Australian Journal of Crop Science AJCS
AJCS 9(11):1126-1130 (2015) ISSN:1835-2707

Somatic embryogenesis and plant regeneration in Brazilian rice genotypes

Glacy Jaqueline da Silva[1,2], Railson Schreinert dos Santos[1,2], Tatiane Medeiros Souza[1], Natã Dienes Machado[2], José Antônio Peters[3], Antonio Costa de Oliveira[1,2*]

[1]Plant Genomics and Breeding Center, Federal University of Pelotas, Brazil
[2]Biotechnology Center, Federal University of Pelotas, Brazil
[3]Department of Botany, Federal University of Pelotas, Brazil

[*]Corresponding author: acostol@terra.com.br

Abstract

The establishment of an efficient protocol for callus induction is extremely important as a first step towards genetically modified rice (*Oryza sativa* L.). Since this step is highly dependent on genotype response, research on various cultivars used in southern Brazil, the major rice-producing region of the country, is fundamental. In this paper, protocols for somatic embryogenesis and regeneration were tested in five rice genotypes, four *indica* (BRS Atalanta, BRS Querência, BRS Sinuelo and IRGA424) and one *japonica* (Nipponbare), to evaluate the efficiency of two concentrations of 2,4-D (2.0 and 4.0 mg L^{-1}) in the induction media. The following parameters were assessed: number of large calli (NLC), mean size of large calli (SLC), number of granular calli (NGC), percentage of callus formation (CF), and number of regenerated plants (NRP). It is shown that callus formation was significantly lower at a 2,4-D concentration of 2.0 mg L^{-1} than at 4.0 mg L^{-1}. However, plant regeneration at 2.0 mg L^{-1} 2,4-D was better. Significant differences were found between large calli among Sinuelo and Nipponbare genotypes, ($p \leq 0.05$). Despite the frequently better responses of japonica genotypes to tissue culture procedures, in this study the regeneration of Nipponbare plants was worse than that of indica cultivars, particularly of Sinuelo. These results, presented here, contribute to determine the best conditions for callus induction and *in vitro* culture for the relevant indica cultivars of Brazil, the largest rice producer outside Asia.

Keywords*: Oryza sativa* L., indica subgroup, *in vitro* culture, callus induction, 2,4-D concentration.
Abreviations: 2,4-D_2,4 dichlorophenoxyacetic acid; ANOVA_analysis of variance; BAP_benzil amino purine; CF_callus formation; GMOs_genetically modified organisms; GS_genomic selection; GT_genetic transformation; HSD_honest significant difference; low eeo_low electroendoosmosis; MS_Murashige and Skoog medium; MAS_marker assisted selection; NAA_1-Naphthaleneacetic acid; NGC_number of granular calli; NLC_number of large calli; NRP_number of regenerated plants; SLC_mean size of large calli; T-DNA_transfer-DNA.

Introduction

Rice (*Oryza sativa* L.) is an important cereal, for being a major staple food for two-thirds of the world population. Around 750 million tons are harvested annually, making rice the second most-produced cereal worldwide. Asia is accounts for approximately 670 million tons per year, while Brazil, the largest rice producer outside Asia, mostly with indica (*O. sativa* ssp. *indica*), produces approximately 12 million tons per year (FAO, 2013). The population of the planet is said to rise continuously from 7 billion today to 11 billion in 2100 (Gerland et al., 2014). This situation leads to an increased demand on the grain market and represents a great challenge for plant breeders, who are interested in using novel technologies to develop higher-yielding genotypes in shorter periods of time. Some of the most important biotechnological tools among these technologies are marker-assisted selection (MAS), genomic selection (GS), genetic transformation (GT), and many others (Phillips, 2008; Abe, et al., 2012; Desta and Ortiz, 2014; Mallick et al., 2015; Moore, 2015; Takagi et al., 2015). Genetically modified organisms (GMOs) have been contributing to plant breeding, not only for allowing the inclusion of new traits in elite cultivars, but also enabling fundamental research for a better understanding of the roles and mechanisms of different genes and their importance in plant metabolism and physiology (Homrich, et al, 2012). Here we highlight the use of *Agrobacterium tumefaciens*-mediated transformation as the best-suited method for genetic modification of plants. This preference is mainly due to its stable and low copy number integration of transfer-DNA (T-DNA) into the host genome, along with its ability to carry larger DNA segments with defined ends (Hiei and Komari, 2008). However, to make this method efficient, different protocol steps, such as efficient callus induction and plant regeneration, have to be validated (Nishimura et al., 2007; Sahoo et al., 2011). Calli are unorganized cell masses that can be induced *in vitro* by the combination of two growth-promoting hormones, auxin and cytokinin (Ikeuchi et al., 2013). The formation of these masses is an important step in different protocols of *Agrobacterium*-mediated GT, whereby these undifferentiated cells regenerate new plants containing T-DNA, along with the gene of interest, inserted in their genome (Hiei and Komari, 2008; Sahoo et al., 2011). However, the molecular basis of callus formation was not understood for a long time, and only recently research finally made headway towards the comprehension of this mechanism (Sugimoto et al., 2010; Ikeuchi et al., 2013).

