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Callusing and Regeneration Potential of Rice (*Oryza sativa* L.) Genotypes Towards the Development for Salt Tolerance

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The response to tissue culture of mature seeds of rice from three genotypes-- PSB Rc28, PSB Rc58, and LX278--- was studied to identify which genotype could be used as source of explants for future genetic transformation endeavors in improving rice yield on saline soil. Callusing ability, structure of the callus, and regeneration capability were used as bases in determining suitability. Results showed that while the three genotypes were all capable of forming calli, PSB Rc28 and PSB Rc58 were not able to sustain growth and generally differentiated roots (55.3%) or became necrotic (76.9%), respectively. LX278 was the most amenable to tissue culture, forming 78.8% of embryogenic calli. When half of this was transferred in regeneration medium, 69.8% regenerated plantlets. Among the plantlets, 48.9% developed by somatic embryogenesis and 51.1% by organogenesis. Thus, LX278 will be used as source of explant for genetic manipulation.

Key Words: Embryogenic calli, genetic transformation, *in vitro* culture, *japonica*/*indica* rice, LX278 rice, salt tolerance

INTRODUCTION

Plant genetic transformation is the introduction of exogenous DNA or gene into a plant cell in which the foreign DNA, derived from a certain species or from a different taxon, may be transferred to another, regardless of whether they are sexually cross-compatible or not. Application of this technique for trait improvement is considered successful if the recipient plant has integrated the foreign gene into its genome and the gene is stably expressed and inherited over generations. Genetic transformation is a powerful tool for improving crops. Using this tool, pest-resistant (Mehlo et al. 2005; Yarasi et al. 2008), herbicide- resistant (Inui et al. 2001; Hirose et al. 2005), high-yielding (Ku et al. 2001 as cited in Lu and Snow 2005; ISB 2004 as cited in Lu and Snow 2005) and more nutritious (Ro¨mer et al. 2000; Ye et al. 2000; Vasconcelos et al. 2003) crops were developed.

In vitro culture is an important component of any genetic transformation protocol because it provides sources of materials that can be used as recipient of introduced foreign genes. The ability of the targeted plant cell to regenerate into plantlet and subsequently develop into a mature plant is a prerequisite for genetic transformation. The starting material, the explant, could originate from different plant parts.

Explants from different species demonstrate varying callusing ability. In the case of rice, it is generally known that *japonica* type readily forms callus while *indica* type is more recalcitrant. The resulting calli may follow different

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pathways of development—they may remain as is, or regenerate shoots, roots, or entire plantlets. For genetic transformation studies, however, plantlets from calli are desired. This can happen only if the calli are embryogenic, i.e., the calli are capable of forming embryos.

The long term goal of this research is to generate salt tolerant rice by genetic transformation, specifically, by particle bombardment. Salt affected areas in the Philippines have grown to more than 100,000 ha in the last decade. To improve rice production, the use of salt tolerant varieties is sought as an option. As a first step, this paper aims to determine the tissue culturability of three genotypes of rice—PSB Rc28, PSB Rc58, and LX278. The quality of callus, the embryogenic response and regeneration potential are said to be influenced by genotype. Previous studies on *Saccharum sp* (Gandonou et al. 2005a; 2005b), *Sorghum bicolor* (Mishra and Khurana 2003), and *Triticum* (Zale et al. 2004) demonstrated this. Similar findings were also shown in *Oryza sativa* (Hoque and Mansfield 2004; Rachmawati and Anzai 2006). Thus, results of this study will identify the genotype that could serve as recipient of exogenous DNA in subsequent transformation study.

MATERIALS AND METHODS

Plant materials

The three genotypes of rice used in this study, namely, PSB Rc28, PSB Rc58, and LX278 were chosen for the following reasons. Among farmers, PSB Rc28 is one of the ten most popular varieties for planting (Launio et al. 2008; Pablico 2008) because it is high-yielding, is resistant to molds, and blasts and matures early (IRRI-CORRA 2003; 2005). Like PSB Rc28, PSB Rc58 has good eating quality and aroma (Pablico and Gado 2007). Both are *indica* rice, which are preferred by Filipinos, while LX278, the third genotype, is an elite line from a *japonica/indica* cross. LX278 rice has bold grains which phenotypically resembles *japonica* rice than *indica.*

Surface sterilization and callus induction

In vitro culture method of mature seeds used at Philippine Rice Research Institute, Muñoz, Nueva Ecija was followed (Philippine Rice Research Institute 1999). Seeds were dehulled and surface sterilized for 1 min with 70% ethyl alcohol then twice with 50% sodium hypochlorite for 30 min each. After rinsing three times with sterile distilled water, seeds from the same source were plated in Gerber bottles containing 30 mL RS medium supplemented with 2 mg L^{-1} 2,4-D and 1 mg L^{-1} NAA and solidified with 2 g L^{-1} phytagel and 2 g L^{-1} Gelrite agar. Thirty seeds were placed in each bottle. For 21 days, the bottles were kept in the dark at 25° C until calli appeared. These calli were subcultured in the same medium for another 21 days. The structure of the resulting calli was compared. The different types were distinguished based on their external features. The number of calli under each type was counted after 21 days and expressed as a percentage of the total.

