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ScienceDirect

Rice Science, 2015, 22(6): 283–289

Proline and Glutamine Improve *in vitro* Callus Induction and Subsequent Shooting in Rice

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Abstract: This study was conducted to evaluate the effects of proline and glutamine on *in vitro* callus induction and subsequent regeneration and to develop a reproducible and highly efficient plant regeneration protocol in four rice genotypes, viz. Pawana, Jaya, Indrayani and Ambemohar. Considerable variation in response to plant growth regulators and amino acid supplements used was observed in all the four genotypes. Medium supplemented with proline and glutamine was shown to be superior to medium without proline and glutamine. The best callusing from mature embryo was observed on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 500 mg/L proline and 500 mg/L glutamine. Shoot induction was higher in the callus obtained from medium supplemented with 500 mg/L proline and 500 mg/L glutamine. The highest shoot regeneration frequency (83.2%) was observed on MS medium with 2.0 mg/L benzylaminopurine, 0.5 mg/L 1-naphthaleneacetic acid, 500 mg/L proline, and 500 mg/L glutamine in the callus obtained from MS medium supplemented with 2.0 mg/L 2,4-D, 500 mg/L proline and 500 mg/L glutamine. Among the four genotypes, Pawana has the highest regeneration efficiency (83.2%), whereas the regeneration efficiency of the rest three rice genotypes was in the range of 32.0% to 72.3%. This optimized regeneration protocol can be efficiently used for *Agrobacterium* mediated genetic transformation in rice.

Key words: callus induction; glutamine; proline; rice; *Agrobacterium* mediated genetic transformation; 2,4-dichlorophenoxyacetic acid; Murashige and Skoog medium

Rice (*Oryza sativa* L.) is an economically important staple food for more than 80% of people in Asia and the most extensive cultivated cereal crop in the world. Its growth and productivity are adversely affected by various abiotic and biotic stresses, which prevents crop from reaching its full genetic potential and limits crop yield worldwide. With constant increase in global population, it became essential to develop new rice genotype tolerant to abiotic and biotic stress with high yield potential to fulfill the increasing demand for food. Genetic manipulation contributes to the agronomic improvement of rice by conquering some of the limitations in traditional breeding methods. There is a great potential for genetic manipulation in rice to enhance productivity by increasing the resistance to pest and disease and the tolerance to environmental stress.

Development of a reliable and efficient regeneration system, including callus induction and differentiation as well as plant regeneration, is pre-requisite for a tissue culture-based transformation system for developing transgenic rice genotypes with useful genes. *In vitro* regeneration in rice from various explants, such as mature seed (Ge et al, 2006), immature seed (Lee and Huang, 2013), leaf (Karthikeyan et al, 2011), shoot apex (Dey et al, 2012), and root (Hoque and Mansfield, 2004), has been reported previously. However, highly genotype specific morphogenetic response is a major limitation with rice tissue culture. Therefore, optimization of regeneration protocol for desired genotype is essential.

In vitro regeneration depends on the composition and concentration of the basal salt, growth regulators and the organic components (Ge et al, 2006). Proline and glutamine are supplemented in the culture medium as an organic nitrogen source. Inclusion of

Received: 16 February 2015; **Accepted:** 29 June 2015

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Peer review under responsibility of China National Rice Research Institute

http://dx.doi.org/10.1016/j.rsci.2015.11.001

proline and glutamine into callus medium has been shown to promote callusing in rice (Chowdhry et al, 1993; Ge et al, 2006; Shahsavari, 2011). Quality of calli might be a key factor for the success of regeneration and transformation, and selecting the most suitable medium to improve the quality of calli might be a key step for the success of transformation (Lin and Zhang, 2005). In view of these factors, the present investigation has been undertaken to evaluate the effects of proline and glutamine on callus induction and subsequent regeneration to develop an efficient plant regeneration protocol using rice mature embryo explant in four rice genotypes.

MATERIALS AND METHODS

Rice materials

Mature seeds of four indica rice genotypes, namely Ambemohar, Indrayani, Jaya and Pawana, were obtained from the Agriculture Research Station, Radhanagari, India. Manually dehusked seeds were surface sterilized with 70% ethanol for 30 s, followed by 0.1% mercuric chloride ($HgCl_2$) solution for 6 min. The seeds were further rinsed five times with sterile distilled water.

