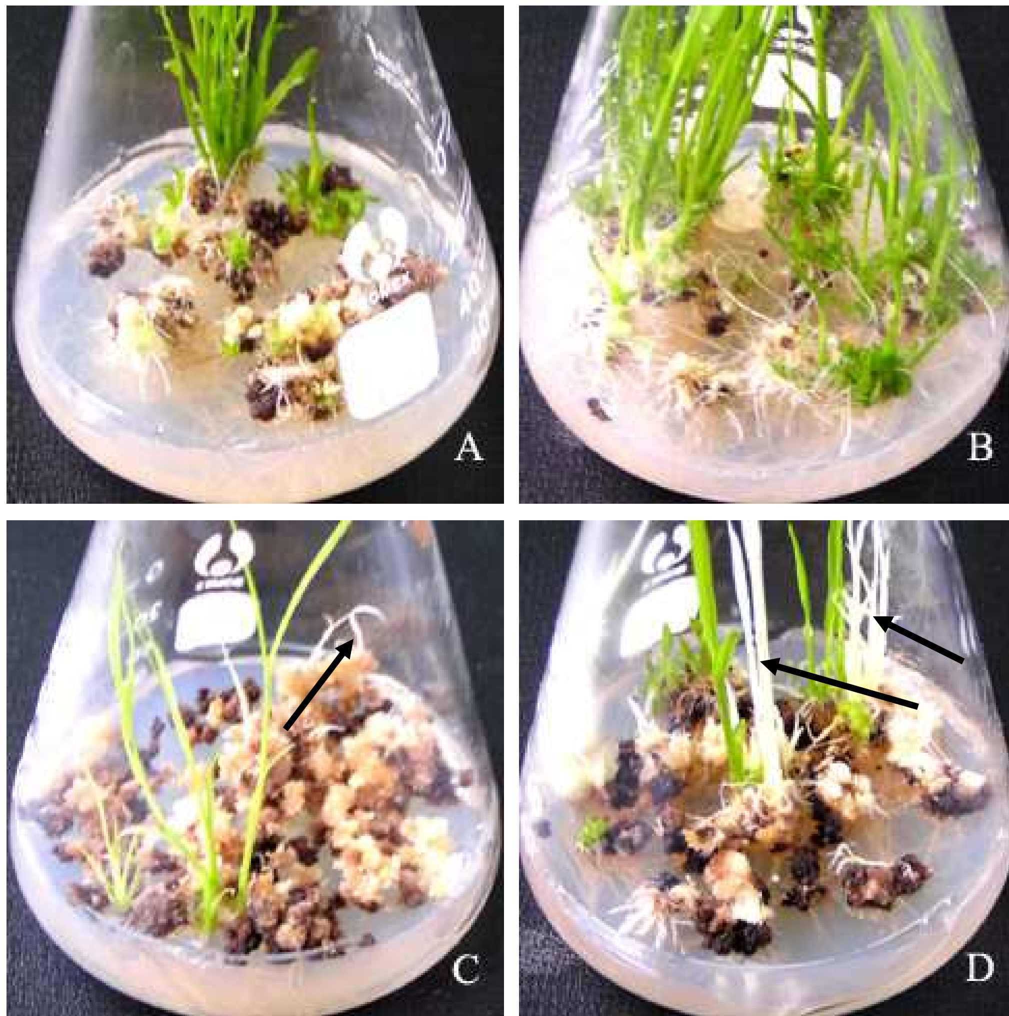

Figure 02: A-D Effects of 3% maltose in the pre-culture media on regeneration of shoots from old calli cultured on subsequent regeneration media containing no BA. Photos were taken after 30 days of regeneration culture initiation. A. Old calli of Quiguiai 11 treated with 3% sucrose in the pre-culture medium (control) showed little effects on regeneration. B. However, the old calli of the same variety on the same regeneration medium produced more elongated plants when the calli were treated with 3% maltose in the pre-culture medium. C. Old calli of Taipei 309 treated with 3% sucrose in the pre-culture media (control) did not produce stronger shoots. D. Old calli of Taipei 309 treated with 3% maltose in the pre-culture medium produced better shoots comparing with the control. Arrows point the albino plants.

Treated calli were transferred onto regeneration media after one month of restoring the regeneration ability in old calli on pre-culture media. Two kinds of regeneration media with or without 1 mg/l BA were tested with calli obtained from each and every pre-culture medium separately. Table 04 shows the results of regeneration efficiencies of old calli in regeneration media after treating them in the pre-culture media for one month. Results clearly show that the regeneration efficiency of the japonica variety, Taipei 309, is still significantly poor comparing with that of the indica variety, Qiuguiai 11, even after using pre-culture media ($P \leq 0.05$). With all the media combinations, Taipei 309 has produced more albino plants, which indicate that there is more somaclonal variations with the calli of Taipei 309 in prolong cultures. Although, all the pre-culture media have improved the regeneration efficiency of old calli in subsequent regeneration media, regeneration efficiency was significantly higher (30.05%) when the calli, which had been obtained from the pre-culture media supplemented with 3% maltose, was transferred onto the regeneration medium containing no BA in both varieties ($P \leq 0.05$). With this treatment, more number of much stronger and early-elongated plants could be obtained even though calli on this medium produced some albino plants (Figure 02). The results also indicated that the use of BA in the regeneration media had little effects for increasing plant regeneration frequency in this situation and would inhibit the initiation and growth of the root as the pre-culture media had supplemented with high level of BA (Table 04). Hence, carry-over effects of BA could enhance the regeneration efficiency in subsequent regeneration media.

