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Assessment of Tissue Culture and Antibiotic Selection Parameters Useful for Transformation of an Important Indica Rice Genotype Karjat-3

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ABSTRACT

We assessed the tissue culture conditions and antibiotic selection of embryogenic callus and report an efficient, simple and reproducible system for mature embryo-derived embryogenic callus induction, its proliferation, its sensitivity against antibiotics such as hygromycin B and cefotaxime, and indirect plant regeneration through callus in a high-yielding, early maturity indica rice genotype 'Karjat-3'. Whitish-yellow, compact, hard and globular embryogenic callus was produced on MS media fortified with 2 mg/l 2,4-dichlorophenoxyacetic acid, 500 mg/l proline, 500 mg/l casein hydrolysate. The threshold limit for survival of callus was observed under hygromycin B (20 mg/l) and cefotaxime (250 mg/l). Multiple shoots (4-5 shoots per callus) were achieved on MS media supplemented with 4 mg/l kinetin and 1 mg/l α-naphthaleneacetic acid. These Microshoots were rooted on half strength MS media without any plant growth regulator and these rooted plantlets were transferred to soil after gradual acclimatization. The present study may be useful for genetic transformation of this indica rice genotype.

Keywords: callus induction, cefotaxime, hygromycin B resistance, plant regeneration
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BAP**, benzylaminopurine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kin**, kinetin; **NAA**, α-naphthaleneacetic acid

INTRODUCTION

Rice is the world's most important cereal crop providing staple food for 50% of the global population (Kathuria *et al*. 2007) and Asian farmers produce about 90% of the total rice, with two countries, China and India, growing more than half the total crop (IRRI 2006). To meet the ever-increasing demand for more food grains, conventional breeding programmes supplemented with recent biotechnology tools to produce rice varieties with higher yield potential, durable resistance to diseases and insects and tolerance to abiotic stresses are needed (Bajaj and Mohanty 2005). Historically, monocots including rice have proven to be less responsive to *in vitro* regeneration techniques than dicots (Repellin *et al*. 2001). Genetic engineering is strongly dependent on the genotype and the regeneration method and because of poor regenerating capabilities, indica cultivars are supposed to be recalcitrant to various biotechnological advances. Indica rice varieties are comparably less responsive in terms of callus induction, regeneration and consequent transformation efficiencies as compared to japonica varieties, which have severely hindered the application of transformation in indica rice (Abe and Futsuhara 1986; Lin and Zhang 2005).

Efficient and reproducible plant regeneration protocols are prerequisite for successful application of available genetic transformation methods available for the particular cultivar and their consequent genetic improvement programs (Abe and Futsuhara 1986; Jain 1997). Even, within the Indica group, there are considerable variations towards *in vitro* culture responses among different genotypes (Khanna and Raina 1998). Therefore, the identification and screening of useful cultivars for embryogenic callus growth and subsequent plant regeneration *in vitro* are of immense importance for rice genetic manipulations (Hoque and Mansfield 2004). The ordinary *in vitro* cultures and tissue culture for transformation differs from each other significantly, as the latter requires prolonged subculture series on antibiotic containing medium for selection purposes.

In this study, we report an efficient and reproducible method for mature embryo-derived embryogenic callus production, its antibiotic selection (kill-curves) and subsequent regeneration through callus in an important high yielding non-basmati indica rice cultivar 'Karjat-3'.

MATERIALS AND METHODS

A high yielding (4-5 t/ha), early maturity (110-115 days), insect-resistant (resistance to blast and moderately resistant to stem-borer) indica rice cultivar 'Karjat-3' was obtained through crossing cvs. 'IR 36' and 'Karjat 35-3' at Balasaheb Sawant Konkan Agriculture University's Regional Rice Research Station, Karjat (Maharashtra, India) (http://agricoop.nic.in/dacdivision/seed/Paddy/PaddyKarjat-3.htm). The certified seeds were collected from this research station and dehusked mature embryos were used for callus induction. Seeds were first washed with 0.1% Tween-20 for 5-10 min followed by washing with sterile distilled water until froth was completely removed. The seeds were then sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 10 min by continuous shaking. Seeds were rinsed three times with sterile distilled water for periods of 2-3 min each to remove any traces of $HgCl_2$ from the surface of seeds. All the chemicals and reagents used in this study were purchased from Himedia (Mumbai, India), plant growth regulators (PGRs) from Sigma-Aldrich (Bangalore,

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Table 1 Different media used for callus induction and shot induction and their composition.

