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Somatic embryogenesis from mature caryopsis culture under abiotic stress and optimization of *Agrobacterium*-mediated transient GUS gene expression in embryogenic callus of rice (*Oryza sativa* L.)

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Abstract
Induction and development of embryogenic callus from mature caryopsis culture of rice (*Oryza sativa* cv. ADT41) was performed by placing sterilized seeds on MS medium supplemented with 2,4-dichlorophenoxyacetic acid or 2,4-D (2.5, 5.0 and 10 mg/l). Morphogenesis in terms of somatic embryogenesis was recorded and 53% embryogenic callus formed by caryopsis culture indicates high chance of regenerating plants eventually. Furthermore, for water stress treatments, sterilized caryopses were cultured on semisolid MS medium supplemented with (5.0mg/l) of 2, 4-D and various concentrations of mannitol (2.5M, 5M and 10M) were added. Present study indicates that potentials of tissues for callus induction and embryo differentiation gradually decline if the level of mannitol is being increased in the nutrient media. Significantly, at high level of mannitol (5M), direct somatic embryo differentiation from the epicotyl tissues was also evident along with embryogenic callus formation at the basal region of the seedling. Similarly for salt stress treatments, MS medium was supplemented with various concentrations of NaCl (50 mM, 100 mM and 250 mM) along with 2, 4-D (5.0 mg/l). Results obtained on salt–stress treatments indicate that cultured rice cells may respond variously depending on the concentration of salt-stress present in the culture medium. Also, transformation experiments were conducted to optimize the transient GUS gene expression in mature caryopsis derived embryogenic callus by employing the various strains of *Agrobacterium tumefaciens* carrying the same plasmids or others. Calli were co-cultivated with *Agrobacterium* strains GV2260 (p35SGUSINT), LBA4404 (p35SGUSINT) and LBA4404 (pCAMBIA 3301) in the presence of acetosyringone (200 µM). Transformation events were best recorded in calli treated with *Agrobacterium* strain LBA4404 harbouring the plasmid p35SGUSINT, as evidenced by maximum frequency (29%) of transient GUS gene expression on histochemical assay and it was followed by strain GV2260 (p35SGUSINT), however, *Agrobacterium* strain LBA4404 (pCAMBIA3301) could be proved the least effective in terms of frequency of transient transformation and expression of GUS reporter gene in target tissues.

Keywords: caryopses, somatic embryos, abiotic stress, agrobacterium, acetosyringone, plasmids, GUS reporter gene

INTRODUCTION

Rice a cereal crop, is a grain belonging to the grass family and currently supports nearly one half of the world population as a staple food source [1]. The ever-increasing human populations and various abiotic and biotic stresses have posed a challenge to boost the rice production in limited cultivable land. Thus there is a need to improve upon the yield of the local varieties/cultivars, because loss in production could lead to hunger and famine.

The basic prerequisite for the potential use of biotechnology in rice improvement is the regeneration ability of cell, tissue and organ of rice plant. *In vitro* culture is an important component of any genetic transformation protocol because it provides sources of materials that can be used as recipient of introduced foreign genes. The ability of the targeted plant cell to regenerate into plantlet and subsequently develop into a mature plant is a prerequisite for genetic transformation.

Successful regeneration of plant tissue culture mainly depends on genotype, explant type, medium composition, plant growth regulator and culture environment [2]. There were numerous reports on callus formation and plant regeneration from mature seeds of *indica* rice especially the non-glutinous type [3-5]. Explants from different species demonstrate varying callusing ability. In the case of rice, it is generally known that *japonica* type readily forms callus while *indica* type is more recalcitrant. Compared to *japonica* rice, *indica* rice has been less responsive to *in vitro* culture [6] and high frequency of somatic embryogenesis is limited [7]. Moreover, the resulting calli may follow different pathways to regenerate shoots, roots, or entire plantlets. For genetic transformation studies, however, plantlets from calli are most suitable target and that could be possible if the calli are embryogenic in nature.

Significantly, a technique with efficient degree of somatic embryo production would be useful in genetic manipulation studies aimed at rice improvement. Therefore, present study involves establishing an efficient protocol for regeneration of a local rice cultivar and also efforts have been made on efficacies of different abiotic stress treatments on morphogenesis during mature caryopsis culture in order to regenerate abiotic stress- tolerant plants.

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Furthermore, in recent years, transgenic production has been a *sine qua non* approach for the crop improvement program. The majority of reports where transformation is successful have relied upon a biolistic approach, even though *Agrobacterium*-mediated transformation has been accomplished recently [8]. The long periods of selection needed for the recovery and regeneration of putative transgenic plants often has hampered optimization of conditions for transformation [9] and hence keeping these objectives, experiments were also undertaken for the optimization of transformation protocols by employing the various strains of *Agrobacterium tumefaciens* harbouring the different plasmids.

MATERIALS AND METHODS

Sterilization of Seeds

Seeds of a local cultivar of rice (*Oryza sativa* L.cv. ADT41) were procured from the Karaikal region of Puducherry, India and were carefully dehusked, washed with detergent and rinsed thoroughly with tap water to remove dust and other surfactants.