Table 1. Analysis of variance for the parameters assessed in this study.

Variation Source	DF	Mean Square				
		NLC	SLC	NGC	CF	NRP
Rate	1	23.22*	0.16**	4.75 ^{ns}	1043.44**	92.897*
Genotype	4	19.70**	0.08 ^{ns}	25.46. ^{ns}	1908.16*	13.45 ^{ns}
Rate*Genotype	4	8.25 ^{ns}	0.07 ^{ns}	27.73 ^{ns}	725.12**	4.85 ^{ns}
Error	39	5.72	0.03	20.67	233.37	8.40
Mean		4.39	0.60	6.16	65.47	1.73
CV%		54.54	32.04	73.77	23.33	167.07

*Significant at $p \leq 0.01$ by the F test; ** Significant at $p \leq 0.05$ by the F test; ^{ns} Non-significant by the F test at $p \leq 0.05$; CV= Coefficient of variation. NLC: Number of large calli; SLC: Mean size of large calli; NGC: Number of granular calli; CF: Callus formation; NRP: Plant Regeneration.

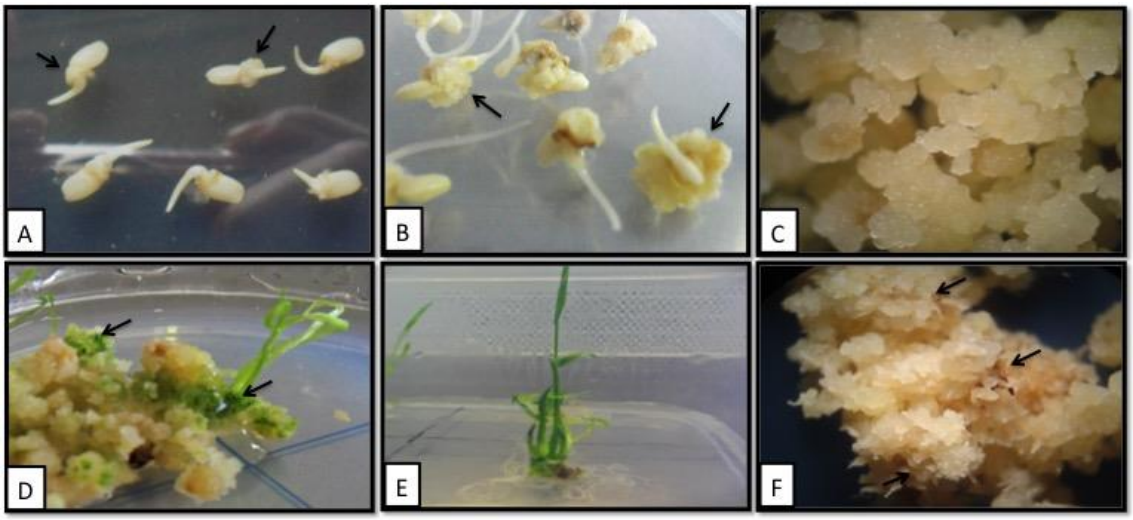

Fig 1. A: Seeds after 7 d in induction medium. Calli generated from the scutellum, emerging directly from the seed. Arrows indicate emerging calli. B: Seeds after 21 d in induction medium. Larger calli in final formation stage, but still not separated from the seed. Arrows indicate callus formation. C: Calli separated from seeds after 30 d in induction medium. Calli sub-cultured for 7 d in pre-regeneration medium. D: Calli after 16 d in regeneration medium. Arrows indicate calli turning green and differentiation into shoots. E: Plant regenerated from calli, in root medium. F: Calli type IV and V which are not useful. Arrows indicate root formation in calli.

The effects of *in vitro* application of growth regulators vary tremendously between species (Goren et al., 1979; Hu et al., 2000) and it is also important to note that methods for tissue-culture and GT of rice are highly genotype-dependent, making studies that test different *Oryza* species and cultivars indispensable (Tie et al., 2012; Ghobeishavi et al., 2015).

Most studies involving genetic transformation of rice are focused on japonica subspecies, especially on cv. Nipponbare, which has a significantly complete map-based sequence (IRGSP, 2005). Part of the preference for japonica cultivars in many studies is due to the fact that they show better responses to different transformation and regeneration protocols, when compared to indica genotypes (Kumar et al., 2005; Lin et al., 2005; Sahoo et al., 2011). Although *in vitro* cultivation and genetic transformation of indica genotypes with the currently available protocols are not easy (Rashid et al., 1996; Mohanty et al., 2002; Wang et al., 2002; Tie et al., 2012; Hiei et al., 2014; Siddique, 2015), its cooking features, i.e., high