Medium	Composition
MS basal	MS salts and vitamins, 30 g/l sucrose, 7 g/l agar, pH 5.8
CIM I	MS basal, 2 mg/l 2,4-D, 30 g/l sucrose, 7 g/l agar, pH 5.8
CIM II	MS basal, 2 mg/l 2,4-D, 500 mg/l proline, 500 mg/l casein hydrolysate, 30 g/l sucrose, 7 g/l agar, pH 5.8
CIM III	MS basal, 2 mg/l 2,4-D, 500 mg/l proline, 500 mg/l casein hydrolysate, 30 g/l maltose, 1 g/l glucose, 50 mg/l tryptophan, 7 g/l agar, pH 5.8
SIM I	MS basal, 4 mg/l Kin, 0.5 mg/l NAA, 30 g/l sucrose, 7 g/l agar, pH 5.8
SIM II	MS basal, 4 mg/l Kin, 1.0 mg/l NAA, 30 g/l sucrose, 7 g/l agar, pH 5.8
SIM III	MS basal, 4 mg/l Kin, 2.0 mg/l NAA, 30 g/l sucrose, 7 g/l agar, pH 5.8
SIM IV	MS basal, 4 mg/l Kin, 3.0 mg/l NAA, 30 g/l sucrose, 7 g/l agar, pH 5.8

MS = Murashige and Skoog (1962); CIM: callus induction medium; SIM: shoot induction media

India) and antibiotics from Duchefa Biochemie (Haarlem, The Netherlands).

Surface sterilized seeds were inoculated in Petri dishes (Axygen, New Delhi, India) containing various compositions of callus induction media as given in **Table 1**. The pH was adjusted to 5.8 prior to autoclaving (Equitron, Mumbai, India) at 1.04 kg cm^{-2} and 121°C for 20 min. Petri dishes were sealed with Parafilm "M" (Pachiney, Chicago, USA) and cultures were kept at 25 ± 2°C in dark for 30 days before callus induction responses were assessed. Only compact hard embryogenic callus cultures were transferred to fresh media with the same compositions for multiplication after every 21 days. Data of frequency of callus formation and its characteristics were recorded after 30 days of culture.

Hard, compact and embryogenic callus obtained on CIM II was used for hygromycin B (0-50 mg/l) and cefotaxime (0-500 mg/l) selection. The calluses were placed on petri dishes containing CIM II media supplemented with variable concentrations of either hygromycin B or cefotaxime. The cultures were kept in dark at 25 ± 2°C and responses were observed on 10 days after inoculation.

Hard, compact and embryogenic callus obtained on CIM II were used for shoot organogenesis. Shoot induction media (SIM) consisted of MS basal media fortified with 4 mg/l Kin and (0.5, 1.0, 1.5, 2.0) mg/l NAA, 30 g/l sucrose, gelled with 7 g/l agar and pH 5.8. The cultures were then shifted to 16 h/d photoperiod, with 25 μmol $m^{-2}s^{-1}$ light intensity provided by cool-white fluorescent tubes (Philips, India) and relative humidity around 80-85%. Cultures were transferred to fresh media after every 21 days. Elongated healthy shoots (4 to 5 cm) were excised and cultured in glass tubes (150 × 25 mm, Borosil, India) containing rooting media (RM) comprising half strength MS basal salts with full strength vitamins (½-MS) with 30 g/l sucrose, 2.5 g/l phytagel, and pH 5.8. The cultures showing root induction were transferred to fresh media after 20 days and basal calluses were removed prior to subculturing. All the tissue culture experiments were carried out in a laminar air flow hood fitted with ultraviolet tubes (Microfilt, India).

Rooted plantlets were removed from RM and the shoots were washed in sterile distilled water to remove all the traces of agar and basal callus. The plantlets were then transferred to plastic pots (6 cm diameter) containing garden soil mixed with vermiculite and sand (1:1:1). The plastic pots were covered with polyethylene bags to maintain the relative humidity of about 70-80%. These pots were maintained at 25 ± 2°C and with a 16-h/d photoperiod. Plantlets were exposed to full sunlight for 4 h in the 4^{th} week, and this period was gradually increased to 6 h in the 5^{th} and 8 h in the 6^{th} week. The plantlets were planted in pots and pots were kept in the field in the 7^{th} week.