These seeds were then sterilized with 95% alcohol for about 1-2 minutes and followed by a sterilization treatment with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. The sterilized seeds were thoroughly rinsed with distilled water to remove possible traces of mercuric chloride.

In Vitro Phyto-nutrition

The basal medium used throughout the present study was that of Murashige and Skoog (MS) [10] salt solution supplemented with various growth hormones as outlined in the result section. Sucrose at 2% was used as the sole carbon source. The pH of the medium was adjusted at 5.8 with either 1.0 N HCl or NaOH and the medium was solidified with 0.8% (w/v) agar.

The medium was then autoclaved at 15 psi with a temperature of 121^{o}C for 15 minutes. Growth hormones were added to the medium before autoclaving. The cultures were then incubated with 16 hours of light cycle in a 24 hours cycle and the temperature regulated at 25 ± 1^{0}C. Following nutrient media were employed in present study; (a) Callus induction medium (b) Medium for Somatic embryogenesis (c) Medium supplemented with various stress agents

Callus Induction Medium

These sterilized seeds were then soaked for 24-36 hours in sterilized distilled water before inoculation. Induction of rice callus was performed by placing sterilized seeds on MS medium containing 2% (w/v) sucrose and supplemented with 2,4-dichlorophenoxyacetic acid or 2,4-D (2.5, 5.0 and 10 mg/l) and 2,4-D-free medium. The seeds were incubated at 25 ± 1^{0}C in culture room. Calli formed from basal region of the young coleoptile of a seedling were transferred onto new fresh media and sub culturing was performed at every 2-3 weeks intervals.

Medium for Somatic Embryogenesis

In order to induce or differentiate somatic embryos on callus derived form the explants, the derived calli were sub-cultured on the MS media supplemented with either low 2,4-D (1mg/l) or 2,4-D- free MS basal media. On MS basal media, somatic embryos were allowed to germinate into plantlets.

Medium for Stress Treatments

Water Stress

To conduct the experiments based for drought stress or water stress treatments, sterilized seeds were cultured in culture tubes (15 ml and 30 ml) containing semisolid MS medium supplemented with 2,4-D (5.0 mg/l)+sucrose (20 g/l) + agar (8 g/l)and various concentrations of mannitol (2.5M, 5M and 10M) were added.

Salt Stress

In order to observe the effects of salinity on morphogenic responses, the experiments were undertaken and sterilized seeds were cultured in culture tubes (15 ml and 30 ml) containing semisolid MS medium supplemented with 2,4-D (5.0 mg/l) + sucrose (20 g/l) + agar (8 g/l) and various concentrations of NaCl (50 mM, 100 mM and 250 mM) were added.

After two weeks of incubation, the induced calli were sub-cultured under the same growth conditions, and in the same MS medium to which various concentrations of mannitol or NaCl were incorporated.

Co-cultivation of Embryogenic Callus

Agrobacterium tumefaciens strains GV2260 (p35SGUSINT), LBA4404 (p35SGUSINT) and LBA4404 (pCAMBIA3301) were freshly grown from the glycerol stocks on YEB medium (1 g/l yeast extract, 5 g/l beef extract, 5 g/l bacto-peptone, 5 g/l sucrose, 0.5 mg/l MgSO4, pH 7.0) for 48 h with shaking at 200 rpm, 28^{0}C. For transformation, 100 µl of freshly grown culture was inoculated in Erlenmeyer flasks containing 25 ml YEB medium supplemented with appropriate antibiotics and 200 µM of acetosyringone (AS).When the culture was in the log phase with OD600 0.25 to 1.00, the cells were pelleted in SS-34 Sorvall tubes at 2000 g, 4^{0}C for 10 min.

Bacterial cell density was calculated (1 absorbance unit at 600 nm = 8 x 10^{8} cells/ml) and cell density was adjusted to 5 x 10^{8} cells/ml with liquid MS medium supplemented with acetosyringone (200 µM), according to the protocol described by Chugh *et al.*[11]. 25 calli were incubated with 25 ml of *Agrobacterium* suspension for 1h.

Agrobacterium strains GV2260 (p35SGUSINT) and LBA4404 (p35SGUSINT) conferring kanamycin resistance, whereas, LBA4404 (pCAMBIA3301) for herbicide (bar) resistance. Incubated caryopses derived calli were placed on MS + 2,4-D (2.5mg/l) medium for two-three days for co-cultivation. *Agrobacterium* cells were washed off with sterile MS liquid medium (3-4 washes); the final wash was supplemented with cefotaxime (500 µg/ml) and given for 15-20 minutes to completely remove excessive *Agrobacteria.*

GUS Histochemical Assay

GUS histochemical assays were employed as the preliminary screening methods for detecting putatively transformed calli after *Agrobacterium* treatment. Activity of the *gus* reporter gene was histochemically detected in the callus tissues according to the protocol described by Jefferson [12].

Statistical Analysis

For each treatment, 25 tubes were raised and each experiment was conducted two times. The medium was changed at every 10-12

days of intervals. The data on callus induction efficiency measured as the number of calli or embryogenic calli obtained/total number of mature caryopses cultured × 100. Same statistical procedure was followed for histochemical assay treatments also, where frequency of calli was recorded which exhibited the GUS gene expression.