amylose content, still ensure its wide market popularity. Since the insertion of a gene of interest into a japonica cultivar followed by its transfer to other indica genotypes requires long cycles of backcrosses, making the process time-, cost- and labor-consuming, more efficient protocols for somatic embryogenesis and plant regeneration of indica genotypes are required. In this study, we tested different conditions and media in five rice genotypes: four indica (BRS Atalanta, BRS Querência, BRS Sinuelo, and IRGA424) and one japonica (Nipponbare). The purpose of this study was to facilitate future introgression of useful traits into rice of different Brazilian indica genotypes by GT.

Results

Callus formation

Seeds of every analyzed genotype started the formation of embryogenic calli of pale yellow-white color after 20 days in induction media. Most seeds formed one or two large compacted white and creamy calli (large calli - type I) and many loose friable calli (granular calli - type II) (Figure 1C).

Calli of type III (yellow or brown), IV (white, yellow or brown) and V (with small roots) were also detected (Figure 1F). As these calli are usually recalcitrant, they were excluded before data collection, i.e., after 30 days in induction media. The statistical analysis shows that auxin rate had an impact on every evaluated parameter except on the NGC (number of granular calli), suggesting a narrow genetic basis for this trait. The analysis of variance (ANOVA) (Table 1), detected significant differences for NLC (number of large calli) and CF (callus formation) between genotypes and the relation between rate and genotype was significant only for the evaluation of CF. The analysis of different auxin (2,4 dichlorophenoxyacetic acid - 2,4-D) concentrations (Table 2) in this study, indicates that 4.0 mg L^{-1} of 2,4-D induces the production of higher NLC, CF and SLC (mean size of large callus**)**, when compared to 2.0 mg L^{-1}. Even though higher concentrations of 2,4-D produced similar effects in other rice studies, these effects are closely related to genotype and not always consistent (Tariq et al., 2008; Revathi and Arumugam, 2011).

Table 2. Tukey's HSD (honest significant difference) test ($p \leq 0.05$) for the parameters assessed in this study.

Rate	NLC	CF	SLC	NRP
2.0 mg L^{-1} 2,4-D	3.68[b]	60.00[a]	0.54[b]	3.08[a]
4.0 mg L^{-1} 2.4-D	5.06[a]	70.00[b]	0.66[a]	0.32[b]

NLC: Number of large calli; SLC: Mean size of large calli; CF: Callus formation; NRP: Number of Regenerated Plants.

