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Regeneration Enhancement in Tissue Culture of *Indica* Rice's through Partial Desiccation and Chemical Supplements

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Abstract

Indica rice genotypes are known to be recalcitrant to culture and several efforts have been made over years to enhance their callus induction and regeneration rates. Some of the effective approaches employed include use of phytohormones in a proper combination and proportion, amino acids like tryptophan, proline or supplements like casein hydrolysate and yeast extract etc. which have shown positive improvements in callus induction but enhancement of regeneration rates stills remains a major bottleneck in *indica* rice tissue culture. Keeping in view that the rates of callus induction and regeneration are not related, an effort was made to enhance regeneration potential of the *indica* rices through supplementation of media with chemicals like silver nitrate and adenine sulphate, sugar alcohols like sorbitol and subjecting the calli to stress through partial desiccation prior to regeneration. The results suggest that significant improvement of callus regeneration rates is feasible that is genotype independent as a two-fold increase of regeneration rates can be induced in most of the genotypes through partial desiccation. The results can widen the scope of *indica* genotypes that are used for development of transgenic *indica* rices.

Keywords: *Indica* rice; Regeneration; Benzylaminopurine; Osmolytes

Introduction

Rice is one of the major food sources and is third to wheat and maize in the world's total production of food grains [1]. It is the staple food for one third of the world's population and occupies one-fifth of the total area covered under cereals. About 92% of the planet's total rice is produced and consumed in [2]. World rice production in 2012 was 486 million tons [3]. World population is estimated to reach 10 billion by 2050. The conventional plant breeding methods alone cannot address the yield potential of rice due to limited availability of donors in the rice gene pool for several biotic and abiotic stresses [4]. This prompts the researchers to rely on transgenic technology which can address these problems effectively as it allows transfer of genes across the species.

Well established tissue culture protocols are essential for successful application of transgenic technology. High frequencies of callus induction and regeneration in most popular cultivars is the critical requirement in cereal tissue culture [5,6]. Choice of explant Bhaskaran et al. [7] is also an important factor and in general, scutellum derived embryogenic callus from the germinating seeds is employed. However, the calli generated have limited totipotency for successful regeneration [8,9] which further depends on a number of biophysical characteristics including osmotic regulation [10]. Sugars like sucrose and maltose [11] and sugar alcohols like sorbitol and mannitol [12] are involved in maintaining osmotic requirements for the dividing cells. In addition, physical environment of the cells is also found to have a profound influence on the regeneration efficiencies of various rice genotypes. Physical treatments like partial desiccation of callus have been reported to be beneficial for embryogenesis and plant regeneration in several plant species [13-17]. Keeping in view of these issues, the present study is an attempt to enhance the regeneration efficiency of *indica* rices by using chemicals like adenine sulphate and silver nitrate, sugar alcohols like sorbitol and inducting of physical stress through partial desiccation of calli.

Materials and Methods

Genotypes

The rice genotypes selected for the study were eight elite *indica* rice cultivars Swarna, Gayatri, Samba Mahsuri, Pooja, Pusa Basamti1, CR Sugandh Dhan 907, Basmati-370 and Dubraj. Out of them, the first four are the highly popular non-aromatic varieties while the other four are popular aromatic varieties. Swarna is widely grown variety in eleven states of India and is highly popular with a yield potential of 8.0 t/ha [18]. It is also being widely grown in Bangladesh and Myanmar suggesting its wide adaptability [19]. Gayatri is a high yielding cultivar released from NRRI, and is widely grown in shallow and medium low land ecology in eastern India. Samba Mahsuri (BPT 5204) is one of India's most popular and highly prized rice varieties because of its high yield 4.5 to 5.0 t/ha and excellent cooking quality [20]. Pooja is a high yielding cultivar released from NRRI, which is widely grown in shallow and medium low land ecology in eastern India. Pusa Basmati 1 is a Basmati variety, which is extensively grown in the Basmati region of India with a yield potential of 4.5 t/ ha [21]. It is highly popular on account of its long slender grains, pleasant aroma and the cooked rice is endowed with desirable traits like soft texture and tenderness. Basmati 370, is the well-known Basmati variety, and is the bench mark for quality of aromatic rices. CR Sugandh Dhan 907, which was released from NRRI, is a high yielding aromatic variety. Dubraj is highly popular short grain aromatic rice mostly grown in Chattisgarh region of India.

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Callus induction

Mature dehusked grains of the eight genotypes were washed with sterile distilled water and were surface sterilized successively with, 70% ethanol for two min, sodium hypochlorite (contains 4% (v/v) active chlorine) for 15 min and with 0.1% (w/v) aqueous mercuric chloride solution for 5 min with intermittent repeated washings with sterile distilled water [22]. The kernels were inoculated in culture tubes containing semisolid callus induction (CI) medium [MS medium supplemented with 2, 4-dichlorophenoxy acetic acid (2, 4-D) (2 mgl), maltose (30 gl) and solidified with of gel-rite (2.6 gl)] [23] and the cultures were incubated in dark at 25 ± 1°C for three weeks. Callus induction (CI) and regeneration frequencies (RF) were recorded and statistical analyses were performed using SAS software [24].