RESULTS

Establishment of Callogenesis

Initially unsterilized seeds were grown on wet cotton in order to test the seeds viability in terms of germination frequency and almost 95% of the seeds could demonstrate healthy germination into normal seedlings. Hence, sterilized seeds were brought for the current study and inoculated on basal MS medium for *in vitro* morphogenesis.

The seeds germinated after 3 days of inoculation on basal hormone-free nutrient media and another week, these germinated seeds developed into young seedlings and further a complete plant formation without any callusing could be observed (Fig.1A). Moreover, after four weeks of inoculation these plants gradually necrosed. When sterilized seeds were inoculated on 2,4-D supplemented MS nutrient media, within a week of culture initiation, callus induction could be seen at basal region of the developing coleoptile (Fig.1B). The frequency and nature of callus induction was absolutely dependent on the concentration of 2,4-D incorporated into the media. At lower concentration (2.5mg/l), the frequency of callogenesis was found to be very low 42% (Fig.3) and calli appeared to be semi-compact. This callus was soft, friable and brown colored. Most of these calli generally turned out to be non-embryogenic in nature.

Significantly, the maximum frequency (73%) of callus induction was observed at high concentration (5mg/l) of 2,4-D supplemented nutrient media. Moreover, calli grown on high concentration of 2,4-D were relatively compact, yellowish colored and mostly nodular and embryogenic (Fig.1B). However, further increase of 2,4-D concentration in nutrient medium could support callus initiation but at very low quantities and frequencies (Fig.3).

Differentiation of Somatic Embryos

Further proliferation was observed when friable or non-embryogenic calli were sub-cultured on the same medium, however, these calli failed to show embryo differentiation. To induce embryogenesis, when the caryopses derived calli were sub-cultured onto MS containing 2,4-D (1.0 mg/l) or 2,4-D- free nutrient media, a yellowish-white, compact and somewhat embryogenic callus got induced after about 3-4 weeks of culture initiation(Fig. 1C). Nonetheless, a soft, friable and non-embryogenic callus formation was also recorded on medium containing 1.0 mg/l each of 2,4-D and BAP (results not shown).

The maximum frequency of somatic embryogenesis (53%) was observed in calli grown at nutrient medium supplemented with high (5.0 mg/l) concentration (Fig. 3) and further increase in 2,4-D concentration in the nutrient medium didn't help in induction of somatic embryos rather it proved inhibitory and therefore, at higher level (10 mg/l) of 2,4-D, merely 8% of somatic embryogenesis was observed. Significantly, all these calli when subcultured on the same fresh media, which subsequently exhibited reasonable proliferation and maintained their embryogenic (Fig. 1D) or non-embryogenic nature for a long duration.

Direct Somatic Embryogenesis on Epicotyls

In present study, it was quite interesting to observe the differentiation of direct embryos in a clustered form without exhibition of callus formation on elongating epicotyl regions of the seedling during caryopsis culture. In 2-week-old culture, the young seedlings exhibited direct differentiation of various globular structures in a clustered form or also on all over the epicotyl regions of the young seedling (Fig. 1E). However, the frequency of such direct embryogenic responses was variable and it was more apparent in explants which were growing on high 2,4-D supplemented nutrient media (results not shown).

Germination of Somatic Embryos

The embryogenic callus growing on 2,4-D (1.0 mg/l) or 2,4-D-free medium was good enough to induce germination of somatic embryos into young plantlets. However, majority of calli exhibited some browning after 3-4 weeks of culture. Nonetheless, it exhibited good growth on fresh medium and germinated few after 4-5 weeks.

Germination of somatic embryos was evident from the emergence of coleoptile and coleorhiza simultaneously on regeneration media containing either the 2,4-D at very low concentration (0.1 mg/l) or 2,4-D-free (Fig. 1F) and further complete plantlets formation could be observed (Fig. 1G) after 6-7 weeks of culture initiation.

Morphogenesis under Abiotic Stress Treatment

Mature seeds were cultured on MS media supplemented with various concentrations of abiotic stress causing agents along with 2,4-D in order to evaluate their efficacy on *in vitro* morphogenesis by induction of callus and furthermore differentiation of somatic embryos.

Drought or Water Stress

This experiment was performed to check the inherent capacity of calli to regenerate on medium which induced drought stress. During stress treatment, explants response was measured in terms of seeds potentials to germinate and further on the callus induction from germinating seeds was assessed. Moreover, the response of calli to elevated levels of a mannitol was recorded as visual growth pattern. The response of the induction of callus and differentiation of somatic embryos was directly dependent on to the levels of mannitol in the nutrient media. Tolerance was carried out by growing mature caryopses on callus induction medium supplemented with 2.5, 5 and 10M mannitol for two to three weeks. Caryopses derived calli exhibited normal proliferation on nutrient medium without mannitol.

As the mannitol concentration in the medium increases, a significant reduction in callus induction and proliferation tendencies was noticed. In comparison to control experiments, the frequency of callus induction and further differentiation of somatic embryos exhibited to decline with the increase in the concentrations of mannitol in the nutrient media. Moreover, the maximum frequency 55% of callus induction (Fig. 4) was recorded at the very low level of mannitol (2.5M) stress whereas at higher level 10M of mannitol, this frequency was very poor (13%) and inhibited both the ability to germinate the seed as well as the potentials of explants for callus induction.

