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Regeneration Protocol for Whole Plants from Embryogenic Callus of Commercial Rice (*Oryza sativa* L.) Variety PR 116

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ABSTRACT

The selection of highly regenerable rice varieties is a pre-requisite for success in rice biotechnology. In this report we developed a reproducible and effective plant regeneration system through somatic embryogenesis for *Indica* rice var. PR 116. Embryogenic calli derived from mature seeds served as explants. Callus was induced from mature seeds cultured on MS medium containing 30 g/l sucrose supplemented with 560 mg/l proline, 1.5-3.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5-1.5 mg/l kinetin (Kin). The regeneration efficiency was tested on Murashige and Skoog (MS) medium containing 30 g/l sucrose fortified with 1.0-3.0 mg/l 6-benzyl aminopurine (BAP), 0.5-1.5 mg/l Kin and 0.5 to 1.5 mg/l α-naphthalene acetic acid (NAA). Highest callus induction (44.4%) was on MS medium supplemented with 2.5 mg/l 2,4-D, 0.5 mg/l Kin, 560 mg/l proline and 30 g/l sucrose. The highest percentage shoot regeneration (42.5%) was on MS medium supplemented 2.0 mg/l BAP, 0.5 mg/l NAA and 0.5 mg/l Kin. The plantlets were hardened and transferred to soil in earthen pots. The developed method was highly reproducible.

Keywords: callus induction, hardening, plant regeneration, tissue culture
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BAP**, 6-benzyl aminopurine; **Kin**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-napthaleneacetic acid

INTRODUCTION

Rice (*Oryza sativa* L.) is a very important cereal crop globally. It is estimated that rice production has to be increased 50% by 2025 (Khush and Virk 2000) to meet global demands. Considerable improvement has already been made by exploiting natural variation through conventional breeding. Despite the success made in the last century, traditional breeding efforts alone cannot meet the increasing demand of rice consumers in the 21st century. Therefore, tissue culture remains a basal technique for the genetic improvement of rice plant throughout the world (Komari *et al*. 2007). Various biotechnology tools are directed towards improvement of conventional plant breeding processes, such as introduction of novel genes by genetic transformation, protoplast fusion for production of male sterile lines, haploid generation for attaining rapid homozygosity and somaclonal variation for introducing increasing trait variability (Hoque *et al.* 2007; Ramesh *et al.* 2009). A routine tissue culture protocol, including callus induction and regeneration, is a fundamental requirement for successful genetic transformation in rice (Seraj *et al.* 1997; Li *et al.* 2007; Dabul *et al.* 2009). Due to their poor regeneration ability, *Indica* cultivars are recalcitrant to various biotechnological advances (Kumar *et al.* 2008). Screening of elite cultivars for embryogenic callus development and subsequent plant regeneration *in vitro* are key steps in a rice genetic improvement program through the application of biotechnology (Hoque and Mansfield 2004). Among many factors which influence callus induction and plant regeneration, genotype and nutrient media composition are the most important. In rice, *in vitro* plant regeneration has been achieved from almost all explant types (Jain *et al.* 1996). However, significant variation was observed in embryogenic callus production, somatic embryogenesis and subsequent plant regeneration from different origins. The use of mature seed embryos has a distinct advantage over other explants as starting material for *in vitro* regeneration because they are available throughout the year from harvested seeds. Moreover, embryogenic calli obtained from mature seed embryos are efficient in *Indica* rice transformation (Kant *et al.* 2007; Kumar *et al.* 2010). This report describes the establishment and optimization of callus induction and regeneration media for the commercial *Indica* rice variety PR 116.

MATERIALS AND METHODS

Experimental material and chemicals

The experiment was conducted on high yielding commercial rice (*Oryza sativa* L.) variety PR 116 obtained from the Rice Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India. The research work was carried out in the Tissue Culture and Transformation Laboratories, School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India during 2006-2009.

All reagents and tissue culture-related chemicals were purchased from Hi-Media (Mumbai, India) unless indicated otherwise.