Table 3. Number of regenerated plants (NRP) from embryogenic callus of indica rice cultivars at both tested 2,4D concentrations.

Genotype	NRP	
	2.0 mg L^{-1} 2,4-D	4.0 mg L^{-1} 2,4-D
Nipponbare	5	0
BRS Atalanta	16	0
BRS Querência	15	2
BRS Sinuelo	30	6
IRGA 424	11	0
Total	77	8

Table 4. Mean comparison of number of large calli (NLC) and percentage of callus formation (CF) from embryogenic calli of indica rice cultivars indicated for planting in southern Brazil.

Genotype	NLC	CF
BRS Sinuelo	6.0[a]	74.0[a]
BRS Querência	4.5[ab]	74.7[a]
BRS Atalanta	5.3[ab]	71.0[a]
IRGA 424	3.6[ab]	65.9[a]
Nipponbare	2.4[b]	41.5[b]

Means followed by the same letter do not differ in the column by the Tukey test at 5% probability. NLC: Number of large calli (number of calli between <4 mm and > 15 mm); CF: percentage of callus formation.

Table 5. Growth culture of pre-regeneration and regeneration used for embryogenic calli regeneration.

Pre-regeneration		Regeneration	
Base for NB medium		Base for NB medium	
Sucrose	30.0 g L^{-1}	Sucrose	30.0 g L^{-1}
ABA	5.0 mg L^{-1}	BAP	3.0 mg L^{-1}
BAP	2.0 mg L^{-1}	NAA	0.5 mg L^{-1}
ANA	1.0 mg L^{-1}	Agarose	7.0 g L^{-1}
Agarose	7.0 g L^{-1}		
	pH=5.8		pH=5.8

Plant regeneration

After regeneration, the calli produced at the two rates were compared and showed higher rates of plant regeneration in calli subjected to 2.0 mg L^{-1} of 2,4-D during induction, compared to the 4.0 mg L^{-1} rate. These results agree with previous reports on other japonica genotypes (Tariq et al., 2008; Revathi and Amurugam, 2011). In both studies, the NRP (number of regenerated plants) was significantly higher at low 2,4-D (2.5 and 2.0 mg L^{-1} respectively) than at higher auxin concentrations (Table 2 and Table 3). This result could be attributed to the fact that since the process of regeneration is very genotype-dependent, each cultivar has a specific 2,4-D rate requirement to allow the most effective regeneration.

Discussion

It is long known that recalcitrance in monocots is lower when auxins and cytokinins are used in the culture media (Ho et al., 1983). However, due to the fact that each cultivar has specific features determined by its genotype, specific concentrations of growth regulators are required. It is shown here that this can also impact plant regeneration derived from embryogenic calli. This regeneration problem can worsen after a genetic transformation process, due to the genetic incompatibility between indica genotypes and *A. tumefaciens*, as genes involved in defense responses can lead to necrosis after infection (Veena et al., 2003). The results presented here are consistent with others, which show that high 2,4-D concentrations can increase callus formation and proliferation, but decrease plant regeneration (Joyia et al., 2013). Although a large callus number is required to improve the probability of plant regeneration, a protocol that produces a high number of calli, with a low rate of regenerated plants is not ideal, since the ultimate aim is plant regeneration. Therefore, the modified protocol of callus formation induced with 2.0 mg L^{-1} of 2-4 D (Hiei et al. 1994), as used in this study, has a higher regeneration efficiency in indica rice plants adapted to the southern region of Brazil.

Materials and Methods

Plant material

The cultivars used in this study were selected according to their agronomic and genetic potential in the southern region of Brazil. Four indica cultivars were used: BRS Atalanta, BRS Querência, BRS Sinuelo, and IRGA 424. The japonica cultivar Nipponbare was also included due to its extensive use in basic and applied research around the world.

