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Efficient Callus Induction and Regeneration Protocol for the Rice *Indica* Genotype Sonali

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ABSTRACT

Most of the *indica* rice varieties are recalcitrant for regeneration and genetic transformation that makes it difficult to achieve effective genetic improvement. The prerequisite for genetic enhancement of *indica* rice genotypes by transgenic approach is to develop an effective protocol for callus induction and plant regeneration. We have developed an easy, rapid and highly efficient *agrobacterium* mediated transformation and regeneration protocol using mature seeds as explants. The combination of 2.5 mgL^{-1} of auxin 2,4-D and 2.0 mgL^{-1} of cytokinin BAP in MS II media induced higher callus in sonali variety and the regeneration percentage of 82.2 per cent was obtained when the media is supplemented with 0.2 mgL^{-1} NAA, 1.0 mgL^{-1} BAP and 2.5 mgL^{-1} Kinetin. The transformants were successfully selected on hygromycin selection media. The method may be used in other elite cultivars.

Keywords : Rice, Transformation, Regeneration, Mature seed-derived calli, *Agrobacterium*

RICE (*Oryza sativa* L.) is one of the most important cereal crops, providing a staple diet for almost half of the world's population (Nisarga *et al*., 2016). There is an enormous need to improve the yield of local rice varieties in order to combat the food demands of increasing population. Rice production has slowed down and it is estimated that rice production has to be increased 50 per cent by 2025 (Khush *et al.,* 2000). In recent years a considerable improvement has been made by exploiting the natural variation through conventional breeding. However, traditional breeding efforts alone cannot meet the increasing demand of rice production. Several biotechnological approaches including transformation*, in situ* and *in vitro* hybridization to introgress new genes from different genotypes, transformation has been successfully developed *via* several techniques including *Agrobacterium* mediated method, electroporation, particle bombardment and polyethylene glycol treatment since 1980s (Chen *et al.*, 2009). *Agrobacterium* mediated gene transfer, is one of the most common rice transformation methods, has been extensively used for developing transgenic rice to study gene function and also to improve agricultural traits like, resistance to disease and insect pest, tolerance to drought, salt and higher quality and yield. Transformation of commercially important *indica* cultivars remains challenging for the scientific community even though *Agrobacterium* - mediated transformation protocols for a few *indica* rice lines have been well established.

The frequency of callus formation and plant regeneration is influenced by the interaction of genotype and its *in vitro* culture conditions. The most sensible approach is to improve callus induction frequency and green shoot regeneration by using appropriate media for production of number of embryogenic calli and regenerated plants. Hence, the establishment of a robust and reproducible tissue culture system is prerequisite for successful transformation in *indica* rice.

Most of the *indica* rice genotypes, the world's most cultivated rice types, still remain less amenable to genetic modifications due to their poor regeneration potential. The existing protocols for transformation and regeneration of *indica* rice are tedious, lengthy and highly genotype-specific with low efficiency of

transformation (Hiei *et al.,* 1997; Khanna *et al.,* 1999; Kumar *et al.,* 2005). Considering the significance of genetic transformation in functional genomics and crop improvement, the need of the hour is to develop an easy, rapid, reproducible, widely applicable and highly efficient transformation and regeneration protocol for various *indica* rice genotypes which does not necessitate further genotype specific standardization. To achieve the above objective, one needs to consider the factors affecting successful transformation and regeneration of transformed calli.

In the present study, a highly efficient and reproducible *A. tumefaciens* mediated transformation protocol for *indica* rice using mature seeds as explants was developed. To improve the regeneration frequency, the proportion of growth regulators, kind and concentration of the gelling agents were optimized and studied their effect individually. Comprehensive protocol where all these modifications were combined to attain maximum transformation efficiency. Very high regeneration frequency of transformed as well as untransformed calli was obtained, thus overcoming the main hurdle in genetic manipulation of rice.

Material and Methods

Plant Material

The *indica* type rice *Oryza sativa* L. *cv.* Sonali was used in this study. The seeds were obtained from the Centre for Plant Molecular Biology, Osmania University, Hyderabad.