Significantly, along with 2,4-D, mannitol at very low level 2.5 M proved to be effective in induction of somatic embryos directly on developing epicotyl tissues. This result indicated the stimulatory effects of mannitol at low level in terms of somatic embryos differentiation (Figs.1E&4) but higher levels of mannitol was always inhibitory for the morphogenic responses.

Salinity Stress

The effect of salt stress on growth of callus was determined by adding NaCl to the MS medium at concentrations of 50 mM, 100 mM and 250 mM. The results revealed that growth of the callus as measured by visual growth and in terms of frequency was not significantly different between salt and non -salt stressed or control cultures during the first week of exposure to stress treatment. However, after a week of exposure to stress, growth of the salt-stressed tissue was slightly decreased as compared to the control.

The callogenic potentials of the salt-stressed explants were also slightly declined after a week of exposure to salt stress. With gradual increase in NaCl concentration in callus induction medium, the frequency of callus induction and differentiation of somatic embryos was declined in comparison to the control experiments without stress treatments (Fig.5) and at higher concentration (250 mM) of salt, it proved inhibitory for both callogenesis as well as somatic embryogenesis.

Screening of Putative Transgenic Calli (GUS histochemical Assay)

Subsequent to *Agrobacterium* co-cultivation, calli were screened for *gus* gene expression in the treated callus tissues (Fig. 2. B, C, D). Control callus or non-treated callus did not show GUS expression (Fig. 2A) during the GUS histochemical assay. It was observed that frequency and intensity of transient GUS gene expression was highly variable amongst the various strains of agrobacteria and also with in the same bacteria carrying different plasmids. Moreover, the results were recorded in terms of frequency of calli exhibited GUS gene expression. It was clear from the experiments that calli treated with LBA4404 (p35SGUSINT) exhibited maximum frequency (29%) of GUS reporter gene expression followed by GV2260 (p35SGUSINT) whereas the strain LBA4404 (pCAMBIA3301) showed the GUS expression at very low frequency (Fig. 6).