Callus induction and subculture

Mature seeds were carefully dehusked and treated with 0.1% (w/v) Bavistin (BASF, India) for 3 h and washed three times with sterile distilled water (SDW). They were then surface sterilized in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 10 min, followed by three rinses in SDW under a laminar air flow cabinet. Callus cultures were initiated from surface-sterilized seeds by placing them onto

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Research Note

Table 1 List of media used in the present study for callus induction and plantlet regeneration.

Combination number (CN)	Media combination
Callus Induction	
1	MS + 1.5 mg/L 2,4-D + 0 mg/L Kin
2	MS + 2.0 mg/L 2,4-D + 0 mg/L Kin
3	MS + 2.5 mg/L 2,4-D + 0 mg/L Kin
4	MS + 3.0 mg/L 2,4-D + 0 mg/L Kin
5	MS + 3.5 mg/L 2,4-D + 0 mg/L Kin
6	MS + 1.5 mg/L 2,4-D + 0.5 mg/L Kin
7	MS + 2.0 mg/L 2,4-D + 0.5 mg/L Kin
8	MS + 2.5 mg/L 2,4-D + 0.5 mg/L Kin
9	MS + 3.0 mg/L 2,4-D + 0.5 mg/L Kin
10	MS + 3.5 mg/L 2,4-D + 0.5 mg/L Kin
11	MS + 1.5 mg/L 2,4-D + 1 mg/L Kin
12	MS + 2.0 mg/L 2,4-D + 1 mg/L Kin
13	MS + 2.5 mg/L 2,4-D + 1 mg/L Kin
14	MS + 3.0 mg/L 2,4-D + 1 mg/L Kin
15	MS + 3.5 mg/L 2,4-D + 1 mg/L Kin
Plant Regeneration	
1	MS + 0.5 mg/L BAP + 0 mg/L NAA + 0 mg/L Kin
2	MS + 1.0 mg/L BAP + 0 mg/L NAA + 0 mg/L Kin
3	MS + 1.5 mg/L BAP + 0 mg/L NAA + 0 mg/L Kin
4	MS + 2.0 mg/L BAP + 0 mg/L NAA + 0 mg/L Kin
5	MS + 2.5 mg/L BAP + 0 mg/L NAA + 0 mg/L Kin
6	MS + 0.5 mg/L BAP + 0 mg/L NAA + 0 mg/L Kin
7	MS + 1.0 mg/L BAP + 0.5 mg/L NAA + 0 mg/L Kin
8	MS + 1.5 mg/L BAP + 0.5 mg/L NAA + 0 mg/L Kin
9	MS + 2.0 mg/L BAP + 0.5 mg/L NAA + 0 mg/L Kin
10	MS + 2.5 mg/L BAP + 0.5 mg/L NAA + 0 mg/L Kin
11	MS + 0.5 mg/L BAP + 0.5 mg/L NAA + 0.5 mg/L Kin
12	MS + 1.0 mg/L BAP + 0.5 mg/L NAA + 0.5 mg/L Kin
13	MS + 1.5 mg/L BAP + 0.5 mg/L NAA + 0.5 mg/L Kin
14	MS + 2.0 mg/L BAP + 0.5 mg/L NAA + 0.5 mg/L Kin
15	MS + 2.5 mg/L BAP + 0.5 mg/L NAA + 0.5 mg/L Kin

Abbreviations: BAP, 6-benayleaminopurine; 2,4-D, 2,4- dichlorophenoxy acetic acid; Kin, kinetin; MS, Murashige and Skoog; NAA, α-naphthalene acetic acid

Murashige and Skoog (1962) medium supplemented with various concentrations of 2,4- dichlorophenoxy acetic acid (2,4-D) and kinetin (Kin) (**Table 1**), 3% sucrose, 560 mg/l proline (Pro) and 8 g/l agar (Cho and Zapata 1988). The pH of all media was adjusted to 5.8 with 1N NaOH prior to the addition of agar. Then, the media was sterilized by autoclaving at 121°C and 15 lbs psi pressure for 20 min. The 20-25 ml of medium-filled in borosilicate test tubes and were kept in a laminar airflow cabinet under UV light for 20 min. The seeds were inoculated in the laminar airflow cabinet and then tubes were placed in a growth room incubated in the dark at 25 ± 2°C. Callus was induced from the seeds within 3 weeks. Callus was sub-cultured every 3 weeks onto the same medium and under the same growth conditions. After a total of 6 weeks from seed inoculation, the percentage embryogenic callus (embryogenic callus number/total number of calli cultured) was scored. Based on visual observation, callus which was watery and friable was considered to be non-embryogenic and creamy–yellow, compact nodular callus was classified as embryogenic.