Seed Sterilization

Healthy seeds were manually dehusked and rinsed in 70 per cent (v/v) ethanol for 90 seconds and then surface sterilized in 0.1 per cent (w/v) Mercuric chloride with mild shaking for 30 sec. to one minute. Seeds were washed thrice by vigorous shaking in sterile distilled water and dried on a sterile blotting paper.

Callus Induction

Twelve seeds were plated in each 90 mm petri plates containing 30-40 ml of one of the callus induction media. The callus induction medium developed for this study denoted as LS media (Linsmair and Skoog, 1965) MS I and MS II (Murashige and Skoog, 1962). Composition of the callus induction media with special reference to the plant growth promoters, amino acids and additional supplements were provided (Table 1). The pH of the media was adjusted to 5.8 and 8 per cent agar was added before autoclaving it for 15 min at 121 ºC. Dehusked rice seeds were placed on the callus induction medium with the scutellum surface facing upward. Petri plates were sealed with 'Parafilm' (Whatman, England) and incubated in the dark at 27 ± 2 °C. After 2-3 weeks, swollen scutella were separated from the endosperm and roots and placed on fresh callus induction medium with the scutellum surface up. The white, friable embryogenic calli were excised and transferred to the fresh medium containing same concentration of growth regulators for further growth. The callus induction frequency on different callus

Table 1

Different callus induction medium with various concentrations of growth regulators

Media	Growth Regulators (mg L^{-1})			Amino acids (mg L^{-1})				Supplements		
	2,4-D	BAP	Kinetin	Proline	Tryptophan	Glycine	Casamino acid (mgL^{-1})	Sucrose (g L^{-1})	Maltose (g L^{-1})	Myo-inositol (mg L^{-1})
LS I media	2.5	0.15	1.0	500	50	-	500	30		-
LS II media	2.5	2.0	-	-	50	2.0	1000	-	30	100
MS I media	2.5	0.15	1.0	500	50	-	500	30		-
MS II media	2.5	2.0	-	-	50	2.0	1000	-	30	100

induction media was calculated by using the following formula (Islam, *et al.,* 2005).

$$\text{Callus induction frequency} = \frac{\text{Number of calli produced from seeds}}{\text{Number of seeds planted}} \times 100$$

Agrobacterium Mediated Callus Transformation

A single colony/10 µl of *A. tumefaciens* strains LBA4404 harboring pRGEB32 (specific vector for CRISPR-Cas9 mediated gene editing which has a Cas9 expressing region with a ubiquitin promoter and sgRNA cloning region with U3 promoter) carrying *MLH1* (*Mut L homolog 1*) gene was cultured in Luria Bertini (LB) broth medium supplemented with kanamycin (100 mg/ ml) and rifampicin (10 mg/ ml) are grown at 28 ºC and 250 rpm shaking for 48 hrs until OD600 = 1.0. *Agrobacterium* cells were pelleted by centri--fugation at 6000 rpm for 15 min at 4 ^{0}C.

The cells were resuspended in MS resuspension medium containing 150 µM acetosyringone (MS salts 68g / l sucrose, 36g / l glucose, 3g / l KCl, 4g / l $MgCl_2$, pH: 5.2) to adjust the OD of the bacterium suspension to 0.3. The calli were infected with *Agrobacterium* culture for 20-25min with intermittent gentle shaking. They were then co-cultivated on the callus induction medium for 48 hrs. After co-cultivation, the seeds calli were washed thoroughly in sterile distilled water containing 250 mg / l cefotaxime, dried on sterile Whatman No. 3 filter paper and transferred onto callus selection medium (CSM: MS salts and vitamins supplemented with 30 g/l maltose, 0.3 g / l Casein hydrolysate, 0.6 g / l Proline, 3 mg/ l 2, 4-D, 0.25 mg / l BAP, 200 mg / l Cefatoxime and 30 mg / l Hygromycine, pH: 5.8) and incubated for 12 days at 27 ± 1°C in dark. After the first selection, brown or black calli were removed and only creamish healthy calli were shifted to the fresh CSM media for second selection and maintained at 27 ± 1 °C in dark. After second selection for 10 days, microcalli could be observed which were finally transferred to fresh MSM media for third selection and allowed to proliferate for 5 days at 27 ± 1 °C in dark.