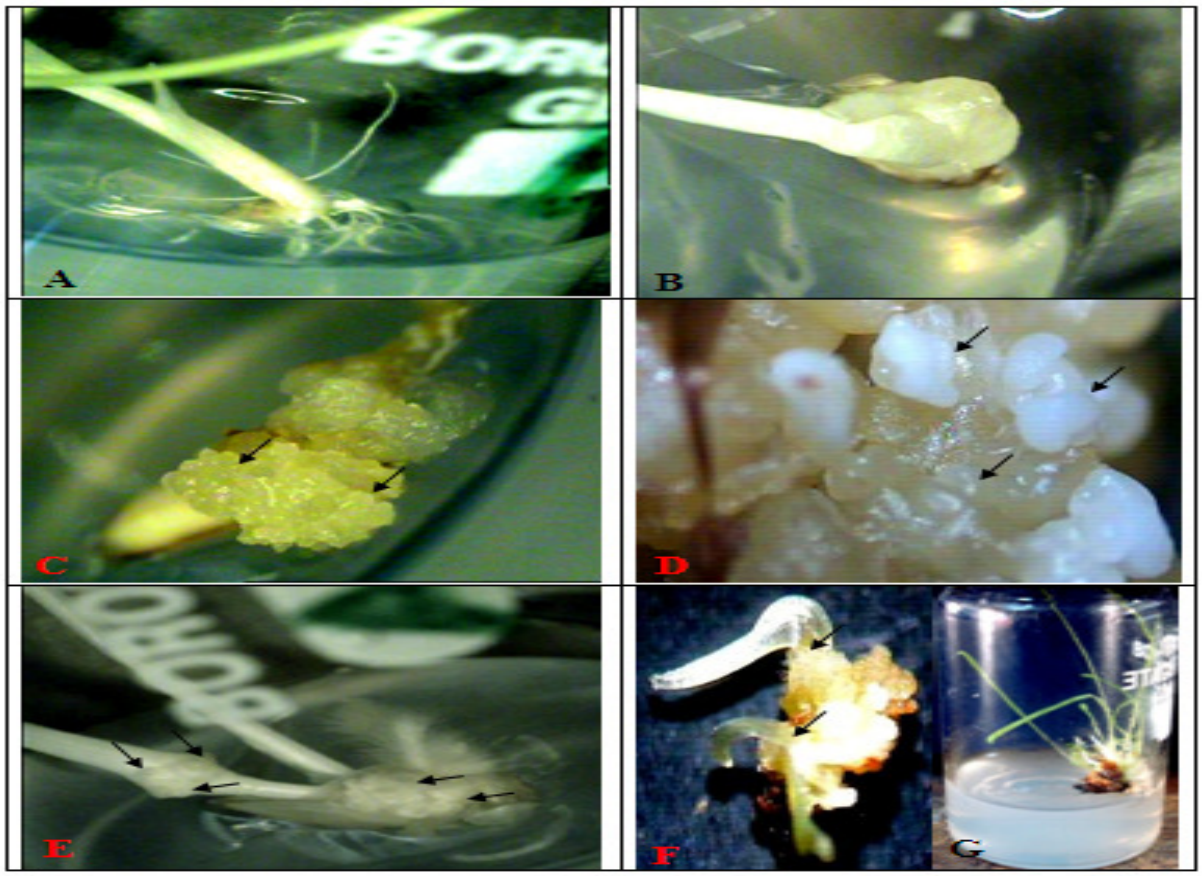

Fig 1. A-G: Oryza *sativa*, Caryopsis Culture
A. Two-week-old culture on basal medium showing normal seedling formation.
B. Compact callus formation at the basal region of coleoptile in one-week-old culture.
C. Caryopsis with profuse callusing exhibits induction of many nodular embryoids like structures (arrows).
D. Embryogenic callus with many typical defined whitish somatic embryos (arrows).
E. 2 to 3-week-old culture on mannitol supplemented nutrient medium exhibits the direct differentiation of somatic embryos (arrows) without callus formation on epicotyl region of the seedling. Also, a cluster of typical somatic embryos (arrows) differentiated on embryogenic callus formed at the basal region of seedling are seen.
F. Germination of somatic embryos is evident by emergence of coleoptiles (arrow) in 6-week-old culture.
G. Almost Two-month-old culture exhibits regeneration of plantlets during culture.

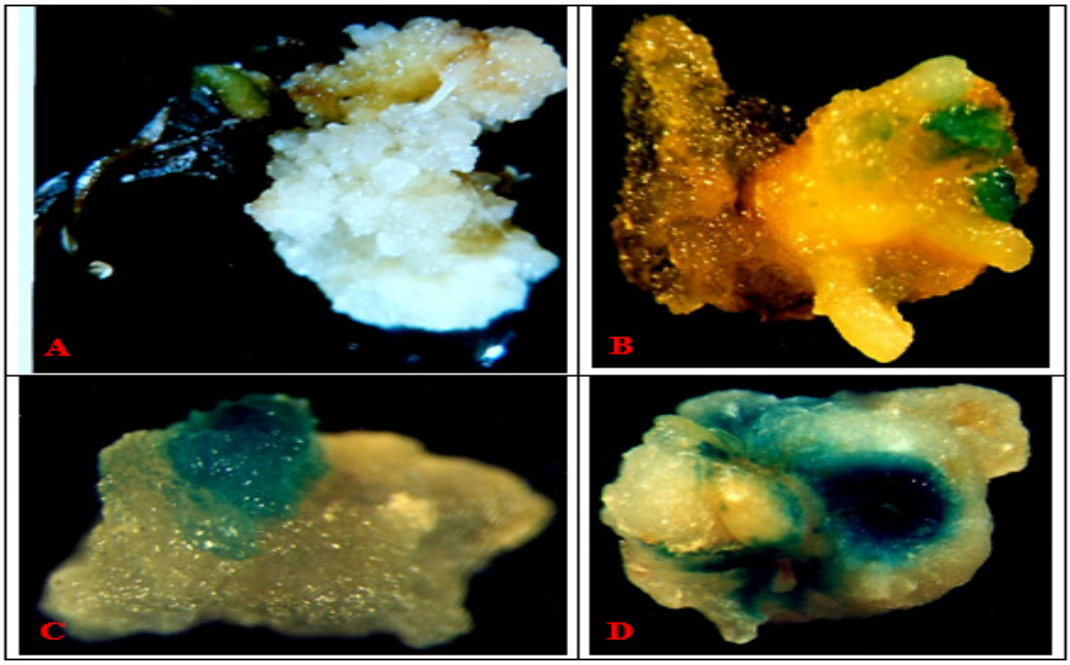

Fig. 2. A-D: *Oryza sativa,* Transient GUS gene expression mediated by *Agrobacterium tumefaciens* in mature caryopsis derived embryogenic callus.
A. Control callus without *Agrobacterium* treatment, GUS gene expression is not seen.
B. Callus treated with LBA4404 (pCAMBIA3301)
C. Callus treated with GV2260 (p35SGUSINT)
D. Callus treated with LBA4404 (p35SGUSINT)

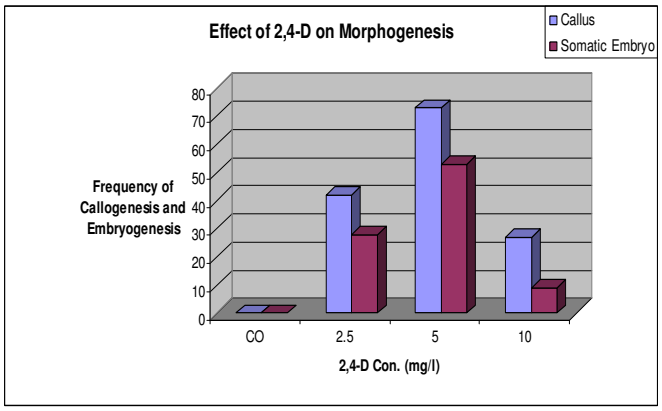

Fig 3. Effect of various concentrations of 2,4-D on morphogenesis in terms of callogenesis and somatic embryogenesis on caryopsis culture of *Oryza sativa*.