Plant regeneration

Embryogenic calli, after two subcultures, were transferred to Linsmaier and Skoog (LS) regeneration medium containing LS inorganic salts, 30 g L^{-1} sucrose, 100 mg L^{-1} inositol, and 10 mL L^{-1} thiamine-HCl (Linsmaier and Skoog 1965), supplemented with 30 g L^{-1} sorbitol, 1 g L^{-1} MES buffer, 2 g L^{-1} casein hydrolysate, 2 mg L^{-1} BA, and 1 mg L^{-1} NAA and solidified with 4 g L^{-1} phytagel (Solis et al. 1998). Calli were exposed to 16 h of light and 8 h of dark period daily. Every month, calli were transferred in fresh medium until regenerants appeared. Regenerated shoots were induced to root in MS medium supplemented with 1 mg L^{-1} picolinic acid (R33 medium) (Dr. Nenita Desamero, personal communication, 2002) while regenerated plantlets with poor rooting were grown in MS medium (Murashige and Skoog 1962) at half-strength concentration. The resulting regenerants were eventually removed from agar, grown in water for one to three days under culture conditions and then in cups filled with sterile soil in a cool place for a week. The number of calli that regenerated plants at the end of a three-month period was counted and expressed as a percentage of the total number of calli that were induced to regenerate. The number of plantlets regenerated at the end of a three-month period was also counted. Those that regenerated by organogenesis were counted separately from those that regenerated by somatic embryogenesis.

RESULTS

Callusing ability of the rice genotypes

As shown in Table 1, the seeds of PSB Rc28, PSB Rc58, and LX278 had varying responses to tissue culture such as germination (Figure 1A) and callus formation; others demonstrated no response. In PSB Rc28, out of 884 seeds plated, 855 (96.7%) formed calli. Of the calli formed, 159 (18.0%) showed hard, extremely dry and non-nodular structure (Figure 1B), 207 (23.4%) were necrotic (Figure 1C) and 489 (55.3%) developed fine roots (Figure 1D). From 654 seeds of PSB Rc58, 583 (89.1%) developed calli.

Table 1. *In vitro* germination and callusing of rice seeds from varieties PSB Rc28 and PSB Rc58 and line LX278.

Variety/ Line	Total no. of seeds plated	Germination	Callus formation							No response
			Total	Type of callus						
				Nodular	Watery, compact	Very hard, dry	Necrotic calli		With differen-tiated roots	
							within 21 days after explanting	within 21 days after subculture		
PSB Rc28	884	6 (0.7)	855 (96.7)	0 (0.0)	0 (0.0)	159 (18.0)	207 (23.4)	**	489 (55.3)	23 (2.6)
PSB Rc58[a]	654	13 (2.0)	583 (89.1)	0 (0.0)	503 (76.9)	0 (0.0)	0 (0.0)	503 (76.9)	80 (12.2)	58 (8.9)
PSB Rc58*	512	41 (8.0)	183 (35.7)	0 (0.0)	150 (29.3)	0 (0.0)	3 (0.6)	150 (29.3)	30 (5.8)	288 (56.3)
LX278	160	34 (21.2)	126 (78.8)	126 (78.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

* Second set of seeds plated to check cause of necrosis of watery compact calli after subculture of the first set[a]
**No longer subcultured
Note: Given numbers are actual counts from the total followed by percentages in parentheses