Callus culture and plant regeneration

For callus induction, sterilized seeds were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.8% agar and different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) (Table 1). All cultures were maintained at (28 ± 2) °C under a 16 h photoperiod provided by cool white fluorescent lamps. Additionally, to investigate the effects of proline and glutamine on callus induction, various combinations of proline and glutamine were used (Table 1). Thirty days after incubation on callus induction medium, calli were transferred on MS medium supplemented with different concentrations and combinations of benzylaminopurine (BAP), kinetin, thidiazuron (TDZ), 1-naphthaleneacetic acid (NAA), proline and glutamine (Table 1). During the course of culture, the explants were sub-cultured after every 15 d to fresh callus induction medium or shooting medium of the same composition. The shoots (5–7 cm) were transferred to MS medium devoid of growth regulator for root formation. Plantlets with well-developed roots were transferred to plastic pots containing perlite. Those were initially covered with plastic bags for 5–7 d, and kept at polycarbonated polyhouse. Plants were irrigated with 1/2 MS solution for 14 d and finally transferred to pot containing soil and cow dunk (4:1), and those plants were irrigated with water at regular intervals.

Statistical analysis

The effects of genotype and hormone treatment on callus induction were tested by using two-way analysis of variance (ANOVA) in the INDOSTAT program. Further effects of genotype, callusing medium and shooting medium on *in vitro* regeneration were tested by using three-way ANOVA. All treatments of regeneration experiments had three replicates with 40 explants in each replication.

RESULTS

Optimization of 2,4-D concentration for callus induction

Callus induction of rice is known to depend on 2,4-D in the induction medium. Therefore, MS medium supplemented with five different concentrations of

Table 1. Media tested for callus induction and shoot regeneration in rice.

Medium number	Callus induction				Medium number	Shoot regeneration						
	MS	2,4-D (mg/L)	Proline (mg/L)	Glutamine (mg/L)		MS	Kinetin (mg/L)	BAP (mg/L)	TDZ (mg/L)	NAA (mg/L)	Proline (mg/L)	Glutamine (mg/L)
C_1	1	0.5	0	0	S_1	1	2.0	0	0	0	0	0
C_2	1	1.0	0	0	S_2	1	0	2.0	0	0	0	0
C_3	1	1.5	0	0	S_3	1	0	0	2.0	0	0	0
C_4	1	2.0	0	0	S_4	1	2.0	0	0	0.5	0	0
C_5	1	2.5	0	0	S_5	1	0	2.0	0	0.5	0	0
C_6	1	2.0	500	0	S_6	1	0	0	2.0	0.5	0	0
C_7	1	2.0	0	500	S_7	1	2.0	0	0	0.5	500	500
C_8	1	2.0	500	500	S_8	1	0	2.0	0	0.5	500	500
					S_9	1	0	0	2.0	0.5	500	500

MS, Murashige and Skoog; 2,4-D, 2,4-dichlorophenoxy acetic acid; BAP, Benzylaminopurine; TDZ, Thidiazuron; NAA, 1-naphthaleneacetic acid.

