Studies on growth dynamics of embryogenic cell suspension cultures of commercially important *Indica* rice cultivars ASD16 and Pusa basmati

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Abstract

Significant efforts are being directed towards the improvement of rice using genetic manipulations. A good and reproducible system for recovering fertile *Indica* rice plants is imperative. The aim of this study was to study the influence of initial cell density on growth dynamics of suspension cultures. The cultures were initiated from 3-week-old embryogenic calli derived from mature seeds of *Indica* rice cultivars ASD16 and Pusa basmati. Growth kinetics of the rice cell suspensions were measured and the obtained data reveal that viable cells at 3% PCV using 20 ml liquid (N6D) medium and sub-culturing at 7-day intervals resulted in rapid increase in fresh and dry weights and the embryogenic competency of the cells were found to be high. The growth kinetics analysis revealed that ASD16 showed better efficiency for high frequency and viable somatic embryo formation as compared to Pusa basmati. The technique was found to be suitable for developing somatic embryos for both cultivars ASD16 and Pusa basmati, which can be used for many important applications including micropropagation and secondary metabolites production.

Keywords Rice · Somatic embryogenesis · Packed cell volume · Cell viability

Introduction

Rice is an important model system with economic and nutritional importance grown around the world (Khush 1997; Harlan 1998). Rice can be genetically modified by genetic transformation methods (Zhang and Wu 1988; Christou et al. 1991) such as electroporation, particle bombardment and agrobacterium-mediated genetic transformation. Efficient transgenic plant production has been reported for *Japonica* rice varieties, whereas the responses of *Indica* rice varieties have been reported scarcely due to its recalcitrance in tissue culture (Datta et al. 1992; Peng et al. 1992; Biswas et al. 1994). It is reported that cell suspension cultures were regarded as one of the potent explant sources to recover prodigious number of transgenic rice plants (Peschke and Phillips 1992; Zhang et al. 1996; Sathishkumar et al. 1997) and these cell suspension cultures of rice have been examined

from the physiological and cytological perspectives (Maeda 1969; Ohira et al. 1973; Igaue et al. 1980). Rapid cell proliferation of suspension cultures is an major advantage for genetic transformation studies in rice (Hiei et al. 1997). Several advantages of the plant cell culture includes, reduced risk of viral and bacterial toxin contamination, no chimeric cells, steady availability of proficient embryogenic cells, easy to purify, low cost, cheaper downstream processing and purification of metabolites secreted from the cells (Fischer et al. 1999). Hilliou et al. (1999) suggested that the viability of suspension cultures is an important aspect for cell kinetic studies and metabolite production. Synchronized homogeneous population can be achieved in cell suspension cultures. However, in general, suspension-cultured cells have been reported to have low genetic transformation efficiency as compared to large clusters of cells (Hiei et al. 1997; Urushibara et al. 2001). Compared to *Japonica* cultivars, applicability of the suspension culture system to a wide range of commercially important *Indica* cultivars has not been demonstrated so far due to its recalcitrance nature. Hence, the present investigation was aimed at understanding the growth dynamics of cell suspension cultures over periodical subcultures from seed derived embryogenic calli in two popular

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Indica rice cultivars, ASD16 and Pusa Basmati. This study was also aimed to find out the implication of inoculating 3 different packed cell volumes (PCV), so as to optimize the potent concentration that can result in maximum somatic embryogenesis. The outcome of this study will have direct implications on the genetic transformation efficiency in these commercially popular recalcitrant *Indica* rice cultivars.

Materials and methods

Plant materials

Two *Indica* commercial rice varieties, ASD16 and Pusa basmati were selected for the establishment of cell suspension cultures in this study. The seeds were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. Mature seeds of the two rice cultivars were used as explants.

Callus initiation

Rice mature seeds were dehusked manually and disinfected in 70% ethanol for 3 min, then in a 4% sodium hypochlorite solution with few drops of Tween 20 for 5 min followed by wash with sterile distilled water for 5 times. The surface sterilized seeds were blot dried using sterile filter paper on a plastic Petri dish. Fifteen seeds were placed in a Petri dish containing 30 ml of N6 culture medium (Chu 1978) supplemented with sucrose 30 g l^{-1} , casein hydrolysate 1.0 g l^{-1} , proline 0.5 g l^{-1} , glutamine 0.5 g l^{-1} and 100 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction. The cultures were incubated at $25 \pm 2^\circ \text{C}$, 16-h photoperiod for 3 weeks.