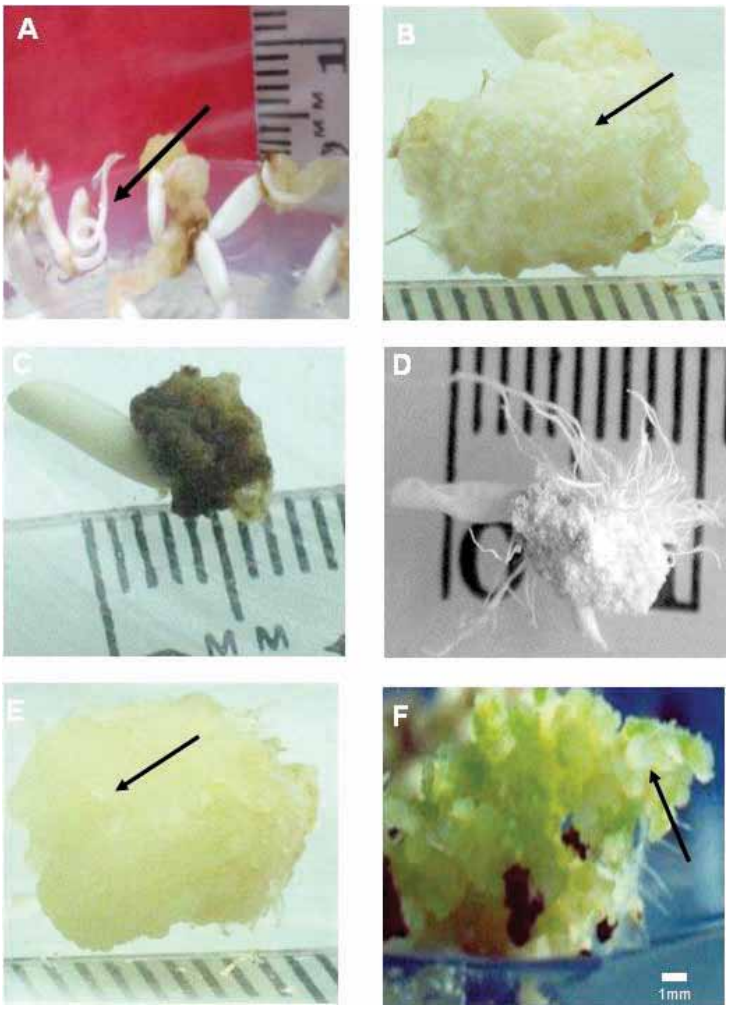

Figure 1. *In vitro* responses of mature seeds of rice plated in RS medium for 21 (A-E) and 45 (F) days. A. Germination of seed with shoot pointed by arrow; B. Hard, dry, non-nodular (pointed by the arrow) callus; C. Necrotic callus; D. Very dry callus with differentiated long roots; E. Compact, non-nodular (pointed by an arrow) callus; F. Glossy, creamy white, nodular callus (pointed by an arrow)

Among these, 80 (12.2%) formed many roots (Figure 1D). The remaining 503 (76.9%) exhibited wet, non-nodular, and compact structure of calli (Figure 1E). When the latter calli were divided into smaller pieces (approximately 2 to 3 mm diameter) during subculture (21 days after), all the calli became necrotic. Just to be sure that necrosis of calli was not brought about by cutting calli into extremely small sizes, another set of PSB Rc58 seeds (512) was plated. From this, 150 watery, compact calli samples resulted and were directly transferred to a fresh medium during subculture without subdividing them. Like the first set of seeds, these 150 calli, which represented 29.3% of the total, turned black after one to two weeks. This is an indication that this genotype was not able to sustain growth. Compared with PSB Rc28 and PSB Rc58 seeds, all the calli formed from LX278 (78.8%) were embryogenic, that is, they demonstrated creamy white, highly nodular, and compact structure (Figure 1F). Embryogenic calli are desired in genetic transformation studies because they could be induced to regenerate complete plantlets.

Plant regeneration in rice line LX278

Half (50.0%) of the 126 embryogenic calli of LX278 were transferred in regeneration medium. Among them, 44 (69.8%) formed plantlets as shown in Table 2. A total of 92 plantlets (Figure 2) were generated – 45 (48.9%) by somatic embryogenesis and 47 (51.1%) by organogenesis. Of the 45 plantlets developed from somatic embryos, 21 (22.8%) had well-developed roots

Table 2. Regeneration response of rice line LX278.

Number of calli subjected to regeneration	Number of responsive calli	Regenerants				
		Total	By somatic embryogenesis	No. of somatic embryos/callus	By organogenesis	No. of shoots/ callus
63	44	92	45	1-4	47	1-13
	(69.80%)		(48.90%)		(51.10%)	

Note: Given numbers are actual counts followed by percentages in parentheses.