Plant regeneration

The embryogenic callus was carefully excised and transferred to MS medium supplemented with various concentrations of BAP, Kin and NAA (**Table 1**) for regeneration. Shoots were initiated from the embryogenic calli on different media combinations with a variable response. Rooting was achieved on half MS medium without plant growth regulators. The regenerated plantlets having well developed roots were taken out carefully from the tubes with the help of forceps and the medium attached to roots was thoroughly washed to avoid any contamination. These plantlets were then hardened by placing them on cotton soaked with tap water in the test tube for one week where they resumed growth. After one week on moistened cotton, the plantlets were then kept directly in test tubes in the water supported by autoclaved filter paper for another week where profuse rooting occurred and the plantlets got ready for their transfer to the soil. The hardened plantlets were finally transferred to the soil in the plastic pots and were kept in glasshouse and maintained at temperature approx. 30°C with relative humidity 80%.

Data analysis

All the data recorded were analyzed according to completely randomized design analysis (Snedecor and Cochran 1967) using statistical software CPCS-1 package developed by Cheema and Singh (1990). Percentage data was converted to an arc sine value for the analysis of variance (ANOVA). The significance of variation among the treatments were observed by applying the F test and critical differences (CD) at the 5% level of significance were calculated and used to compare the means of treatments; interpretations were made accordingly.

RESULTS AND DISCUSSION

Callus induction

Experiments were conducted to determine the optimum concentration of the growth regulators 2,4-D and Kin or their combinations on the induction and quality of the embryogenic calli produced from mature seeds. The scutellum region of the seeds swelled in 7–8 days and the swollen scutellum developed into irregular callus after 3 weeks (**Fig. 3A**). Initially, most of the calli thus formed were non-embryogenic and did not have any nodular structures that are characteristic of embryogenic callus. After 3 more weeks of subculture in the dark, pockets of embryogenic calli with nodular structures appeared on the surface of the non-embryogenic callus (**Fig. 3B**). These calli were slightly different from the non-embryogenic callus which grew in size on the callus induction medium. Morphologically, embryogenic callus was usually light yellow to white, dry, compact and nodular, while non-embryogenic callus appeared watery, light yellow to tan and non-nodular. MS medium containing 2,4-D alone without the addition of Kin generally produced less embryogenic callus than those on medium containing 2,4-D plus Kin (**Fig. 3A**). The highest frequency of callus induction (54.1%) was recorded on callus induction medium 8 (**Table 1**) but the response to callusing reduced as the level of 2,4-D increased in the medium (**Fig. 1**).

Much work has been done on developing regeneration systems for rice using various explants including immature embryos (Christou *et al.* 1991; Li *et al.* 1993; Cooley *et al.* 1995) embryogenic calli derived from immature embryos (Li *et al.* 1993), embryogenic cell suspensions (Jain *et al.* 1996; Zhang *et al.* 1996), and mature seed-derived embryogenic call (Jiang *et al.* 2000; Oh *et al.* 2005; Kant *et al.* 2007; Karthikeyan *et al.* 2009). However, the preparation of a large quantity of immature embryos or embryogenic cell suspensions is labour intensive, whereas mature seed embryos are available all-year round (Jiang *et al.* 2000) and amenable to transformation (Tyagi *et al.* 2007). Scutellar-derived embryogenic callus from mature seeds is the best explant for *in vitro* regeneration and even for the production of transgenic rice (Hiei *et al.* 1994; Rashid *et al.* 1996). The regeneration ability of a cell is hindered by many factors like genotype and *in vitro* culture conditions like nutrients, hormone composition and explant type (Ramesh *et al.* 2009). Hence, an effort was made to develop a regeneration protocol using mature seed-derived embryogenic callus for *Indica* rice cv. 'PR 116'. Sivamani *et al.* (1996) reported the induction of embryogenic calli from the seeds of *Indica* cv. 'TN1'. The initiation of callus started on the scutellum region of the seed embryo within one week and grew in size over the next 3 weeks on callus induction medium. A similar reduction in quality and quantity of callus induction with an increase in 2,4-D was also observed in Japanese lawngrass (*Zoysia japonica*) (Liu *et al.* 2009). Moreover, Ramesh *et al.* (2009) reported necrosis of rice callus with an increasing concentration of 2,4-D. Inducing callus on MS