Shoot Regeneration

To standardize the shoot regeneration media for rice genotype Sonali, five different media combinations with different growth regulators and gelling agents were used. Granualar creamish healthy calli collected after third selection were transferred to maintenance media (MS salts and vitamins supplemented with 30 g / l sucrose, 2.0 mg / l NAA, 0.5 mg / l Kinetin, 0.5 mg / l BAP, 200 mg / l Cefatoxime, pH: 5.8) for the better growth of calli. Actively growing calli were later transferred to the five different shoot regeneration medium containing different hormonal combination and gelling agents (SRM 1, SRM 2, SRM 3, SRM 4, SRM 5). The combination of hormones used in the media was listed in Table 2. The plant regeneration frequency

TABLE 2

List of media used for shoot regeneration

Media	Growth Regulators (mg L^{-1})			Gelling agent
MS basal + Vitamin	NAA	BAP	Kinetin	
SRM 1	0.2	-	2	Agar
SRM 2	0.2	3	1	Agar
SRM 3	0.2	1	2.5	Clerigel
SRM 4	0.5	3	-	Clerigel

was calculated by the following formula (Islam, *et al.,* 2005).

$$\text{Shoot regeneration frequency} = \frac{\text{Number of shoots produced from calli}}{\text{Number of calli plated}} \times 100$$

Root Induction and Plant Hardening

Healthy shoots with defined stem were transferred to MS rooting medium (MS medium supplemented with 0.5 mg L-1 IBA, 0.4 per cent clerigel, 250 mg / L Cefotaxime, pH–5.8) and incubated at 28 °C under continuous light for complete initiation of roots. The rooted plants were taken out from the medium and washed under running tap water and then transferred to soilrite and the pots were covered

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with polythene bags for initial plant hardening. The plantlets were established in such a way that the clump of plantlets originated from each callus was separated carefully and planted in several pots. The hardened plants were finally transferred to the soil in the mud pots and were kept in green house and maintained at 30 °C with relative humidity 80 per cent.

Results and Discussion

Callus Induction and Regeneration

Efficient callus induction, transformation and regeneration depend on the composition and concentration of different components in culture media. Choice of explant, hormonal composition of the medium used and nutritional supplements, antibiotic concentration, highly affect the callusing and regeneration (Katiyar *et al.,* 1999; Pipatpanukul *et al.,* 2004 and Sawant *et al.,* 2018). The concentrations of plant growth regulators majorly influence the process of callus induction (Revathi & Pillai, 2011 and Reddy *et al.,* 2013). Different crops respond to different hormones and their concentration. Within the crop, between the sub-species, there is a large variation in callus induction response at same hormone concentration (Shanthi *et al.,* 2010). Sahoo *et al*. (2011) reported that scutellum derived callus was the most amenable explant for *Agrobacterium* mediated transformation. Therefore, it is necessary to identify the optimized hormone concentration required for the efficient callus induction and regeneration. The different stages of callus was induced from scutellum by using the mature embryo as explants on two different media, *i.e.,* LS (Linsmaier & Skoog Medium, 1965) and MS (Murashige & Skoog, 1962) media with different hormonal combinations (Fig. 1a-d). To optimize callus induction in Sonali *indica* rice variety, four different combinations of 2, 4-D with varying concentrations of BAP (0.15 and 2.0 mgL^{-1}) and Kinetin (0.0 and 1.0 mgL^{-1}) was tested (Table 1). The embryo of healthy seeds germinated within 2-3 days and produced shoots and roots. However, further development of shoots and roots was inhibited and the scutellum enlarged. Within 7-10 days, callus formation was observed on scutellum surface. In ungraded seeds many embryos showed black coloration and did not grow further. Black coloration of embryos was also seen when seeds were surface sterilized in mercuric chloride ($HgCl_2$) for 10 minutes. After 20 days of incubation under dark at 26 ± 2 °C, calli initiated from scutella were sub cultured on fresh callus induction medium. The callus induction frequency on different callus induction media was calculated by considering the percentage callus produced from the number of seeds inoculated. Among the four combination of hormones used, 2.5 mgL^{-1} of 2, 4-D and 2.0 mgL^{-1} of BAP supplemented with the aminoacids, tryptophan (50.0 mgL^{-1}) glycine (2.0 mgL^{-1}) and casamino acid (1000 mgL^{-1}), induced more number of callus produced from the seed inoculated. The other hormonal combinations induced comparatively less callus and lowest callusing (53.75%) was recorded in LS II media (Fig. 2). The results suggest that the combination of 2.5 mgL^{-1} of 2, 4-D and 2.0 mgL^{-1} of BAP in MS II media has induced higher callus in Sonali variety and same concentration was used for further callus induction and sub-culturing. The resulting calli were creamish, nodular, compact and highly prolific. However, seeds in LS media produced non-embryogenic rhizogenic calli.