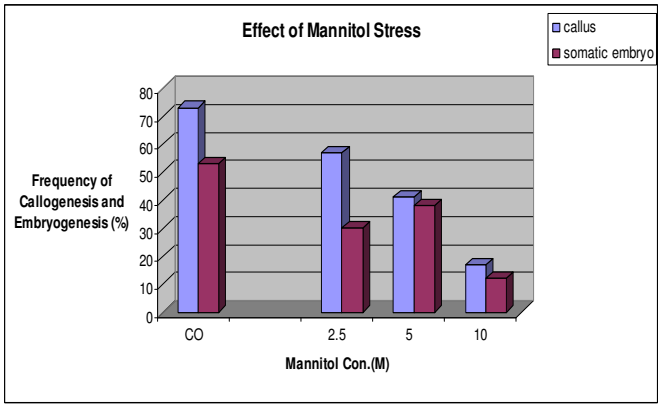

Fig 4. Effect of various concentrations of mannitol along with 2,4-D (5mg/l) on morphogenesis in terms of callogenesis and somatic embryogenesis on caryopsis culture of *Oryza sativa.*

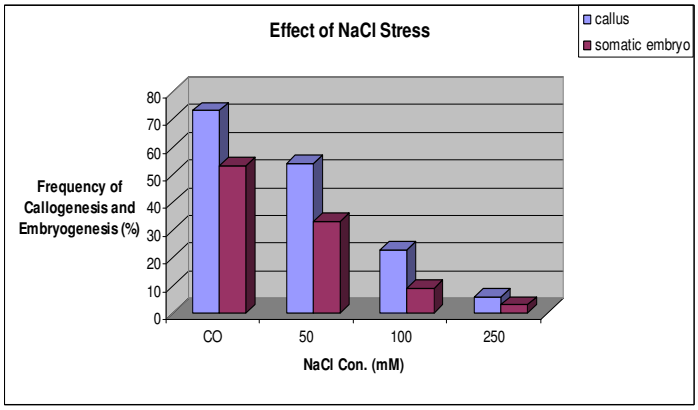

Fig 5. Effect of various concentrations of NaCl along with 2,4-D(5mg/l) on morphogenesis in terms of callogenesis and somatic embryogenesis on caryopsis culture of *Oryza sativa.*

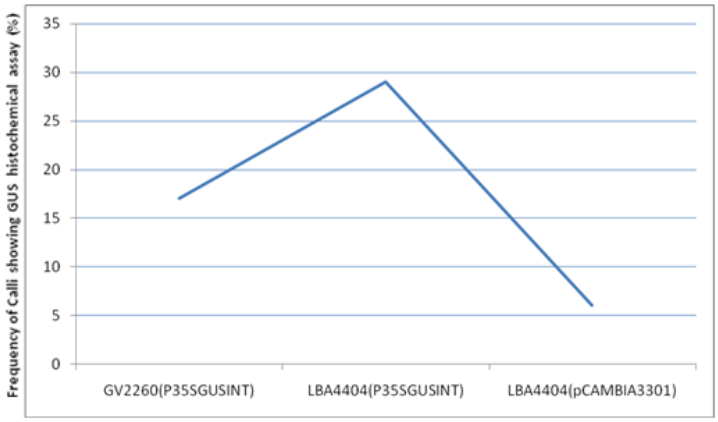

Fig 6. Frequency of Calli exhibits transient GUS gene expression mediated by *Agrobacterium tumefaciens.*

DISCUSSION

In vitro regeneration of monocots is difficult compared to dicots [13]. Maeda [14] induced callus in rice seedlings on medium containing 2,4 -D. Nishi *et al*. [15] obtained callus from rice roots on LS medium fortified with 2, 4 -D and IAA. Any regenerable cell, tissue, or organ can be used as explants. Among members of family Poaceae, plant regeneration has been achieved using young inflorescence [16], leaf, root [17] and mature embryo [18]. In present study on a local cultivar of rice, mature seeds have been employed to evaluate the *in vitro* regeneration potentials of the explants and further investigation of the various abiotic stress exposures to the explants in order to achieve stress tolerant regenerants of rice. Simultaneously, transient transformation of GUS gene via *Agrobacterium* strains has been optimized in calli derived from mature caryopsis culture.

Induction and Proliferation of Embryogenic Callus

The present study was initiated to achieve reproducible embryogenic callus production from the mature caryopsis culture and further differentiation of callus into somatic embryos. As evident from the results, a reasonable callus was induced when MS was supplemented with 2,4-D alone. These studies are in contrast with the reports of [19] where high callusing was obtained on a medium containing an auxin and cytokinin. Moreover, this combination has been proved excellent for callus proliferation and regeneration in many crop plants. However, in present study, a soft, friable and non-organogenic callus formation occurred in rice caryopsis when the MS medium contained 1.0 mg/l 2, 4-D along with BAP or NAA alone.

Nutrient medium composition and moreover the hormonal balance is an important factor influencing *in vitro* culture initiation and plant regeneration from explants tissues [20]. The auxin 2,4-dichlorophenoxy acetic acid (2,4-D) alone or in combination with cytokinins, is widely used to enhance callus induction and maintenance [21]. Genetic factors are considered to be a major contributor to the *in vitro* response of cultured tissues. Differences in the production of embryogenic calli and the regenerated plantlets have been observed, depending on the genotype and source of the explants [22].