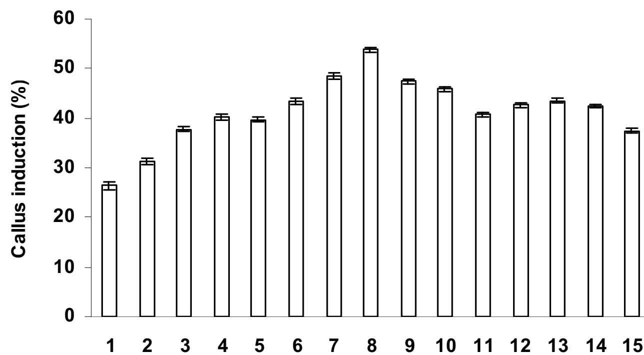

Fig. 1 Percent callus induction in *Indica* rice variety PR 116. n = 25. X-axis, 1-15 = Combination No. (**Table 1**).

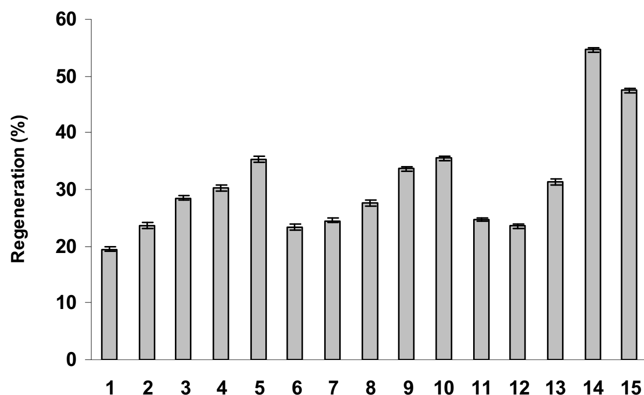

Fig. 2 Percent plant regeneration in *Indica* rice variety PR 116. n = 20. X-axis, 1-15 = Combination No. (**Table 1**).

medium supplemented with 2.5 mg/l 2,4-D in combination with 0.5 mg/l Kin enhanced callus growth and increased regeneration capacity (Al-Khayri *et al*. 1996). Tariq *et al.* (2008) observed the highest frequency of callus induction in three *Indica* rice varieties on N6 medium supplemented with 2.5 mg/l 2,4-D. In another study, Aqeel Ahmed *et al.* (2009) showed callus induction in *Indica* rice on MS medium supplemented with 2.5 mg/l 2,4-D and 0.5 mg/l Kin. Induction of embryogenic calli in rice is considered to be the most critical step. Several different media, including MS medium, have been used for rice tissue culture. Mostly 2,4-D (2-3 mg/l) has been used as the growth regulator for callus induction in rice (Visarda and Sarma 2002; Saharan *et al*. 2004; Lin and Zhang 2005; Tariq *et al*. 2008; Syaiful *et al*. 2009; Wani and Gosal 2010). The auxin 2,4-D, alone or in combination with cytokinins, is also widely used to enhance callus induction and maintenance (Castillo *et al.* 1998). The use of the amino acid Pro in the medium has been reported to be effective for the initiation and maintenance of embryogenic calli (Kavi Kishor *et al.* 1999; Saharan *et al.* 2004).