Establishment of cell suspension cultures

The actively proliferating calli of both ASD16 and Pusa basmati were used for cell suspension initiation. Calli (21-day-old) from both the cultivars were observed under the microscope and the compact embryogenic regions loosely attached to the surface of the calli were carefully removed and inoculated in the liquid medium. Approximately 100 mg of tissues (fresh wt) was transferred into 100 ml Erlenmeyer flasks, each flask containing 25 ml liquid N6 medium supplemented with 2 mg l^{-1} 2,4-D. The cultures were incubated on a rotary platform shaker (125 rpm) at $25 \pm 2^\circ \text{C}$, 16 h photoperiod. Suspension cultures were subcultured by replacing with 10–15 ml of fresh medium for every 4 days along with 10 ml of spent medium. After 4 weeks, the suspension cultures were subcultured on a 7-day interval. Separation of primary calli from the fine filtrate of suspension initiated after the second week was done using a $500 \mu\text{m}$ metallic mesh.

During every subculture, observations on fresh weight, dry weight, PCV was recorded by taking a subset sample from each suspension culture. A total of 10 subsets were observed and recorded over a 10-week period.

Growth kinetics of cell suspension cultures

Absorbance

To perceive the cell density of the suspension culture, absorbance values were recorded at 485 nm using UV spectrophotometer (Thermo Fisher, USA). The measurements were carried out every 7 days. Suspension cultures (1 ml) from experimental flasks were pipetted out to the cuvette having 2 cm light path. The control suspension cultures were also measured under the same intervals. All the experiments were carried out at the same time point at each interval.

Fresh weight and dry weight analysis of suspension cultures

A pre-weighed sterile Whatman No. 3 filter paper was used to filter the cell suspension and the cells retained on the filter paper were then air dried till the entire media drained out. After drying, the weights of the filter paper along with the cells were recorded. The actual weight of the cells was calculated by deducting the weight of filter paper from the total weight recorded. The samples were then dried in an oven at 60°C for 4–6 h to record the dry weight. The weight of oven dried filter paper with cells was recorded. By deducting the weight of dry filter paper from the value of oven dried paper with the cells actual dry weight of cells was calculated. All the experiments were carried out at the same time points at each interval.

Cell growth assessment

To estimate the growth rate of a culture, the PCV was measured. A fixed volume of suspension culture was transferred to a 15-ml graduated centrifuge tube. Spun it down in a clinical centrifuge at $200 \times g$ for 5 min. PCV is the volume of the pellet and is expressed as a percentage of the volume of the culture used. Initial inoculum densities of PCV (1.0, 3.0 and 5.0%) were used for culturing in N6 media. For each cell density, five replicates were used. The change in cell volume was recorded every 3 days. All experiments were carried out at the same time points at each interval. The percentage of PCV (% PCV) was measured by regularly transferring the content of Erlenmeyer flasks to sterile centrifuge graduated tubes and using the following formula,

$$\% \text{ PCV} = \frac{\text{Volume of packed cells} \times 100}{\text{Total volume (cells + medium)}}$$

Determination of embryogenic cells and cell viability

A drop of 3–4-weeks-old suspension cells were transferred onto a clean slide, covered with a cover slip. Both embryogenic and non-embryogenic types of cells were identified and recorded. Viability of suspension cell cultures was evaluated using fluorescein diacetate (FDA) staining. FDA solution was prepared by dissolving 5 mg of FDA in 1 ml acetone (stored at -20°C). A drop of diluted stain (prepared by adding 0.1 ml stock solution to 5 ml basal N6 medium) and a drop of suspension culture was placed on a glass slide and mixed. After 2–3 min, stained culture was observed under a fluorescent microscope (Nikon, Japan) with filter system B (excitation range between 405 and 490 nm and emission above 515 nm).

Plantlet regeneration from callus derived suspension culture

Six-week-old microcalli with 2–5 mm diameter, were selected and transferred to 100 ml Erlenmeyer flask. The calli were rinsed thrice with 100 ml sterile distilled water to remove the suspension medium. The calli were blot dried in a Whatman filter paper. The calli of both the cultivars were then plated in 90 mm Petri dish containing 20 ml of regeneration medium (MS medium supplemented with 2.0 mg l^{-1} 6-benzylaminopurine (BAP); 1.0 mg l^{-1} naphthaleneacetic acid (NAA) and 30 g l^{-1} sucrose). The plates were incubated at $25 \pm 2^{\circ}\text{C}$ with 16-h photoperiod until the emergence of green shoots, which occurred between 5 and 14 days. Well grown shootlets were transferred to rooting media (1/2 strength MS medium without growth hormones) and incubated at $25 \pm 2^{\circ}\text{C}$ at 16-h photoperiod.