Pre-regeneration

Three-week-old calli were transferred to the medium supplemented with MS salts and vitamins, along with maltose (30 gl), naphthalene acetic acid (1.0 mg l), kinetin (2.0 mg l), and agar (9 gl), pH 5.8 was maintained and grown in dark for 15 days. To this media, sorbitol (20 gl), was added in one treatment and in another treatment, silver nitrate (5 mg l) and adenine sulphate (10 mg l), were added. In a separate treatment, the calli were subjected to partial desiccation (on a sterile filter paper inside the laminar air flow chamber) for 2-4 hrs prior to their transfer onto regeneration media.

Regeneration

After the three different independent treatments, calli were transferred on to the MS regeneration medium, supplemented with naphthalene acetic acid (0.5 mgl), kinetin (0.5 mgl), and benzylaminopurine (1.5 mgl). Maltose (30 gl) was used as the carbon source and the medium was solidified with agar (9 gl) and a pH of 5.8 was maintained. After plating of calli, the cultures were incubated in dark for 15 days and later transferred to light (~ 2000 lux).

Results

The influence of chemicals, sorbitol and partial desiccation on regeneration frequency was recorded (Table 1). Results reveal an enhancement in regeneration frequencies of all the genotypes compared to control through the addition of silver nitrate and adenine sulphate. In the case of sortibol treatment increase in regeneration frequencies were observed in all the eight genotypes taken under this study in comparison with control (calli grown on sorbitol free media). This increase is clearly observed in the case of the genotypes which had poor regeneration potential. For example, in Dubraj, the frequency has almost doubled (16.9 to 31.7) and considerable increase was observed in case of Pooja (36.2 to 44.5) while in the remaining genotypes there was a marginal increase in regeneration frequency. While in desiccation treatment it was found that four-hour desiccation was superior to two-hour desiccation (Table 1; Figure 1). The analysis of variance of the data (Table 2) suggests that the addition of chemicals (silver nitrate and adenine sulphate) or sorbitol in the medium enhanced the regeneration rates while highly promising results were recorded with partial desiccation (Figure 1).

Discussion

Indica rice cultivars are recalcitrant to culture [25,26] and efforts are being made over years to enhance callus induction and regeneration potential of *indica* rice cultivars [27-30]. Callus induction and regeneration frequency of the genotypes is enhanced by adding adequate amounts of amino acids like tryptophan and proline [31-34]. Earlier studies indicate that rice genotypes having good callus induction rates may or may not show good regeneration rates [24] and for this reason, both the steps of tissue culture of *indica* rices have to be addressed separately. Differentiation of somatic embryo into plantlet is an important step and many studies show that due to physiological changes in the dividing cells, different osmolytes accumulate and cause hindrance to regeneration [35]. This causes water consistency in the medium and if this can be minimized, higher number of plants can be generated and efforts were made in this work to address this issue.

Effect of sorbitol, a sugar alcohol, on regeneration

Sorbitol is readily taken up and metabolized in some species and acts as an osmo- protectant [36] and the regeneration ability of non-regenerable rice callus could be promoted by treatment with an osmotic agent such as sorbitol or mannitol [37,38]. During the period of cell cycles, calli accumulate water probably due to osmolytic action of maltose leading to lower levels of regeneration. To enhance regeneration, medium was supplemented with a combination of sorbitol (20 gl) and phytohormones NAA (1 gl), and kinetin (2 mgl) and calli were incubated in dark for a period of two weeks prior to transfer to regeneration media and later transferred to regeneration media. Sorbitol, acting as a good osmotic agent, might have acted to desiccate the callus and the regeneration levels were higher as seen in our studies (Table 1).

Effect of silver nitrate and adenine sulphate on regeneration

Earlier studies have suggested that addition of compounds with anti-ethylene activity like silver nitrate and adenine sulphate [39] to the

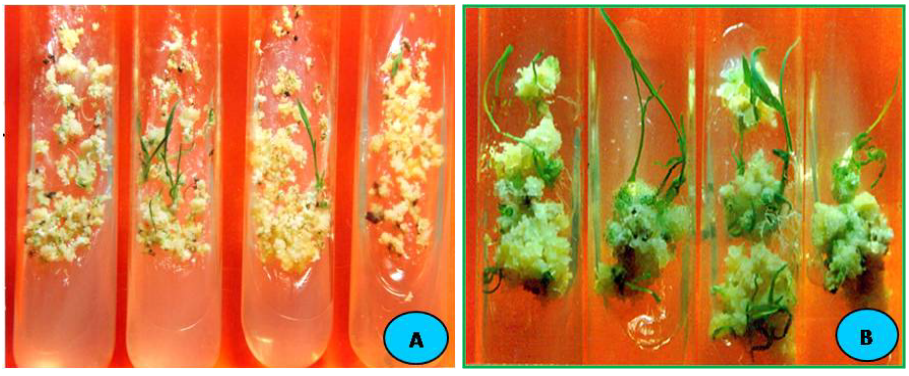

Figure 1: Influence of partial desiccation on regeneration. Regeneration from A) Normal calli (no desiccation-control); B) Partially desiccated calli (4 h).