Callus induction and plant regeneration

For callus induction and plant regeneration, a modified protocol of Hiei et al., (1994) was used. For callus formation, the growth medium NB was supplemented with sucrose (30 g L^{-1}), agarose gel (Type I: low EEO, 7.0 g L^{-1}), and two different concentrations of 2,4-D (2.0 and 4.0 mg L^{-1}). Seeds

were manually shelled and washed with sterile water, transferred to the laminar airflow chamber and then soaked in 70 % ethanol for one minute. Thereafter, the seeds were washed three times with sterile distilled water, immersed in 0.1 % mercuric chloride for 1 minute and washed again thoroughly four times with sterilized distilled water to remove any traces of mercuric chloride. After disinfection, the seeds were carefully placed in semi-solid induction culture and incubated in the dark at 28°C for 20 days (Figure 1B). After this period, calli that formed from the scutellum were separated from the seeds and placed on new Petri dishes containing fresh induction medium for another 10 days in the dark to allow further proliferation.

Trait measurement

After formation, the calli were separated from the seeds and the following traits were evaluated: number of large calli (NLC) (< 4.0 mm and > 15.0 mm), mean size of large calli (SLC), number of granular calli (NGC) and percentage of callus formation (CF). Calli were then moved to pre-regeneration medium for seven days in the dark (Figure 1C) and then transferred to the regeneration medium (Table 5) in a growth chamber at 27°C, with 12/12 hours of light/dark. After 16 days, aerial parts began to emerge from the calli (Figure 1D), and the cultures were moved to larger containers for 21 days. Calli differentiated into shoots were then transferred to regeneration media. When the plants reached a height of 4 cm, the number of regenerated plants (NRP) was evaluated, and these were transferred to rooting culture media (MS+80.0 g L^{-1} sucrose) (Figure 1E) and acclimated for three weeks in a glass house.

Statistical analysis

Five replications consisting of 10 seeds of each cultivar were used for each trait measurement in this study. The data were subjected to ANOVA and means compared using Tukey's HSD (honest significant difference) test at $p \leq 0.05$. Statistical analysis was performed with SAS statistical software version 9.3 (SAS Institute Inc.; Cary, NC).

Acknowledgements

This study was supported by the Brazilian Ministry of Science and Technology, National Council of Technological and Scientific Development (CNPq); Coordination for the Improvement of Higher Education Personnel (CAPES) and State Foundation for Research Support of Rio Grande do Sul (FAPERGS). We would like to thank Surbhi Mehra from the University of Nottingham, for her useful English corrections.

References

Abe A, Kosugi S, Yoshida K, Natsume S, Takagi H, Kanzaki H, Matsumura H, Yoshida K, Mitsuoka C, Tamiru M, Innan H, Cano L, Kamoun S, Terauchi R (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. Nat Biotechnol. 30:174-178.

Desta ZA, Ortiz R (2014) Genomic selection: genome-wide prediction in plant improvement. Trends Plant Sci. 19:592-601.

FAO, Food and agriculture organization. http://faostat3.fao.org/download/Q/QC/E. (April 20, 2015).

Gerland P, Raftery AE, Sevčíková H, Li N, Gu D, Spoorenberg T, Alkema L, Fosdick BK, Chunn J, Lalic N, Bay G, Buettner T, Heilig GK, Wilmoth J (2014) World population stabilization unlikely this century. Science. 346:234-7.

Ghobeishavi H, Uliaie E D, Alavikia S S, Valizadeh M (2015) Study of factors influencing somatic embryogenesis in rice (*Oryza sativa* L.). Int J Adv Biol Biom Res. 3:43-50.

Goren, R., Altman, A., Giladi, I (1979) Role of ethylene in abscisic acid-induced callus formation in citrus bud cultures. Plant Physiol. 63: 280–282.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-dna. Plant J. 6:271-282.

Hiei Y, Komari T (2008) Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. Nat Prot. 3:824-834.

Hiei Y, Ishida Y, Komari T (2014) Rice, indica (*Oryza sativa* L.). In: Wang K (ed) Agrobacterium protocols, 1st ed. Springer-Verlag, New York.

Ho W, Vasil IK (1983) Somatic embryogenesis and plant regeneration from leaf tissues and anthers of *Pennisetum purpureum* schum. Theor Appl Genet. 59:269-273.