Although the use of pre-culture media is not a necessary one for achieving plant regeneration from rice callus, (however, in most of the cases the rice calli do not regenerate plants well) it has been shown that this step is very effective for enhancing plant regeneration ability of the old rice calli.

Plant regulators are known to be the most important substances in callus cultures. Addition of high levels of BA with ABA to the pre-culture media containing weak auxin like NAA enhances the *in-vitro* responses such as proliferation rate and plant regeneration efficiencies in rice. Not only that but also the use of pre-culture media reduces the somaclonal variations and enhances to produce more vigorous and normal plants in subsequent culture procedures (Enoue and Maeda, 1981; Yin *et al*., 1993; Yang *et al*., 1999c) because these plant regulators have their own carry-over effects. The aim of using pre-culture media is to improve and restore *in-vitro* responses both in terms of quantitatively and qualitatively. As proline has a stimulating effect of callus growth, it is frequently being supplemented to the proliferation media. In this study, proline was supplemented even in the pre-culture media as it has no carry-over effect after providing it in the proliferation media used before being transferred the calli onto the pre-culture media. However, there are evidences that proline delays regeneration process if it is added to the regeneration medium in rice.

In this study, different kinds of carbon sources such as sucrose, glucose, sorbitol and maltose were tested with different combinations to restore *in-vitro* responses in old calli. Primarily, sugar is used in the media as a carbon source, which is necessary for various

metabolic activities. It is required for differentiation of xylem and phloem elements in cultured cells. Sugars also represent the major osmotic component of the medium. Different kinds of sugar have different activities and efficiencies of utilization as their molecular sizes and utilizable efficiencies are different in different plant tissues. Glucose and fructose are known to support good growth of some tissues. In general, dicotyledonous tissues grow best with sucrose whereas those of monocots do best with dextrose (glucose) (Bhojwani and Razdan, 1996). Although, sorbitol has been used for maintaining or restoring the plant regeneration ability of rice calli (Kishor and Reddy 1987; Kishor, 1987; Tsukahara and Hirosawa, 1992; Ozawa *et al*., 1996), supplementation of sorbitol to pre-culture media in these experiments was not much effective as maltose and sucrose alone in the media. Even though, it was expected that high level of copper sulfate in pre-culture media would improve the regeneration efficiency, it did not show any favorable effects in these experiments.

Regeneration from callus was achieved long back in japonica varieties (Nishi *et al*., 1973). The potential for callus formation and regeneration have been reported to be varietal characteristic and efficient regeneration in indica rice is still poses a major problem for genetic manipulation through innovative approaches (Toki, 1997). Strategies to improve plant regeneration frequency in cereals, including rice, have been steadily evolving during the last decade (Kyozuka *et al*., 1988; Datta *et al*., 1992; Raman *et al*., 1994). While it has been possible to obtain high plant regeneration frequencies in japonica rice varieties, the success for reproducible fertile plant regeneration has been limited in indica rice varieties with most of the experiments (Kyozuka *et al*., 1988; Raman *et al*., 1994). As a result, progress towards the transfer of useful genes into indica rice has been slow. Many factors have been examined to improve the frequency of plant regeneration in rice. Different reports have shown that many factors affect plant regeneration frequency in rice: genotype, development stage of callus in the explant, and hormonal composition of medium (Jain, 1997; Kyozuka *et al*., 1988). Partial desiccation treatments have been reported to be beneficial for embryogensis and plant regeneration in several plant species. Tsukahara and Hirosawa (1992) have reported that dehydration for 24 h of cell suspension derived calli of japonica rice increased shoot regeneration from 5 to 47%. However, this research protocol clearly indicates that the regeneration efficiency can be improved in fresh and old calli of indica variety, Qiuguiai 11, even though Japonica variety, Taipei 309, showed higher rates of *in-vitro* proliferation. This confirms that the culture conditions, media compositions and different stages of various *in-vitro* protocols play major roles in *in-vitro* responses in different varieties of 2 subspecies of rice.

CONCLUSIONS

It is obvious that *in-vitro* culture responses of rice calli decline after the second subculture. As such, both proliferation rate and regeneration efficiency of fresh calli are higher than those of old long-term cultured calli. Old calli are prone to produce more albino plants indicating more somaclonal variations. These declined *in-vitro* responses in old rice calli can be restored by using pre-culture media containing high level of BA, ABA,

NAA and different kinds of carbon sources before being transferred them to regeneration media. Out of these pre-culture media, medium containing 3% maltose stands out to restore both proliferation rate and regeneration efficiency, especially in the indica variety, Qiuguiai 11. Furthermore, varieties of the 2 sub-species used in this study responded differently for different stages of various culture procedures.

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