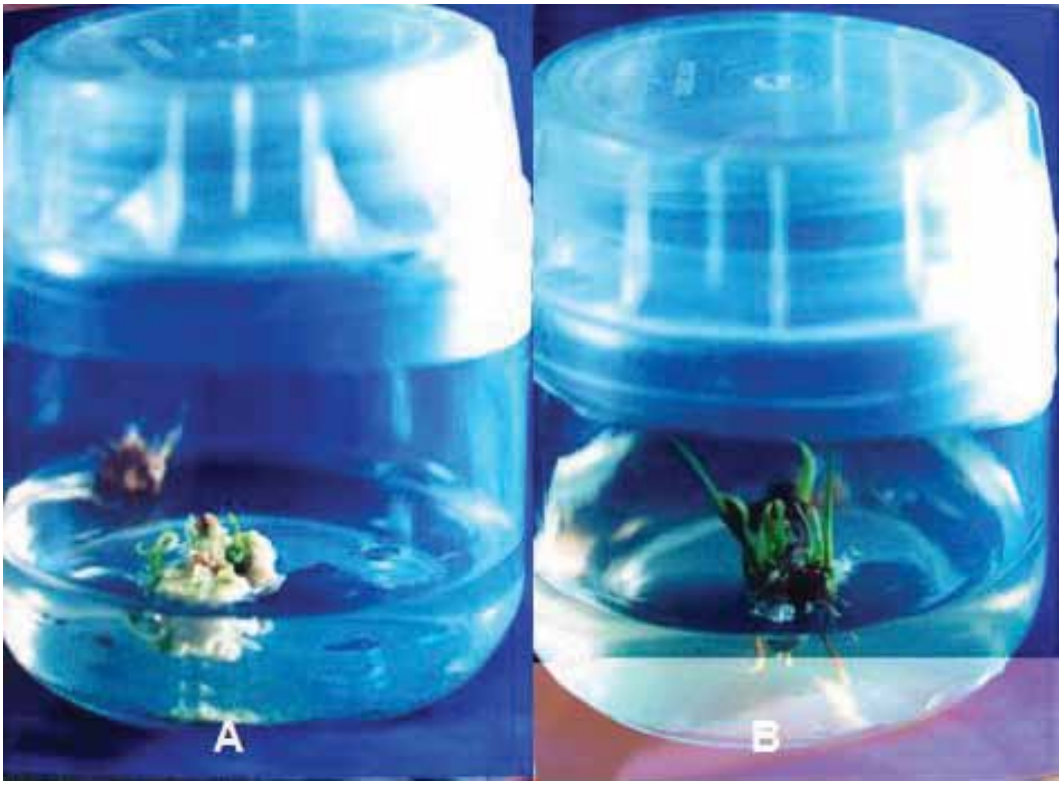

Figure 2. Plant regeneration in *Oryza sativa* line LX278. A. Callus with regenerating shoots. B. A regenerating plantlet with shoots and roots.

while 24 (26.1%) showed improved root growth in half-strength MS medium. At most, four somatic embryos per callus were noted. The remaining 47 plantlets formed by organogenesis developed shoot first in LS regeneration medium and then roots in R33 medium. In some cases, as many as 13 shoots per callus were observed. The resulting plantlets responded well to acclimatization and soil transfer.

DISCUSSION

Oryza sativa L., commonly known as rice, is classified under the tribe Oryzeae, subfamily Oryzoideae of the grass family Poaceae (Lu 1999). Among cereals, it is one of the most studied, serving as staple food of more than half of the world's population (FAO 2004). Rice is composed of two subspecies: *indica* rice, described as tropical long grain, accounts for approximately 80% of cultivated rice, and *japonica* rice, with rounded and shorter grain, is more adapted to temperate climates (Ayres and Park 1994).

Production of rice is limited by abiotic factors like saline soil. One way to improve its yield is by genetic manipulation, i.e., biolistic transformation, wherein genes for salt – resistance is introduced into its genome.

Before embarking on biolistic transformation, however, the capability to grow *in vitro* and the regeneration potential of possible target cell, tissue, or organ must first be determined because the ease of genetic manipulation and transformation of species would depend upon their amenability to tissue culture. The target material must be able to sustain growth *in vitro*, and give rise to a fertile plant for transformation to be considered successful. The resulting transgenic plant could then serve as a breeding parent for the improvement of other varieties which are not responsive to tissue culture and transformation. It is for this reason that, in this study, the culturability *in vitro* of possible transformation targets was tested.

Any regenerable cell, tissue, or organ can be used as explant. Among members of family Poaceae, plant regeneration has been achieved using young inflorescence (Chaudhury and Qu 2000), leaves (Mishra and Khurana 2003; Gandonou et al. 2005b), root (Hoque and Mansfield 2004) and mature embryos (Zale et al. 2004). In rice, mature seeds have been used previously (Rachmawati and Anzai 2006) and in the present study.