Homrich MS, Wiebke-Strohm B, Weber RLM, Bodanese-Zanettini MH (2012) Soybean genetic transformation: A valuable tool for the functional study of genes and the production of agronomically improved plants. Genet Mol Biol. 35:998–1010.

Hu Y, Bao F, Li J (2000) Promotive effect of brassinosteroids on cell division involves a distinct CycD3-induction pathway in Arabidopsis. Plant J. 24: 693–701.

Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: mechanisms of induction and repression. Plant Cell. 25:3159-73.

Joyia F, Khan M (2013) Scutellum-derived callus-based efficient and reproducible regeneration system for elite varieties of indica rice in Pakistan. Int J Agr Biol. 15:27-33.

Kumar KK, Maruthasalam S, Loganathan M, Sudhakar D, Balasubryamaniam P (2005) An Improved Agrobacterium-Mediated transformation protocol for recalcitrant elite indica rice cultivars. Plant Mol Biol Rep. 23:67-73.

Lin YJ, Qifa Z (2005) Optimising the tissue culture conditions for high efficiency transformation of indica rice. Plant Cell Rep. 23:540-547.

Mallick N, Vinod Sharma JB, Tomar RS, Sivasamy M, Prabhu KV (2015) Marker-assisted backcross breeding to combine multiple rust resistance in wheat. Plant Breeding. 134:172-177.

Moore, G (2015) Strategic pre-breeding for wheat improvement Nature Plants 1, Article number: 15018.

Mohanty A, Kathuria H, Ferjani A, Sakamoto A, Mohanty P, Murata N, Tyagi AK (2002) Transgenics of an elite indica rice variety pusa basmati 1 harbouring the codA gene are highly tolerant to salt stress. Theor Appl Genet. 106:51-57.

Nishimura A, Aichi I, Matsuoka M (2006) A protocol for Agrobacterium-mediated transformation in rice. Nat Protoc. 1:2796-802.

Phillips, T (2008) Genetically modified organisms (GMOs): Transgenic crops and recombinant DNA technology. Nature Education 1(1):213.

Rashid H, Yokoi S, Toriyama AK, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in indica rice. Plant Cell Rep. 15:727-730.

Revathi S, Arumugam MP (2011) *In vitro* callus induction in rice (*Oryza sativa* L.). Res Plant Biol. 1:13-15.

Sahoo KK, Tripathi AK, Pareek A, Sopory SK, Singla-Pareek SL (2011) An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. Plant Methods. 7:49.

SAS/STAT® computer software (2013) Version 9.3. SAS Institute Inc.

Siddique R (2015) Impact of different media and genotypes in improving anther culture response in rice. Eur Sci J. 11:164-169.

Sugimoto K, Jiao Y, Meyerowitz EM (2010) Arabidopsis regeneration from multiple tissues occurs via a root development pathway. Dev Cell 18: 463–471.

Takagi H, Tamiru M, Abe A, Yoshida K, Uemura A, Yaegashi H, Obara T, Oikawa K, Utsushi H, Kanzaki E, Mitsuoka C, Natsume S, Kosugi S, Kanzaki H, Matsumura H, Urasaki N, Kamoun S, Terauchi R (2015) MutMap accelerates breeding of a salt-tolerant rice cultivar. Nat Biotechnol. 33:445-449.

Tariq M, Ali G, Hadi F, Ahmad S, Ali N, Shah AA (2008) Callus induction and *in vitro* plant regeneration of rice (*Oryza sativa* L.) under various conditions. Pakistan J Biol Sci. 11:255-259.

Tie W, Zhou F, Wang L, Xie W, Chen H, Li X, Lin Y (2012) Reasons for lower transformation efficiency in indica rice using *A.tumefaciens* - mediated transformation: lessons from transformation assays and genome-wide expression profiling. Plant Mol Biol. 78:1-18.

Veena J, Iang H, Doerge RW, Gelvin SB (2003) Transfer of t-dna and vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. Plant J. 35: 219-236.

Wang LJ, Ming XT, An CC, Yuan HY, Chen ZL (2002) Callus induction and regeneration from mature seeds of indica rice Minghui 63 and anti-fungal assay of transgenic rice plants. Chin J Biotechnol. 18:323-326.