Mature caryopses exhibited the highest percentage of callogenesis on nutrient media that were supplemented with high 2, 4 -D (5 mg/L). The quality of calli formed, however, differed. Calli formed from seeds were able to sustain proliferation, however, some of them tended to differentiate roots whereas calli formed from the explants cultured at very high concentration (10mg/L) were relatively poor to maintain growth and generally became necrotic eventually. These results indicate that calli from seeds were actively dividing as shown by the rapid change in size and were compact and nodular, a primitive features required for somatic embryogenesis.

Differentiation of Somatic Embryos

The ability to form embryogenic structure of calli is an important consideration in transformation studies because it is an

indicator of the capacity of the callus to form somatic embryos and regenerate complete plantlets. According to Seraj *et al*. [23], calli with embryogenic features exhibit high regeneration response. From proliferative callus, embryogenic calli could be stimulated with the right type and concentration of growth regulators. This was shown in bermuda grass [24] and in banana. In some cases, however, callus medium serves both for callus induction and stimulation of embryogenic response. This was demonstrated in the present study on rice and was also shown in sorghum, sugarcane and peach palm [25].

The different stages of development for indirect regeneration of plants involve induction of callus and its proliferation, followed by embryogenic callus production and plant regeneration. A previous study found no correlation between the ability to form callus and the capacity to differentiate embryos and also in between the ability of callus and capacity to regenerate plants. However, a positive relation could be found only in between the ability to produce embryogenic callus and the capacity for plant regeneration [25]. Thus, embryogenic callus percentage constitutes a good index for callus ability to regenerate later on plantlets. In the present study, therefore, the 53% embryogenic callus formed by caryopsis culture indicates high chance of regenerating plants eventually.

Regeneration of Plantlets

Callus with more proembryos/embryos and high plantlets regeneration frequency is an essential prerequisite for developing an efficient tissue culture system. There has been a constant approach to identify suitable explants to produce embryogenic calli under appropriate physical and chemical environments to maximize the callus yield with high plantlets regeneration. Therefore, in this direction, besides mature and immature seeds, young inflorescences, rachilla, anthers and microspores; roots [26] and coleoptiles [27] were reported to be good sources of embryogenic calli production.

Growth regulator concentrations play a critical role in the control of growth and morphogenesis. In general, the regeneration medium is comprised of basal medium or basal medium with BAP or NAA as phytohormones, however, in the present study, calli grown in the regeneration medium with low concentrations of NAA alone or on to BAP, differentiation of callus was not seen.

In view of achieving regeneration of plantlets, the embryogenic callus obtained on MS + 2,4-D was sub-cultured on MS with either very low 2,4-D or 2,4-D-free medium. The embryoids then germinated into plantlets. Ling and Yoshida [28] reported that 1.0 mg/l each of 2,4-D and Kn was necessary for induction of embryogenic callus in the *japonica* rice. However, lower concentrations were required for plantlets development. Similar results were obtained by Carmen *et al*. [29] that higher concentration was necessary for embryogenic callus induction and maintenance, but a lower for plant formation in the embryos. As a result of these studies, a protocol has been developed for embryogenic callus formation and its subsequent regeneration into plantlets. Using mature seed explants in present study, callus formation, embryogenic response, and even regeneration of plantlets were attained. Thus, it will be suitable for use in the future as source of target material for genetic transformation of rice.

Morphogenic Responses under Abiotic Stress

Plant cell and tissue culture has been a useful tool to study stress tolerance mechanisms under *in vitro* conditions [30]. *In vitro* culture techniques minimize environmental variations due to defined nutrient media, controlled conditions and uniformity of stress application. Abiotic stresses can directly or indirectly affect the physiological status of an organism by altering its metabolism, growth and development. A common response of organisms to drought, salinity and low-temperature stresses is the accumulation of sugars and other compatible solutes. These compounds serve as osmo-protectants and, in some cases, stabilize biomolecules under stress conditions.

Production of rice is limited by abiotic factors like saline soil or water crisis. One way to improve its yield is by genetic manipulation wherein genes for stress – resistance are introduced into its genome. Before embarking on transformation, however, the capability to grow *in vitro* and the regeneration potential of possible target tissue must first be determined because the success of genetic manipulation and transformation of species would depend upon their ability to *in vitro* regeneration.

Drought is one of the most common environmental stresses affecting plant growth and productivity [31]. Simulation of drought stress under *in vitro* conditions during the regeneration process constitutes a convenient way to study the effects of drought on the morphogenic responses. Dragiiska *et al*. [32] developed a system for *in vitro* selection during somatic embryogenesis in alfalfa (*Medicago sativa* L.).

Among osmotic agents, mannitol is the most widely used osmoticum to study plant status. Mannitol is inert, non-ionic and non-toxic and of high molecular weight. It is very soluble in water, and it simulates water deficit conditions in cultured cells in a similar manner to that observed in the cells of intact plants subjected to actual drought conditions. It induces water stress in plants by decreasing the water potential of the nutrient solution without being taken up and with no evidence of toxicity.