Statistical analysis

All the samples were analyzed and the values were expressed as means of analyzed samples ($n=10$) $\pm$ SD. All the data were further analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($P<0.05$) with the aid of SPSS (17 version) statistical package program. $P<0.05$ was considered as indicative of significance, as compared to either control or between the varieties.

Results and discussion

Growth parameters

Fresh, dry weight and absorbance

The seeds of both cultivars were plated on to callus induction media and first subculture was done after 7 days. Many reports have highlighted the effectiveness of N6 medium over MS medium for callus initiation as well as for somatic embryogenesis (Koetje et al. 1989; Ozawa and Komamine 1989; Hiei and Komari 2008). The embryogenic calli from 21-days-old explants (Fig. 1c, d) was used for the initiation of cell suspension culture. During weekly routine subcultures the suspension was observed for its friability, compactness and single cell nature. A stable embryogenic cell suspension culture will be established after 2 subcultures (Fig. 2 a, b). The suspension can be maintained by routine subculture at an interval of 7–15 days with the dilution ratio of the suspension:fresh liquid medium at 1:2. It was necessary to change the liquid medium to reduce browning of cells. The cultures were composed of large clumps of highly vacuolated irregular-shaped cells along with small groups of rounded cytoplasmic cells. Even though majority of cells continued to grow in large aggregates in cultures, enrichment of the cells was observed with periodic replacement of the liquid medium and sieving to remove large groups of cells. The increase in fresh and dry weights at different stages of growth of suspension cultures were recorded and are shown in Fig. 3a and b. No growth was observed during the lag phase, which lasted for 2 weeks (day 1–14). This was followed by 4–5 weeks of exponential phase of cell development with rapid weight gain. In 2 weeks preceding, the growth of cells was observed to be in a steady linear phase. The cultures registered highest fresh weight (3350 mg/ml of suspension culture) after eight subcultures (60 days). The doubling phase for growth of cells in suspension from ASD16 and Pusa basmati was recorded between 3 and 5 weeks. The dry weight observations also followed the same pattern as that of ASD16. It can be suggested that the optical density is proportional to cell number in the suspension cultures. The growth of cultures was found to be in correlation with that of fresh, dry weight and the absorbance recorded which followed a similar progression (Fig. 4). It is, therefore, suggested that unless warranted for specific objective, measurement of absorbance can be relied upon to perceive the growth kinetics of suspensions cultures.

Fig. 1 Various growth phases of ASD16 and Pusa basmati. Callus culture from **a** ASD16 and **b** Pusa basmati at 14 days. Callus culture from **c** ASD16 and **d** Pusa basmati at 21 days. Scale bar=0.5 cm

Growth kinetics of cell suspension culture

Indica cell line IR43 has been previously used successfully for cell cycle studies (Biswas et al. 1994; Sauter 1997; Fabian-Marwedel et al. 2002). The callus induced from mature seeds when transferred to liquid medium with 2, 4-D resulted in disintegration of callus mass followed by fine suspension. A typical initial log phase followed by exponential phase, linear phase and progressive declination phase were observed during the cell suspension culture of rice in the cultivars viz., ASD16 and Pusa basmati. Slow growth rate and large formation of clumps were reported to be major obstacle in development and maintenance of suspension cultures (Thompson et al. 1986). In our investigation, slower initiation of suspension cultures as well as a few clump formations were

observed. On careful sieving and selecting promising cell aggregates during the initial subcultures ensured the multiplication or proliferation of pro embryogenic calli from which embryogenic suspension can be established. Soon after acclimatization after the lag phase, there was a rapid proliferation or multiplication during exponential phase which was evident from increase in fresh and dry weights in both the varieties investigated. The exponential phase lasted till 9 weeks in cv. ASD16 and 8 weeks in cv. Pusa basmati. After 8 weeks, growth rates were passive as evident by the linear growth phase which could be a trait to possible nutrient media depletion. In general, continuous growth of the cultures in the same culture medium is not recommended, because of the possibility of accumulation of toxic substances in the culture medium which affects the

Fig. 2 Growth stages of embryogenic cell suspension cultures. ECS of Cvs. **a** ASD16 **b** Pusa basmati at 4 weeks after initiation. Scale bar=2.0 mm

Fig. 3 **a** Fresh weight **b** Dry weight analysis of suspension cultures—ASD16 and Pusa basmati. Results of experiments were expressed as the mean $\pm$ SD (10 replicates)

Fig. 4 Absorbance values of ASD16 and Pusa basmati suspension cultures. Results of experiments were expressed as the mean $\pm$ SD (10 replicates)

growth of the cultures. Hence, sub-culturing of the callus at optimal intervals is apt (Lima et al. 2008).