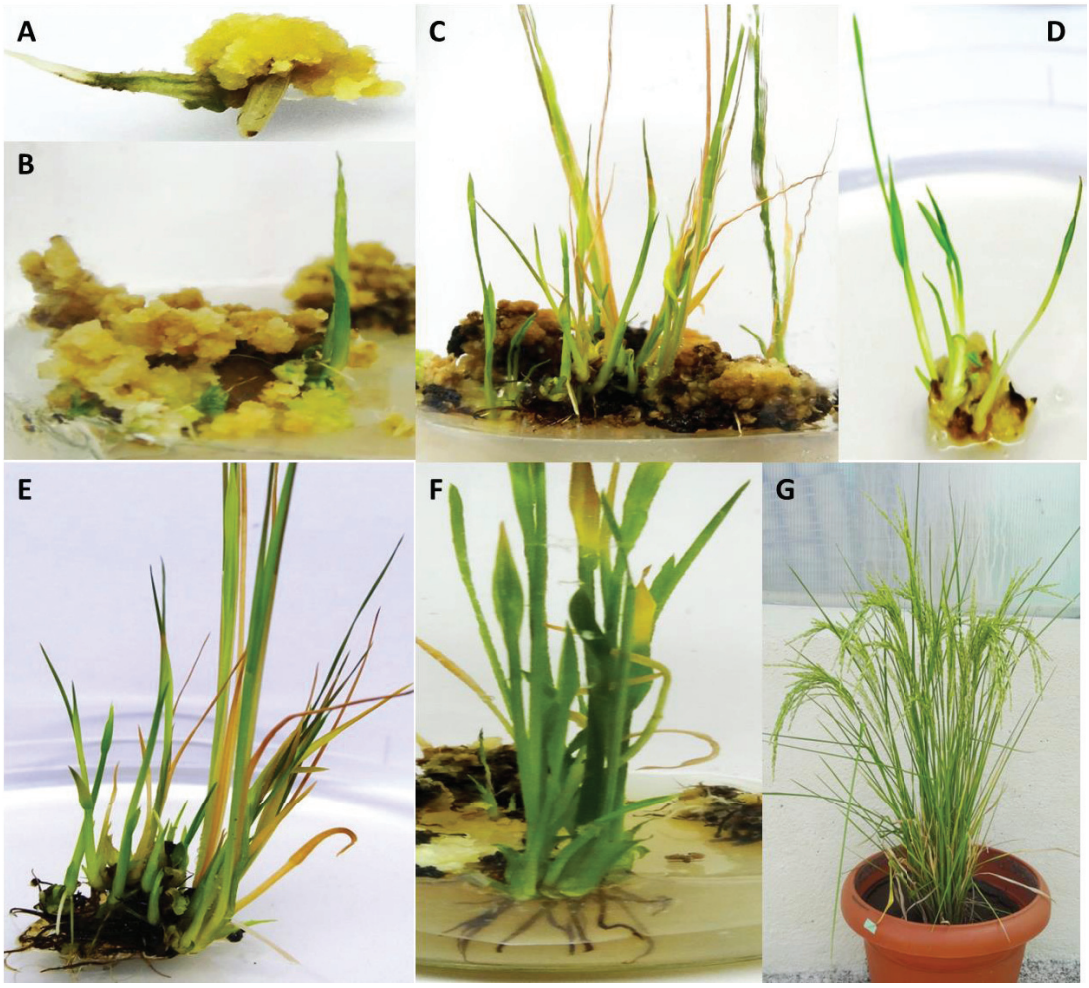

Fig. 1. ***In vitro*** **regeneration in rice using mature seeds of Pawana.**

A, Callusing on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D), 500 mg/L proline and 500 mg/L glutamine (30 d); B, Shoot initiation on MS medium supplemented with 2.0 mg/L benzylaminopurine (BAP) and 0.5 mg/L 1-naphthaleneacetic acid (NAA); C, Multiple shoot induction on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA; D, Subcultring of shoot clumps on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA; E, Shoot elongation on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA; F, Rooting on the same medium; G, Mature rice plant.

2,4-D alone were tested for their efficiency for callus induction from mature seeds (Table 1). Within one week, swelling of the scutellum and formation of mass on the surface of scutellum from cultured mature seeds were observed. After three weeks of incubation, scutellum developed into irregular callus (Fig. 1-A). The highest average callus induction (78.4%) among 2,4-D alone callus induction medium was observed on the MS medium supplemented with 2.0 mg/L 2,4-D, but the response for callusing was slightly reduced to 74.2% as the level of 2,4-D increased to 2.5 mg/L in the medium (Table 2).

Similarly, the highest average fresh callus weight (61.8 mg) among 2,4-D alone callus induction medium was observed on the MS medium supplemented with 2.0 mg/L 2,4-D (Table 3).

Effects of proline and glutamine on callusing and subsequent plantlet regeneration

The effects of proline and glutamine to cultures of rice mature seeds undergoing *in vitro* callusing and subsequent plantlet regeneration was investigated. On MS medium supplemented with 500 mg/L proline and 500 mg/L glutamine, an average 85.3% callusing was observed, whereas on medium devoid of proline and glutamine, average callusing was reduced to 78.4%. Similarly inclusion of proline and glutamine resulted in increasing fresh weight of callus (Table 2). The highest fresh callus weight (94.9 mg) was observed on MS medium supplemented with 2.0 mg/L 2,4-D, 500 mg/L proline and 500 mg/L glutamine in Pawana, whereas the lowest fresh callus weight (34.7 mg) was