S. No	Genotypes	Control	$AgNO_3$ ± AS	D-2 hrs	D-4 hrs	Sorbitol
1.	Swarna	66.6 ± 3.3	68.5 ± 2.8	71.5 ± 1.9	74.2 ± 1.1	72.6 ± 1.1
2	Gayatri	81.3 ± 2.1	81.3 ± 2.9	82.6 ± 2.0	84.6 ± 1.1	82.3 ± 0.9
3	Samba Mahsuri	46.7 ± 2.9	48.3 ± 2.8	50.4 ± 1.8	58.5 ± 0.81	55.7 ± 1.1
4	Pooja	36.2 ± 3.1	38.7 ± 2.2	42.4 ± 2.2	48.5 ± 0.9	44.5 ± 1.1
5	Pusa Basamti1	85.7 ± 1.7	86.7 ± 1.5	86.6 ± 2.0	89.3 ± 0.9	86.3 ± 1.3
6	Basmati370	76.3 ± 1.5	75.5 ± 1.4	80.6 ± 1.9	84.6 ± 1.2	81.5 ± 0.9
7	CR Sugandh Dhan 907	50.3 ± 1.6	50.7 ± 1.4	52.5 ± 2.2	61.8 ± 1.7	53.5 ± 1.2
8	Dubraj	16.9 ± 1.7	25.3 ± 1.6	30.8 ± 1.5	34.5 ± 0.9	31.7 ± 0.9

$AgNO_3$: Silver nitrate; AS: Adenine Sulphate; D-2 hrs: Desiccation for 2 hours; D-4 hrs: Desiccation for 4 hours

Table 1: Influence of chemicals, sugar alcohol and partial desiccation on regeneration rates in *indica* rice tissue culture.

Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.8968	<0.0001	****	Yes	
Treatments	3.011	<0.0001	****	Yes	
Varieties	95.48	<0.0001	****	Yes	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	366.3	21	17.44	F (21, 64)=4.446	P<0.0001
Treatments	1230	3	410.0	F (3, 64)=104.5	P<0.0001
Varieties	38999	7	5571	F (7, 64)=1420	P<0.0001
Residual	251.1	64	3.924		

SS: Sum of Squares; DF: Degrees of freedom; MS: Mean of squares; DFn: degrees of freedom numerator; DFd: degrees of freedom denominator

Table 2: Analysis of variance of different treatments on regeneration frequencies.

culture medium promotes *in vitro* regeneration. It was reported that the addition of silver nitrate in the regeneration medium can influence shoot organogenesis in several species [40]. However, the continued presence of silver nitrate and adenine sulphate in the medium for more than ten days resulted in tip burning of the leaves of the plantlets often leading to mortality of the plantlets thus requiring the transfer of the plantlets from the silver nitrate supplemented media to normal media. However, it was observed that the formation of shoot primordia was early from the calli grown on media supplemented with $AgNO_3$ and adenine sulphate as compared to control. On the media supplemented with $AgNO_3$ and adenine sulphate, the shoot initiation was observed within 3-4 days, while it took a week or more in the control. There is a marginal increase in regeneration frequency of all the genotypes by the addition of silver nitrate and adenine sulphate but comparatively less when compared to desiccation and sorbitol treatments.

Effect of partial desiccation on regeneration

As discussed earlier, the calli in the culture appear to be water soaked through accumulation of osmolytes and such calli take more time to regenerate. In an effort to enhance regeneration, the calli were subjected to partial desiccation (on sterile filter paper) for 2-4 hr prior to their transfer onto regeneration media. The results suggest that the simple partial desiccation treatment can enhance regeneration frequencies when compared to controls (no desiccation) (Figure 1). It was clear that the enhanced levels of regeneration (Table 1) were successfully achieved through simple partial desiccation treatment in all the genotypes tested suggesting its influence is genotype independent thus supporting the previous reports [41-43]. Due to its effectiveness, partial desiccation could be employed to enhance regeneration potential of somatic cell cultures of *indica* rices. The results suggest the necessity for inclusion of partial desiccation as an important step in the tissue culture protocol of *indica* rice genotypes in order to enhance their regeneration potential. The enhancement of regeneration levels will also be of great help in the generation of transgenic *indica* rices.

Conclusion

Keeping in view of the constraints involved in the regeneration of *indica* rice callus, we tried various approaches to enhance regeneration by a) chemical treatment (silver nitrate and adenine sulphate) b) sugar alcohol (sorbitol) and c) desiccation treatments (2 hrs and 4 hrs). Among all the three methods, we found that partial desiccation treatment for 4 hrs had shown good effects on regeneration of the calli. Hence results from this work suggest that partial desiccation could be included as an essential step to achieve higher regeneration efficiencies in *indica* rice callus irrespective of the genotypic differences. This work seems highly promising in developing more putative transgenic plants, where each successful transformation event holds greater significance.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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