Effect of cell density in the multiplication of embryogenic cell suspensions

An initial increase in 3% PCV with the embryogenic cell suspension was maintained at various cell density levels (1, 3, and 5%) of the rice cultivars, ASD16 and Pusa basmati was observed (Fig. 5a, b). With 3% PCV, cell proliferation was better than other two density levels tested. On observation over a period of 30 days, ASD16 and Pusa basmati resulted in highest PCV value of 6.1 and 5.9 ml, respectively, with the initial suspension inoculum of 3% PCV. The overall growth rates of the cell suspension at 1 and 5% PCV were slow and resulted in

Fig. 5 PCV in suspension cultures of rice cultivars. **a** ASD16 **b** Pusa basmati. Results of experiments were expressed as the mean $\pm$ SD (10 replicates)

PCVs of 1.9 and 3.6 ml in cv. ASD16 and 1.9 and 3.3 ml PCV in cv. Pusa basmati, respectively. The present study showed that exponential growth phase without deterioration in the quality of embryonic cells was observed from 18 to 30 days. A rapid growth of the suspension established in the different subculture cycles was observed consistently at 3% PCV in both cvs. of *Indica* rice. It was found that 3% PCV was best with active multiplication of embryogenic cells in suspension as compared to 1 and 5% PCV. Similar results were obtained in the suspension established from male flower buds of banana (Roux et al. 2004). In our study, growth rates of suspension cultures were slower in 1 and 5% PCV when compared to 3% PCV. The cultures initiated with 1 and 5% needed a long period to enter the exponential phase when compared to the 3% PCV inoculum. The present results indicate that to develop an ideal embryogenic cell suspension cultures in recalcitrant rice cultivars ASD16 and Pusa basmati the ideal approach will be initiating cell suspension with 3% PCV and sub-culturing at 7-days interval.

Cell viability

On observing the suspension cultures under microscope, the embryogenic as well as the non-embryogenic cells could be observed. Cells with degenerate nucleus, cells with well-developed cytoplasm and structures were also visualized (Fig. 6). Viable embryogenic clumps stained green were seen when these suspension cultures were examined by FDA staining under a fluorescent microscope (Fig. 7). Viable cells labeled with FDA were visible and exhibited green-yellow fluorescence (Fig. 7b), whereas dead and vacuolated cells failed to stain and remained yellow.

Plantlet regeneration from callus derived suspension culture

The embryogenic calli derived from the cells suspensions of both cultivars ASD16 and Pusa basmati readily regenerated and formed shoots (Fig. 8) within 8–14 days of incubation on plant regeneration media. Once the individual shoots reached a height of 2–3 cm (Fig. 8c), they were transferred to the rooting media (Fig. 8d).

Conclusion

Rice suspension cultures offer prodigious target materials for genetic transformation and this system can help in rice germplasm improvement by introducing agronomically beneficial genes into recalcitrant rice cultivars. After ascertaining the viability by staining procedures, we concluded that the suspension cultures initiated using 3% PCV with N6 medium to attain the high frequency proliferation and somatic embryogenesis. The growth kinetics including fresh, dry weight analysis revealed that ASD16 showed better efficiency when compared to Pusa basmati. Thus, the system described can be used for somatic hybridization studies, genetic transformation and production of pharmaceutical/commercial proteins. The *Indica* rice suspension culture system can be a probable tool as well as a model system for biological and physiological studies that will eliminate the rice from recalcitrance nature list in future.

Fig. 6 Embryogenic and non-embryogenic cells in suspension (20× magnification). **a** Embryogenic cells with distinct cell wall, cytoplasm, and well-developed nucleus **b** Proliferating cells **c** and **d** Cell aggregates 10 days after suspension initiation. Scale bar = 0.1 mm

Fig. 7 FDA staining of somatic embryos. **a** Viable cells emitting green fluorescence. **b** Viable cells stained green (indicated as →) and non-viable cells stained fluorescent yellow (indicated as ←)

Fig. 8 Regeneration from embryogenic suspension derived cells in both cultivars after 2 weeks. **a** ASD16 **b** Pusa basmati **c** individual shoots of both ASD16 and Pusa Basmati plated on to regeneration medium **d** Shoots transferred to rooting media. Scale bar = 1 cm