Addition of amino acid tryptophan had improved callus induction frequency and callus growth in *indica* rice (Table 1) as similar to earlier reports (Chowdhury *et al.,* 1993). Among the exogenously applied hormones, auxins played an active role in the conversion of somatic cells to embryogenic cells and 2, 4, D is the best growth regulators for achieving this (Khalequzzaman *et al.,* 2005; Sridevi *et al.,* 2005; Tyagi *et al.,* 2007 and Hoque *et al.,* 2013). However, in this study, a cytokine (BAP and kinctin) was used along with the auxin 2, 4, D which resulted in generation of higher embryogenic calli compared to cultures grown on 2, 4, D alone. The total amount of callus and the percentage of callus volume composed of embryogenic cells of rice could be increased if 0.1-0.5 $mg \cdot l^{-1}$ kinetin was added to

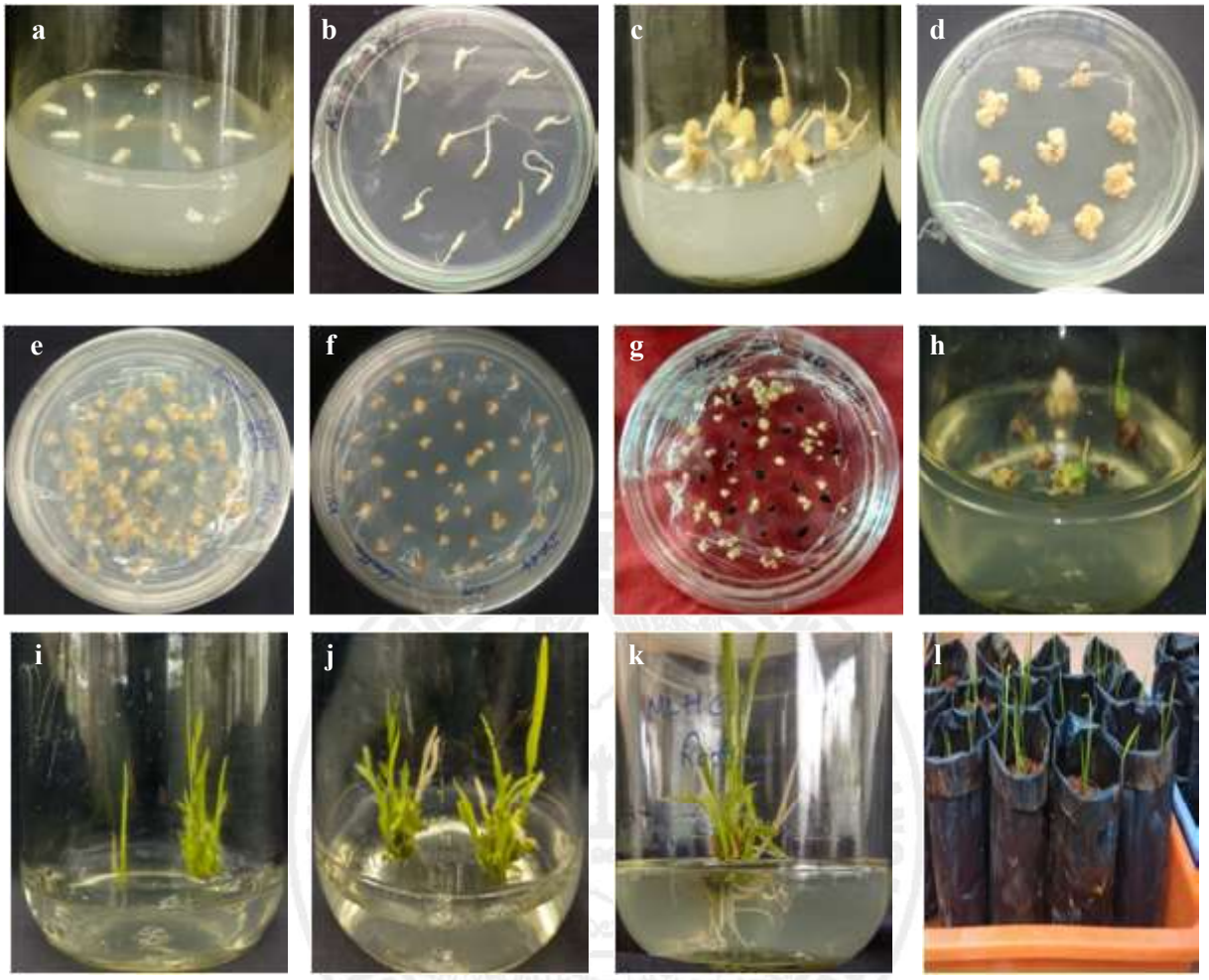

Fig. 1 : Generation of transformants,from scutellum of mature seed of the *indica*ricevariety, Sonali (a-d), callus transformation (e-f), green areas in the transformed calli (g-h), shoot regeneration (i-j), rooting (k), primary hardening (l).

medium containing 0.5 or 1.0 mg·l^{-1} 2, 4-D (Ram and Nabors, 1984). Supplementary effect of the addition of cytokinin along with auxin in callus induction media for rice callus induction was also reported by Raina *et al.* (1987), Hartke and Lorz (1989). A significant amount of embryogenic calli was noted in 2, 4, D (2.0 mg$^{-1)}$ and BAP (2.0 mg-1) compared to cultures grown on other combinations of phytohormones. Further, Yellowish, compact, friable calli were observed in this combination of media and calli exhibiting these type of morphology often considered as embryogenic calli.

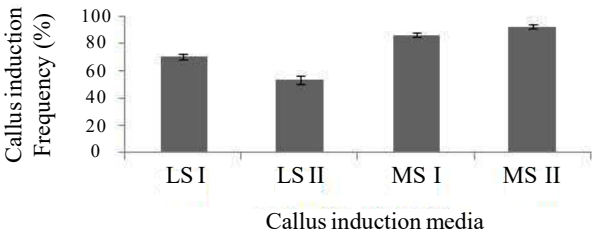

Fig. 2 : Frequency of callus induction on different media, from mature seed embryo rice genotype Sonali (LS: Linsmair and Skoog, MS: Murashige and Skoog media)