Table 2. Effect of culture medium on percent callus induction from mature seed explants in rice (Mean ± *SE*). %

Callusing medium	Ambemohar	Indrayani	Jaya	Pawana	Average (Medium)
C_1	40.0 ± 0.48 a	65.2 ± 0.56 e	68.1 ± 0.52 f	78.1 ± 0.42 i	62.9
C_2	52.1 ± 0.42 b	72.3 ± 0.48 g	72.9 ± 0.49 g	83.7 ± 0.62 j	70.3
C_3	56.2 ± 0.62 c	74.0 ± 0.58 g	77.3 ± 0.78 h	87.2 ± 0.74 k	73.7
C_4	60.3 ± 0.54 d	79.2 ± 0.48 i	82.1 ± 0.62 j	91.9 ± 0.57 l	78.4
C_5	56.0 ± 0.62 c	74.8 ± 0.68 h	75.4 ± 0.56 h	90.4 ± 0.64 l	74.2
C_6	64.8 ± 0.69 e	83.8 ± 0.72 j	86.2 ± 0.48 j	94.8 ± 0.75 m	82.4
C_7	64.1 ± 0.79 e	84.1 ± 0.68 j	84.3 ± 0.62 j	95.2 ± 0.81 m	81.9
C_8	68.7 ± 0.58 f	86.2 ± 0.64 j	89.2 ± 0.72 k	97.0 ± 0.64 m	85.3
Average (Genotype)	57.8	77.5	79.4	89.8	76.1
Significance of two-way ANOVA					
Medium (M)		$P < 0.001$			
Genotype (G)		$P < 0.001$			
M × G		$P < 0.001$			

Means followed by the same letter are not significantly different based on analysis of variance at the 0.05 level.

Table 3. Effect of callusing medium on fresh callus weight after 30 d obtained from mature seed explants in rice (Mean ± *SE*). mg

Callusing medium	Ambemohar	Indrayani	Jaya	Pawana	Average (Medium)
C_1	34.7 ± 0.35 a	54.6 ± 0.54 f	43.9 ± 0.42 c	81.9 ± 0.56 i	53.8
C_2	36.8 ± 0.44 a	56.3 ± 0.49 f	45.8 ± 0.58 c	85.5 ± 0.45 j	56.1
C_3	38.6 ± 0.56 b	58.6 ± 0.71 g	47.5 ± 0.52 d	87.8 ± 0.67 k	58.1
C_4	43.9 ± 0.54 c	60.9 ± 0.57 h	51.5 ± 0.44 e	90.7 ± 0.73 l	61.8
C_5	39.9 ± 0.48 b	59.6 ± 0.54 g	48.7 ± 0.48 d	88.8 ± 0.59 k	59.3
C_6	47.9 ± 0.57 d	63.8 ± 0.36 h	56.1 ± 0.63 f	93.5 ± 0.52 m	65.3
C_7	47.0 ± 0.48 d	62.8 ± 0.55 h	55.0 ± 0.57 f	92.0 ± 0.45 l	64.2
C_8	49.4 ± 0.36 e	65.9 ± 0.46 i	59.6 ± 0.53 g	94.9 ± 0.67 m	67.5
Average (Genotype)	42.3	60.3	51.0	89.4	60.7
Significance of two-way ANOVA					
Medium (M)		$P < 0.01$			
Genotype (G)		$P < 0.001$			
M × G		$P < 0.001$			

Means followed by the same letter are not significantly different based on analysis of variance at the 0.05 level.

observed on MS medium supplemented with 0.5 mg/L 2,4-D in Ambemohar. Callus quality was also improved by this treatment resulting in substantially higher callus regeneration rates. Among the medium supplemented with either proline or glutamine, no significant difference in callusing and fresh weight of callus was observed (Table 3).

To determine the regeneration ability, the initiated calli were transferred to a regeneration medium. Following transfer of the calli onto regeneration media, shoot and root axis began to develop within 2–3 weeks (Fig. 1-B). Regenerated shoots were sub-cultured after every two weeks (Fig. 1-C, -D). We compared plant regeneration ability among calli obtained from four callus induction medium. It was observed that the regeneration frequency was greatly influenced by medium used for callus induction with the best results (83.2%) achieved using the MS medium supplemented with 2.0 mg/L 2,4-D, 500 mg/L proline and 500 mg/L glutamine (Table 4).