The addition of mannitol to medium decreases the water potential of the medium inducing water stress that adversely affects the callus growth and *in vitro* regeneration capacity. Similarly, the role of PEG in creating chemical drought was demonstrated in wheat by exposing seedling to 20% PEG (6000) for one week [33]. However, Al-Bahrany [34] studied the response of *Hassawi* rice (*Oryza sativa*) callus to varying degrees of (PEG 8000) induced water stress including callus growth, water content and proline accumulation. Lagerwerff *et al*. [35] indicated that PEG can be used to modify the osmotic potential of nutrient solution culture and can induce plant water deficit in a relatively controlled manner, appropriate to experimental protocols [36].

The presence of PEG in the regeneration medium exhibits a detrimental effect upon most protocols of plantlet regeneration. There was a gradual decrease in the total number of viable plantlets regeneration under *in vitro* stress conditions in potato [37] and rice [38].

In present work mannitol was used as a selective agent for osmo-tolerance. The procedure involved growing mature seeds on nutrient media supplemented with various concentrations of mannitol along with callus inducing hormone 2,4-D. This study is also in conformity with other earlier reports where potentials of tissues for callogenesis along with embryo formation gradually decline if the level of mannitol is being increased in the nutrient media. However, at optimal level of mannitol (5M), both callus induction and somatic embryo differentiation was clearly evident and moreover, further increase of mannitol concentration proves to be supportive for

compact callus induction along with differentiation of defined typical somatic embryos. Significantly, lower levels of mannitol (2.5M), exhibited differentiation of direct somatic embryos all over the surface of growing epicotyls tissues.

Rice represents a group of plants in which the correlation of salt tolerance, compared at cell and whole plant levels, is negative, i.e., the isolated cells are much more tolerant to salt than the whole plant. Cells with increased salt tolerance can be selected from different rice genotypes by *in vitro* culture. Saline soils are one of the major biotic stresses that adversely affect the overall metabolic activities and cause plant demise [39].

The compounds, which include simple or complex sugars, sugar alcohols, inositols, proline and higher polyamines (PAs), serve as osmo-protectants under stress conditions, maintain membrane structure and act as free-radical scavengers [40]. A wide variety of species also synthesize the phytohormone abscisic acid (ABA) as an adaptive response to reduce transpiration via stomatal closure [41] and express common array of genes and similar specific proteins such as late embryogenesis abundant (LEA) [42] or salt protein which has several proposed protective functions.

Results obtained here indicate that cultured rice cells may respond variously depending on the concentration of stress agents present in the nutrient medium. Embryonic cells have been identified as the salt-tolerant-cell type in the mix population of different cells. The embryogenic cells grew slowly, generating typical globular structures. These cells could give rise to embryogenesis in both salt-free and low salt-containing conditions. While high salt stress (250 mM) inhibits differentiation, the embryogenetic cells can adapt to growing with totipotency retained over subcultures in the continuous stress condition.

Agrobacterium-mediated Transient Transformation

Agrobacterium-mediated transformation (AMT) is the method of choice for transformation as it is a simple, low cost and highly efficient alternative to direct gene delivery methods such as biolistics. The main advantage of AMT is the defined insertion of a discrete segment of DNA into the recipient plant genome. Only a single or fewer copies of the gene get integrated into the plant genome, potentially leading to lesser problems with transgene co-suppression owing to homology, and gene silencing. Since there is no rearrangement of the transgene during integration into the host genome, the resulting stable integration patterns favours high level of transgene expression [43].

The mature caryopses derived embryogenic calli used for transformation with agrobacterial strains GV2260 harbouring p35SGUSINT, LBA4404 pCAMBIA3301 and LBA4404 p35SGUSINT and were subjected to GUS assay after co-cultivation stage. Transient GUS gene expression was observed in all constructs. While *Agrobacterium* strain LBA4404 p35SGUSINT resulted in greater proportion of explants expressing GUS gene exhibited in intense blue colouration of the explants.

Agrobacterium strain LBA4404 p35SGUSINT was more efficient than GV2260 p35SGUSINT for rice transformation whereas LBA4404 pCAMBIA3301 proved to be the least effective for transient transformation amongst the entire constructs tested above in rice cultivar. This result is in contrast of the earlier report where *Agrobacterium* strain EHA 105 was more efficient than LBA 4404 and AGL1 for rice transformation [44]. Hence, these results in a local cultivar of rice would support the successful application of the technology for using high fodder and grain yielding crops.

CONCLUSION

Totipotency in terms of somatic embryogenesis was recorded and therefore, embryogenic callus formed by caryopses indicates high chance of regenerating plants eventually required for genetic transformation programs. Present study also indicates that potentials of tissues for callogenesis along with embryo formation gradually decline if the level of mannitol is being increased in the nutrient media. However, at optimal level of mannitol direct somatic embryo differentiation was clearly evident at the epicotyls tissues. The embryogenic cells grew slowly, generating typical globular structures even at high stress level. While high salt stress inhibits differentiation, the embryogenic cells can adapt to growing with totipotency retained over subcultures in the continuous stress condition. For transformation experiments, *Agrobacterium* strain LBA4404 p35SGUSINT was more efficient than GV2260 p35SGUSINT whereas LBA4404 pCAMBIA3301 proved to be the least effective in terms of delivery of GUS gene in the target embryogenic tissues.

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