All the experiments were set up in a completely randomized design, and repeated 3 times before conducting the statistical analyses. The data was subjected to analysis of variance (ANOVA) to detect significant difference between means. Means differing significantly were compared using Duncan's multiple range test (DMRT) at $P \leq 0.05$. All the statistical analyses were done by using MSTATC statistical software package.

RESULTS AND DISCUSSION

Embryogenic callus production with high regeneration capacity is a prerequisite for highly efficient transformation of rice. Even though indica subspecies is the most widely cultivated type of rice worldwide, the magnitude of genetic manipulations is very less as compared to its japonica counterpart. The main reason behind this is that indica rice appears as recalcitrant or say more specific than japonica rice to tissue culture conditions in *Agrobacterium*-mediated transformation (Visarada *et al*. 2002; Martinez-Trujillo 2003; Ge *et al.* 2006). Even within the indica group, there are significant variations in the *in vitro* culture response among different genotypes (Peng and Hodges 1989). Despite the availability of a plethora of protocols for rice tissue culture, no procedure appears to be universally adaptable when a new genotype is to be considered for *in vitro* manipulation. Though regeneration, rather than callus induction, is limiting in most of the *indica* rice cultivars, introduction of foreign gene(s), selection and multiplication of only the transformed sectors depends upon the embryogenic potential of callus in *in vitro* cultures (Visarada *et al*. 2002). Therefore, development of methods for embryogenic callus production, its antibiotic selection and indirect regeneration is of great importance.

In the present study, we observed that calli initiated from the scutella of germinating seeds had embryogenic potential. Various explants have been used for callus induction in rice, including immature embryos (Chand and Saharawat 2001), mature embryos (scutellum) (Khanna and Raina 1998), roots (Mukhopadhyay *et al.* 1997), anthers (Sugimoto *et al.* 1999), mature endosperm (Bajaj 1991), the stem base (Finch *et al.* 1992) and young coleoptiles (Oinam and Kothari 1993). However, among the several types of explants, scutellum-derived callus has been found to be the most amenable to transformation (Tyagi *et al*. 2007), in addition to its year-round availability. We tried various auxins such as IAA, IBA, NAA and cytokinins like BAP and Kin for callus induction, however, amongst these, no PGR could induce callus from mature embryos (data not shown). Only 2,4-D was observed as the sole PGR suitable for callus induction. Firstly, the optimum concentration of 2,4-D was standardized for callus induction by using 2,4-D from 0 to 4 mg/l (data not shown); 2 mg/l 2,4-D was most suitable for optimal callus induction. Our results are in conformity of the use of 2,4-D (with little variation of 2 to 3 mg/l) for optimal callus induction and proliferation from mature seeds as reported by various researchers (Visarada and Sarma 2002; Saharan *et al.* 2004; Lin and Zhang 2005; Ge *et al.* 2006).

However, MS media fortified with 2 mg/l 2,4-D alone responded poorly in terms of embryogenic nature of callus, and only around 10% of callus cultures showed embryogenic type callus (**Table 2**). Therefore, in addition to this, effects of proline and casein hydrolysate were investigated for improvement of induced callus in terms of its embryogenic nature and fresh weight. Interesting results were observed when proline and casein hydrolysate were used, as high rate of embryogenic callus induction was observed (CIM II and III, **Table 1**). Both these nutritional supplements have been reported to work as a source of amino acids and addition of these supplements to the callusing media proved to enhance the production of embryogenic type callus (Lin and Zhang 2005; Zaidi *et al*. 2006; Tyagi *et al*. 2007). Our results confirmed the findings of these investigations.

Table 2 Callus induction efficiency and growth on different media used.