Effect of growth regulators on plantlet regeneration from callus

No shoot initiation from callus was observed on the medium supplemented with 2,4-D even after 45 d and 60 d of culture, indicating the importance of cytokinins for shoot induction. Among three cytokinins used, the highest plant regeneration was observed on medium supplemented with BAP, followed by medium supplemented with kinetin and TDZ. No significant morphological difference during shoot regeneration among different shooting medium supplemented with BAP and kinetin was observed. However, with only TDZ supplemented medium, blackening of callus after four weeks of culture was observed. Yellowing of shoots on prolong culture (more than eight weeks) was more prominent on TDZ supplemented medium as compared to BAP and kinetin supplemented medium. Addition of NAA in shooting medium resulted in increase in regeneration frequency. The highest plantlet regeneration (83.2%) was observed on MS medium supplemented

Table 4. Effect of culture medium on regeneration efficiency from callus in rice (Mean ± *SE*). %

Shooting medium	Callusing medium			
	C_4	C_6	C_7	C_8
Ambemohar				
S_1	37.0 ± 0.26 b	41.9 ± 0.34 d	40.2 ± 0.28 c	43.7 ± 0.56 d
S_2	42.7 ± 0.32 d	46.2 ± 0.62 e	46.8 ± 0.56 e	51.9 ± 0.45 g
S_3	32.0 ± 0.53 a	35.1 ± 0.58 b	35.3 ± 0.56 b	38.1 ± 0.62 c
S_4	40.1 ± 0.45 c	42.0 ± 0.52 d	42.8 ± 0.43 d	47.0 ± 0.39 e
S_5	50.3 ± 0.33 f	52.1 ± 0.38 g	54.1 ± 0.38 h	58.4 ± 0.47 i
S_6	39.1 ± 0.62 c	42.3 ± 0.44 d	42.2 ± 0.44 d	44.2 ± 0.53 e
S_7	40.4 ± 0.39 c	42.6 ± 0.54 d	43.7 ± 0.52 d	47.1 ± 0.46 f
S_8	51.0 ± 0.46 g	52.8 ± 0.48 g	55.2 ± 0.58 h	59.0 ± 0.48 i
S_9	40.2 ± 0.45 c	43.0 ± 0.68 d	43.2 ± 0.37 d	47.2 ± 0.54 e
Indrayani				
S_1	42.1 ± 0.44 d	45.0 ± 0.68 e	44.1 ± 0.46 d	49.0 ± 0.63 f
S_2	53.2 ± 0.36 g	57.1 ± 0.63 i	56.0 ± 0.44 h	60.2 ± 0.44 j
S_3	40.1 ± 0.48 c	42.0 ± 0.46 d	43.0 ± 0.57 d	46.0 ± 0.57 e
S_4	47.7 ± 0.53 f	54.0 ± 0.48 h	54.0 ± 0.75 g	57.1 ± 0.52 i
S_5	56.9 ± 0.36 i	61.2 ± 0.53 j	59.0 ± 0.48 i	65.1 ± 0.52 l
S_6	45.3 ± 0.38 e	50.9 ± 0.36 g	49.0 ± 0.66 h	53.1 ± 0.46 g
S_7	48.4 ± 0.62 f	55.2 ± 0.48 h	58.8 ± 0.61 i	58.7 ± 0.70 i
S_8	57.4 ± 0.57 i	62.3 ± 0.42 j	60.5 ± 0.39 j	66.4 ± 0.48 l
S_9	46.2 ± 0.70 e	51.9 ± 0.50 g	50.3 ± 0.45 f	54.2 ± 0.51 h
Jaya				
S_1	52.1 ± 0.47 g	55.0 ± 0.46 h	54.0 ± 0.68 h	57.0 ± 0.55 i
S_2	53.0 ± 0.68 g	56.0 ± 0.42 h	56.0 ± 0.54 h	60.3 ± 0.46 j
S_3	47.0 ± 0.62 e	52.1 ± 0.38 h	50.2 ± 0.47 f	55.7 ± 0.63 h
S_4	55.4 ± 0.55 h	60.2 ± 0.66 j	57.4 ± 0.59 i	65.8 ± 0.58 l
S_5	69.7 ± 0.52 j	62.8 ± 0.56 k	62.0 ± 0.63 j	71.1 ± 0.57 n
S_6	50.8 ± 0.39 h	54.9 ± 0.51 h	53.0 ± 0.40 g	60.2 ± 0.42 j
S_7	56.3 ± 0.32 h	61.8 ± 0.50 j	57.8 ± 0.53 i	67.4 ± 0.34 k
S_8	61.1 ± 0.68 j	63.6 ± 0.43 k	62.9 ± 0.62 k	72.3 ± 0.75 n
S_9	52.1 ± 0.59 g	56.0 ± 0.37 h	54.6 ± 0.71 h	61.4 ± 0.54 j
Pawana				
S_1	57.3 ± 0.67 i	60.4 ± 0.44 j	61.2 ± 0.56 j	66.3 ± 0.74 l
S_2	64.1 ± 0.54 k	69.0 ± 0.48 m	67.1 ± 0.53 l	71.7 ± 0.54 n
S_3	50.2 ± 0.59 f	54.1 ± 0.63 h	52.8 ± 0.47 g	58.3 ± 0.46 i
S_4	63.4 ± 0.73 k	71.2 ± 0.33 n	68.0 ± 0.51 m	74.9 ± 0.48 o
S_5	69.3 ± 0.47 m	75.2 ± 0.43 o	72.1 ± 0.42 n	82.1 ± 0.52 p
S_6	57.3 ± 0.47 i	62.2 ± 0.54 j	61.2 ± 0.48 j	65.8 ± 0.67 l
S_7	65.1 ± 0.58 l	72.4 ± 0.48 n	69.4 ± 0.64 m	75.4 ± 0.60 o
S_8	70.8 ± 0.56 n	75.6 ± 0.52 o	73.8 ± 0.61 n	83.2 ± 0.47 o
S_9	58.7 ± 0.65 i	63.1 ± 0.69 k	61.9 ± 0.53 j	67.2 ± 0.56 o
Significance of three-way ANOVA				
Callusing medium (C)		$P < 0.001$		
Shooting medium (S)		$P < 0.01$		
Genotype (G)		$P < 0.001$		
C × S × G		$P < 0.001$		