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Author contributions SS designed the work, executed experiments and prepared the manuscript. VR did the experimental analysis. SN contributed substantially to the manuscript. RS mobilized the funds and critically evaluated the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Biswas GG, Iglesias VA, Datta SK, Potrykus I (1994) Transgenic Indica rice (*Oryza sativa* L.) plants obtained by direct gene transfer to protoplasts. *J Biotechnol* 32(1):1–10
- Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Nat Biotechnol* 9(10):957–962
- Chu CC (1978) The N6 medium and its applications to anther culture of cereal crops. *Proceedings of Symposium plant tissue culture*. Sci Press, Peking, pp 43–50
- Datta SK, Datta K, Soltanifar N, Donn G, Potrykus I (1992) Herbicide-resistant Indica rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplasts. *Plant Mol Biol* 20(4):619–629

- Fabian-Marwedel T, Umeda M, Sauter M (2002) The rice cyclin-dependent kinase-activating kinase R2 regulates S-phase progression. *Plant Cell* 14(1):197–210
- Fischer R, Liao YC, Hoffmann K, Schillberg S, Emans N (1999) Molecular farming of recombinant antibodies in plants. *Biol Chem* 380(7–8):825–839
- Harlan JR (1998) *The living fields: our agricultural heritage*. Cambridge University Press, Cambridge
- Hiei Y, Komari T (2008) *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat Protoc* 3(5):824–834
- Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Mol Biol* 35(1–2):205–218
- Hilliou F, Christou P, Leech MJ (1999) Development of an efficient transformation system for *Catharanthus roseus* cell cultures using particle bombardment. *Plant Sci*. 140(2):179–188
- Igaue I, Shimizu M, Miyauchi S (1980) Formation of a series of myo-inositol phosphates during growth of rice plant cells in suspension culture. *Plant Cell Physiol* 21(2):351–356
- Khush GS (1997) Origin, dispersal, cultivation and variation of rice. *Plant Mol Biol* 35:25–34
- Koetje DS, Grimes HD, Wang YC, Hodges TK (1989) Regeneration of indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. *J Plant Physiol* 135(2):184–190
- Lima EC, Paiva R, Nogueira RC, Soares FP, Emrich EB, Silva ÁAN (2008) Callus induction in leaf segments of *Croton urucurana* Baill. *Ciência Agrotec* 32(1):17–22
- Maeda E (1969) Multiplication of rice cells freely suspended in vitro. *Proc Crop Sci Soc Japan* 38(3):535–546
- Ohira K, Ojima K, Fujiwara A (1973) Studies on the nutrition of rice cell culture I. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiol* 14(6):1113–1121
- Ozawa K, Komamine A (1989) Establishment of a system of high-frequency embryogenesis from long-term cell suspension cultures of rice (*Oryza sativa* L.). *Theor Appl Genet* 77(2):205–211
- Peng J, Kononowicz H, Hodges TK (1992) Transgenic indica rice plants. *Theor Appl Genet* 83(6–7):855–863
- Peschke VM, Phillips RL (1992) Genetic implications of somaclonal variation in plants. *Adv Genet* 30:41–75
- Roux NS, Toloza A, Dolezel J, Panis B (2004) Usefulness of embryogenic cell suspension cultures for the induction and selection of mutants in *Musa* spp. In: *Banana improvement: cellular, molecular biology and induced mutations*. Science Pub Inc., USA, pp 33–43
- Sathishkumar R, Agrawal S, Manoharan K (1997) Particle mediated DNA delivery and transient expression of GUS gene in plated cells of rice. *Biol Plant* 40(2):305–309
- Sauter M (1997) Differential expression of a CAK (cdc2-activating kinase)-like protein kinase, cyclins and cdc2 genes from rice during the cell cycle and in response to gibberellin. *Plant J* 11(2):181–190
- Thompson JA, Abdullah R, Cocking EC (1986) Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. *Plant Sci* 47(2):123–133
- Urushibara S, Tozawa Y, Kawagishi-Kobayashi M, Wakasa K (2001) Efficient transformation of suspension-cultured rice cells mediated by *Agrobacterium tumefaciens*. *Breed Sci* 51(1):33–38
- Zhang W, Wu R (1988) Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. *Theor Appl Genet* 76(6):835–840
- Zhang S, Chen L, Qu R, Marmey P, Beachy R, Fauquet C (1996) Regeneration of fertile transgenic indica (group 1) rice plants following microprojectile transformation of embryogenic suspension culture cells. *Plant Cell Rep* 15(7):465–469