Media	Callus induction [%] [Mean ± SE]	Embryogenic callus [%] [Mean ± SE]	Fresh weight of callus per explant [mg] [Mean ± SE]
MS basal	Nil	Nil	Nil
CIM I	60 ± 2.5 a	10 ± 0.8 a	244 ± 2.0 b
CIM II	82 ± 1.8 c	72 ± 2.5 c	250 ± 2.0 c
CIM III	75 ± 2.0 b	64 ± 1.9 b	235 ± 3.5 a

100 seeds were inoculated per treatment with 3 replicates. Means within a column followed by different letters were significantly different from each other according to Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

Moreover, use of maltose in place of sucrose in addition to glucose and tryptophan did not show any significant effect on embryogenic callus induction frequency (**Table 2**). The results presented in **Table 2** clearly indicate that CIM II was most suitable media for embryogenic callus induction and growth. It induced hard, dry, compact, globular whitish-yellow embryogenic callus from mature dehusked seeds (**Fig. 1A**). Similar to our findings, the use of proline and casein hydrolysate for embryogenic callus induction from indica rice, used for *Agrobacterium*-mediated transformation has been reported by a number of researchers (Saharan *et al*. 2004; Ge *et al*. 2006; Kant *et al*. 2007).

Numerous factors, including culture conditions prior to and during inoculation (Aldemita and Hodges 1996; Mohanty *et al*. 1999), selection marker genes and selective agents (Hiei *et al*. 1997; Yookongkaew *et al*. 2007), genotype of plants and various conditions of tissue culture including a robust system of plant regeneration are of critical importance.

The most widely used selectable markers in monocot transformation are the genes encoding hygromycin phosphotransferase (*hpt*), phosphinothricin acetyltransferase (*pat* or *bar*) and neomycin phosphotransferase (*npt*II) (Shrawat and Lorz 2006). Use of these marker genes under the control of constitutive promoters such as the 35S promoter from *Cauliflower mosaic virus*, or the ubiquitin promoter from maize, works efficiently for selection of *Agrobacterium*-transformed cells (Cheng *et al.* 2004). Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus*, which kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis. It also interferes with translocation and to cause mistranslation at the 70S ribosome. It is the most widely used antibiotic se-lection agent, as it kills the non-transformed cells more quickly than kanamycin and only resistant or in other words, transformed cells survives. This kind of selection is referred to as negative selection (Shrawat and Lorz 2006). Thus, finding the threshold limit of hygromycin B concentration for survival of the calluses will help immensely prior to transformation work, as this concentration of selective agents are needed to avoid development of undesirable numbers of the escapes, post-transformation. In the present investigation, hygromycin B showed significant effect on percent survival of rice calli. The survival of the calli was 100% in the control medium (without antibiotic). As the concentration of hygromycin B increased in the medium, the percent survival of the calli decreased. At 15 to 20 mg/l hygromycin B, the callus tissues gradually started turning brown after 2-3 days of inoculation on selection medium and after 10 days of selection, calluses were observed completely dark reddish-brown or blackish and 100% inhibition of calli or cell death was noticed in presence of 20 mg/l of hygromycin B (**Fig. 1B**), suggesting this as suitable concentration for selecting the putative transformants for future transformation programmes of this genotype. Various researchers have used 50 mg/l hygromycin B for selecting the putative transformants of different indica type rice genotypes (Sridevi *et al*. 2005; Kant *et al*. 2007; Nandakumar *et al*. 2007). On the other hand there a few reports, where hygromycin B has been used as a selective agent below this concentration (Pipatpanukul *et al*. 2004; Tyagi *et al*. 2007). Therefore, our findings are significant as we could employ a significantly lower concentration of hygromycin B to completely inhibit callus growth in 'Karjat-3', which may be exploited for genetic engineering.

In order to eliminate *Agrobacterium tumefaciens* after co-cultivation, the use of antibiotics in culture medium is required. In the present investigation, the susceptibility of callus tissues was standardized for cefotaxime. Survival of the calli was 100% up to 250 mg/l of cefotaxime, however, above this level drastic changes were observed and calli were turned brown and subsequently all the callus tissues were observed to be dead on 10 days after inoculation. The reduced regeneration capacity is in agreement with the results obtained by Pipatpanukul *et al*. (2004), who reported that cefotaxime over 250 mg/l played an inhibitory effect of callus growth and regeneration of indica rice cv. 'RD6'. Therefore, the present results clearly suggest that cefotaxime at 250 mg/l and hygromycin B at 20 mg/l may be used for this cultivar for transformation studies.