Means followed by the same letter are not significantly different based on analysis of variance at the 0.05 level.

with 2.0 mg/L BAP, 0.5 mg/L NAA, 500 mg/L proline and 500 mg/L glutamine in Pawana, while the lowest (32.1%) was observed on MS medium supplemented with 2.0 mg/L TDZ in Ambemohar (Table 4).

Effects of genotype on callusing and subsequent plantlet regeneration

Interactive effects of genotypes with callus induction and regeneration media combinations on callusing and subsequent plantlet regeneration response were studied for the four rice genotypes. Pawana formed the highest frequency (97.0%) of callus followed by 89.2% in Jaya, 86.2% in Indrayani and 68.7% in Ambemohar on MS medium supplemented with 2.0 mg/L 2,4-D, 500 mg/L proline and 500 mg/L glutamine. The fresh weight of callus was greatly increased and significantly higher in Pawana than the other three genotypes. The average fresh weight of callus at 30 d of culture was approximately 89.4 mg in Pawana, however, only 60.3 mg in Indrayani, 51.0 mg in Jaya and 42.3 mg in Ambemohar (Table 3).

The overall regeneration efficiency of the four

genotypes revealed that Pawana had highest regeneration efficiency (83.2%), whereas the regeneration efficiency of the other three rice genotypes was in the range of 32.0% to 72.3% (Table 4). Among all the four genotypes, Ambemohar exhibited the lowest regeneration frequency (32.0%–59.4%).

Most of the embryogenic calli derived shoots develops roots on shooting medium (Fig. 1-E, -F). Remaining shoots were rooted on a plant growth regulator-free MS medium and were then successfully established in soil after hardening (Fig. 1-G).

DISCUSSION

Present investigation revealed the positive effects of proline and glutamine on callus induction and plant regeneration in rice using mature seeds as explants. This study described an efficient and reproducible plant regeneration protocol from mature seed derived callus culture. The genotype, combination of growth regulator employed and supplementation of proline and glutamine showed significant effect on callus induction and subsequent plant regeneration. The ultimate objective of the study was to optimize callus induction from mature seed of rice, subsequent plant regeneration, rooting and acclimatization of plants to maturity.