Shoot regeneration from calli

The embryogenic calli (**Fig. 3B**) were transferred onto regeneration media for shoot induction and incubated at 25°C under a 16-h photoperiod. Multiple shoot apices were observed after a 2-week incubation (**Fig. 3C**) and they grew into multiple shoots after a further 2-week culture (**Fig. 3D**). The regenerated shoots were sufficiently long and strong to be transferred to rooting medium for root initiation 4 weeks after transfer onto regeneration medium (**Fig. 3E**). The induction frequency of shoots was markedly influenced by different concentrations and combinations of PGRs. A higher percentage of shoot induction was achieved on regeneration medium 14 (MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l NAA and 0.5 mg/l Kin) (**Fig. 2**). The role of cytokinins like BAP and NAA in plant regeneration

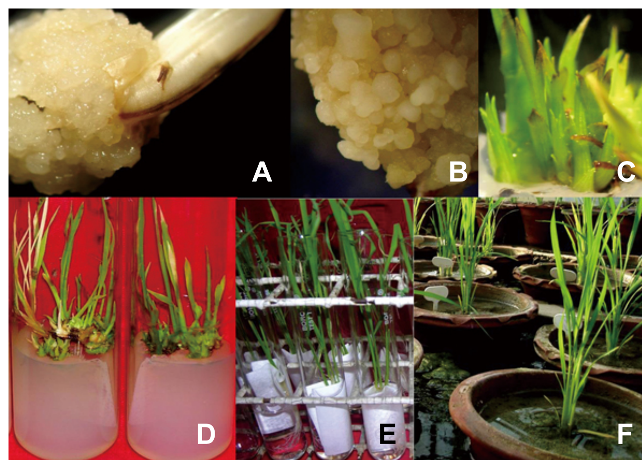

Fig. 3 Callus induction and plant regeneration in rice variety PR 116. (**A**) Scutellar-derived callus with seed; (**B**) Nodular embryogenic callus; (**C**) Shoot initiation from embryogenic callus on regeneration medium; (**D**) Shoot elongation; (**E**) Hardening of rooted plantlets; (**F**) Hardened plantlets transferred to earthen pots showing normal growth.

has been demonstrated in many published reports. The stimulatory effect of BAP in combination with NAA has been reported to facilitate regeneration in rice callus cultures (Boissot *et al.* 1990; Ramesh and Gupta 2005). In a recent report it has been mentioned that plant regeneration was achieved through embryogenic callus on MS medium supplemented with different concentrations of BAP and NAA (Karthikeyan *et al.* 2009; Ramesh *et al.* 2009). *Indica* types are often considered to be sensitive to tissue culture and less responsive to transformation due to poor callus production, low frequency of somatic embryogenesis, and few subsequent whole plants regenerated (Zaidi *et al.* 2006; Kumar *et al.* 2008). The regenerated plants in our study were normal green and no albinos were observed (**Fig. 3F**). For *in vitro* regeneration, the use of mature embryos rather than immature tissues as initial explants has distinctive advantages since embryogenic calli induced from mature seeds are suitable for gene delivery and genetic transformation, actively dividing and capable of regenerating into fertile plants (Jiang *et al.* 2000). The proliferation of embryogenic calli with high regeneration capacity through subculture is a prerequisite for the successful development of transgenic rice plants via callus culture. The occurrence of albino plants is a problem in tissue culture, especially when plants are developed from callus induced through anther/pollen culture (Torrizo *et al.* 1986; Raina *et al.* 1987) but in our case no albino plants were recorded as we used mature seed-derived embryogenic calli as the base material for plant regeneration. Likewise, no albino plants were recorded when plants were regenerated from mature seed-derived embryogenic rice callus (Hoque *et al.* 2007). Plantlets regenerated via tissue culture are usually very fragile and most of these die if directly transferred to field conditions because of the transplantation shock. Rooting was achieved on half-strength MS medium without PGRs (**Fig. 3E**). Regenerated plantlets were hardened and transferred to the soil in earthen pots and were kept in a glasshouse. Transfer of regenerated rice plants into sterile soil after hardening has also been reported earlier (Karthikeyan *et al.* 2009). The transferred plants became acclimatized to the soil and exhibited normal growth and flowering (**Fig. 3F**).

In summary, we have optimized a high frequency callus induction and regeneration protocol for high yielding commercial *indica* rice variety PR 116, which was shown to be a simple and highly reproducible approach with potential applicability for molecular breeding by the production of transgenic plants.

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