Effects of Kin in addition of IAA and NAA were observed on shoot regeneration through callus, though IAA was found unsuitable (data not shown) and optimization of

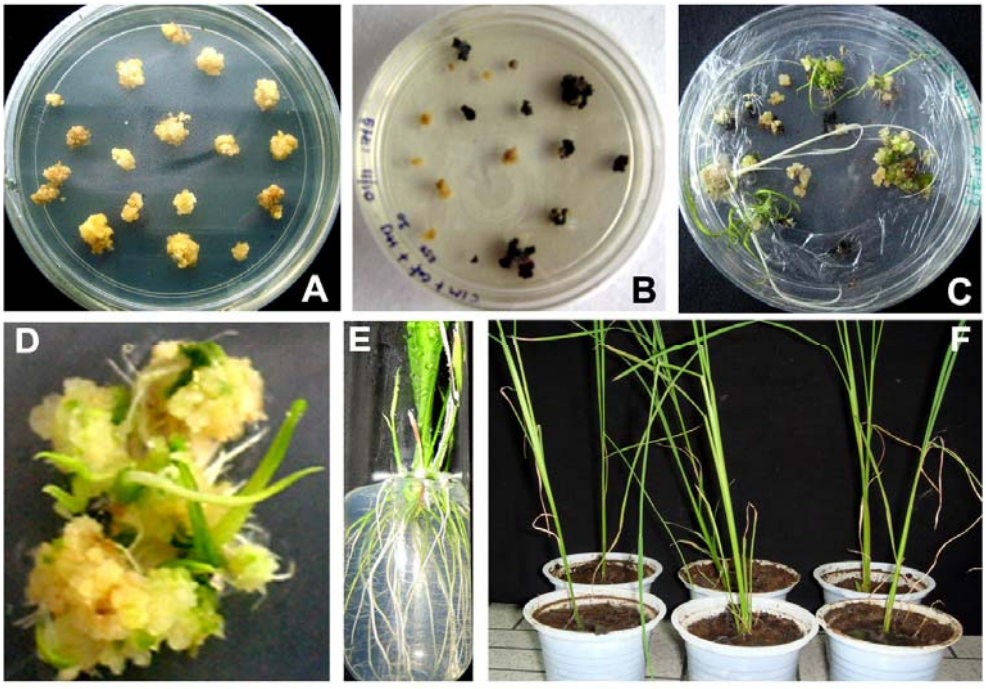

Fig. 1 Mature embryo (scutella) derived-embryogenic callus induction, antibiotic selection and plant regeneration in indica rice cultivar 'Karjat-3'. (**A**) Embryogenic callus production on CIM-II. (**B**) Antibiotic selection of calli on 20 mg/l hygromycin. (**B, C, D**) Shoot organogenesis of antibiotic resistant calli on SIM-II. (**E**) Rooting of microshoots on RM. (**F**) regenerated plants transferred to pots for hardening.

Table 3 Response of 'Karjat-3' regeneration through embryogenic callus.

Media	Shoots regeneration [%] [Mean ± SE]	No. of shoots regenerated per callus piece [Mean ± SE]
SIM 1	15 ± 2.0 a	1.5 ± 0.2 a
SIM 2	75 ± 2.0 c	4.5 ± 0.5 c
SIM 3	30 ± 1.0 b	2.0 ± 0.1 b
SIM 4	30 ± 1.5 b	1.6 ± 0.3 a

The embryogenic callus (100 per treatment with 3 replicates) produced on CIM-II was used for shoot regeneration. Means within a column followed by different letters were significantly different from each other according to Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$

shoot induction media was done by using various combinations of Kin and NAA (**Table 3**). SIM II was found most suitable for shoot organogenesis through embryogenic callus in indica rice cv 'Karjat-3' with more than 75% hygromycin B resistant cultures shown green shoots at an average of 4-5 shoots per callus within 3-4 weeks after inoculation (**Fig. 1C** and **1D**). Well-developed shoots after 3-4 weeks of inoculation induced vigorous roots, when transferred to RM (**Fig. 1E**). These rooted plantlets were transferred to plastic cups containing soil and gradually acclimatized to natural conditions (**Fig. 1F**). The regenerated plants did not show detectable variation in morphological or growth characteristics compared with the parent plant.

Thus, the present report deals with an efficient and reproducible method for indirect organogenesis of an important indica rice cultivar, which may work as key for *Agrobacterium*-mediated transformation and subsequent genetic manipulations of this cultivar. In addition the selection of callus for sensitivity towards hygromycin B and cefotaxime may also be useful as these antibiotics are used for selection and to inhibit the proliferation of *Agrobacterium* during selection and shoot regeneration of putative transformants respectively.

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