The use of mature seed as starting material for *in vitro* regeneration to produce transgenic rice plants over inflorescences and immature embryos has distinct advantages as inflorescences, and immature embryos are available only for a limited period in a year because of photoperiodic sensitivity of the rice genotypes and problems associated with isolation and sterilization of immature embryos. Calli induced from scutellar tissue of mature seed are the excellent source of cells for *in vitro* regeneration and for the production of transgenic rice (Wani et al, 2011).

Induction of embryogenic callus is usually promoted by auxin especially a synthetic plant growth regulator 2,4-D. Use of 2,4-D alone had been reported to better for callus induction than combinations with other auxins and cytokinins (Shahsavari et al, 2010; Zhao et al, 2011). In the present investigation, with increase in 2,4-D concentration, there was corresponding increase in the frequency of callus formation. Of the different concentrations of 2,4-D tested, 2.0 mg/L 2,4-D was found to be the most effective in inducing callus. The present results are consistent with the earlier observation that 2.0 mg/L 2,4-D respond better on callus induction (Tyagi et al, 2007; Shahsavari et al, 2010). Decrease in callus induction was observed when 2,4-D concentration was increased above 2.0 mg/L. Tendency of decrease in callus induction at higher 2,4-D concentration was also reported by Verma et al (2011).

Although, cultured cells are normally capable of synthesizing all of their required amino acids, addition of proline and glutamine to the medium may increase cell growth. The use of amino acid proline and glutamine in the medium has been reported to be positive effect on frequency of callusing and regeneration in rice (Chowdhry et al, 1993; Ge et al, 2006; Shahsavari, 2011). In the present investigation, addition of proline or glutamine either alone or in combination resulted in significant increase in callusing and fresh weight of callus. The enhancement of the callus growth by amino acid could be explained on the basis that amino acids provided a readily available source of nitrogen to the growing growth. Proline and glutamine are relatively non-toxic which will enable the cells to maintain a high growth rate for a longer period. The frequency of plantlet regeneration was higher in callus obtained from medium supplemented with proline and glutamine. Inclusion of proline and glutamine in shooting medium was also found to improve regeneration efficiency. Proline and glutamine have been reported to increase the percentage of cultures showing plant regeneration (Chowdhry et al, 1993; Shahsavari, 2011). In the plant regeneration experiment, all the four genotypes were capable of regenerating plantlets. However, callus induction and plant regeneration frequency are highly diverse among rice genotypes. Significant genotypic variation in callus induction and plant regeneration potential has been reported in rice (Ge et al, 2006).

Regeneration frequency was influenced by callus induction medium, shooting medium as well as the interaction between the callus induction medium and shooting medium. Auxin and cytokinins are considered as key factors to shoot differentiation in callus culture. The type of the growth regulators in the shooting medium was found to be crucial factor controlling the *in vitro* plantlet regeneration. BAP was found to be superior to kinetin and TDZ. Inclusion of NAA in shooting medium enhanced the regeneration frequency of the four rice genotypes. BAP at the concentration of 2.0 mg/L when used in combination with 0.5 mg/L NAA has been found to be most effective for plantlet regeneration from callus. The stimulatory effect of BAP in combination with NAA

has been reported to facilitate regeneration in rice callus cultures (Ramesh and Gupta, 2005). On the contrary, Lee et al (2002) found kinetin to be more effective for shoot regeneration compared with BAP.

In summary, we report high efficience and reproducible regeneration protocol for *in vitro* callus induction and plant regeneration in rice. The optimum indirect plant regeneration was obtained by callusing on MS medium supplemented with 2.0 mg/L 2,4-D, 500 mg/L proline and 500 mg/L glutamine and shoot induction and elongation on MS medium supplemented with 2.0 mg/L BAP, 0.5 mg/L NAA, 500 mg/L proline and 500 mg/L glutamine, followed by rooting on growth regulator-free MS. This optimized regeneration protocol can be efficiently used for tissue culture-based genetic transformation in rice.

ACKNOWLEDGEMENTS

This study was financially supported by the Mahatma Phule Agricultural University, Rahuri, India.

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