The three genotypes of rice – PSB Rc28, PSB Rc58, and LX278 all formed calli with PSB Rc28 showing the highest percentage (96.7). The quality of calli formed, however, differed. Calli from PSB Rc28 were not able to sustain cell division and mostly tended to differentiate roots. Calli from PSB Rc58 were not able to maintain growth and generally became necrotic eventually. These results indicate that both PSB Rc28 and PSB Rc58 are unsuitable sources of explants for subsequent bombardment. Calli from LX278 were actively dividing as shown by the rapid change in size, were compact and nodular– features which are associated with embryogenic tissues. The ability to form embryogenic structure of calli is an important consideration in transformation studies because it is an indicator of the capacity of the callus to form somatic embryos and regenerate complete plantlets. According to Seraj et al. (1997) calli with embryogenic features exhibit high regeneration response.

From proliferative callus, embryogenic calli could be stimulated with the right type and concentration of growth regulators. This was shown in Bermuda grass (Chaudhury and Qu 2000) and in banana (Titov et al. 2006). In some cases, however, callus medium serves both for callus induction and stimulation of embryogenic response. This was demonstrated in the present study on rice and was also shown in sorghum (Mishra and Khurana 2003), sugarcane (Gandonou et al. 2005a; 2005b), and peach palm (Steinmacher et al. 2007).

The ability to form embryogenic calli is genotype-dependent. Previous studies in coffee (Molina et al. 2002) and more recently in sugarcane (Gandonou et al. 2005 b) substantiated this. They report that the genotype effect is a stable trait, in that the embryogenic capacity of a line could be predicted if the embryogenic rate of its ancestors is known. In this study, the three genotypes of rice were of the same age, exposed to the same culture conditions, and incubated in the same media. Of the three, however, only LX278 developed embryogenic calli.

The different stages of development for indirect regeneration of plants involve induction of callus and its proliferation, followed by embryogenic callus production and plant regeneration. A previous study (Gandonou et al. 2005b) found no correlation between the ability to form callus and the capacity to form embryogenic callus and between the ability to form callus and capacity to regenerate plants. Only between the ability to produce embryogenic callus and the capacity for plant regeneration was a high positive correlation found. Thus, embryogenic callus percentage constitutes a good index for callus ability to regenerate later on plantlets (Gandonou et al. 2005 b). In the present study, therefore, the 78% embryogenic callus formed by LX278 indicates high chance of regenerating plants eventually. Indeed, when verified, the embryogenic calli of LX278, when placed in regeneration medium, formed complete plantlets.

The regeneration medium was LS with BA and NAA as phytohormones. The totipotent cells of the calli followed different pathways of development to regenerate entire plants. Some of them developed plants through somatic embryogenesis which means that the calli formed somatic embryos to generate plants. Others regenerated plants through organogenesis which means that plant organs, usually shoots, emerged from the totipotent cells and developed complete plantlets after the shoots were transferred to root-forming medium. Growth regulator concentrations play a critical role in the control of growth and morphogenesis. Generally, a medium with low auxin and high cytokinin concentrations result in the induction of shoot morphogenesis (Evans et al. 1981). Auxin alone or with very low concentrations of cytokinin is important in the induction of root primordia (Evans et al. 1981). In the present study, calli grown in the regeneration medium with low concentrations of NAA, an auxin, relative to BA, a cytokinin, developed shoots. The shoots formed roots when transferred to an auxin-containing medium, R33. It is interesting to note that the same medium (with low NAA relative to BA) also gave rise to somatic embryos that formed complete plantlets.

Line LX278 of rice is responsive to tissue culture. Using mature seed explants, callus formation, embryogenic response, and regeneration of complete plantlets were attained. Thus, it will be suitable for use in the future as source of target material for genetic transformation of rice. Its tissue culturability and known crossability with either *japonica* or *indica* subspecies of LX278 are desirable factors in future breeding endeavors.

CONCLUSION

In vitro culture response of three genotypes--- PSB Rc28, PSB Rc58, and LX278 was examined to be able to find a genotype for use as source of regenerable explants in subsequent genetic transformation studies. The structure of the calli from among three candidate genotypes differed with only line LX278 calli displaying embryogenic features and capacity to regenerate complete plantlets. Both PSB Rc28 and PSB Rc58 were not able to sustain callus growth with the former differentiating into roots and the latter becoming necrotic. Thus, *Oryza sativa* line LX278 is the most suitable genotype for genetic transformation studies. Its calli will be used as recipients of exogenous DNA in genetic manipulation.

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