The aminoacid glycine and casein hydrolysate is reported to enhance the cell growth in culture media by providing nitrogen source (Saad *et al.,* 2012). The supplementation of culture medium with more of casamino acid stimulated formation of nodular embryogenic calli. Casein hydrolysate / casamino acid as a supplement in rice tissue culture medium has been widely used for callus induction from mature seeds and immature embryo (Wang *et al.,*

1987), seedling leaf bases (Sticklen *et al.,* 1991), root (Abe and Futsuhara, 1984), endosperm (Davoyan, 1981), anther (Comejo-Martin and Primo-Milo 1981) and protoplast-derived calli (Gupta and Pattanayak 1993). Casamino acid, when used as a supplement, induces nodular embryogenic calli from scutellum of mature seed and immature embryo (Wang *et al.,* 1987).

Sucrose was used as the energy source to assess response of callusing in these media. Preliminary screening revealed wide variation in callus induction frequency. To select the most responsive energy source, media were supplemented with both sucrose as well as maltose and the callus induction frequency was higher in media supplemented with maltose. MS media supplemented with 30 gL^{-1} maltose induce healthy embryogenic calli from mature seeds (Sahoo *et al.,* 2011).

Maintenance of Calli

Calli produced from mature embryos were sub-cultured regularly to induce friability and to maintain embryogenesis. At first subculture, enlarged scutella with developing calli were replated on callus induction medium and maintained for 15 days. Scutella showed further enlargement and nodular embryogenic and unorganized non-embryogenic calli became more prominent. Embryogenic calli were friable, dry and pale yellow in colour. After about 15 days of re-plating of enlarged scutella, embryogenic calli were separated from non-embryogenic calli and plated on the callus induction medium. Subculturing at 15 days interval and separation of embryogenic calli from non-embryogenic calli was found to be most suitable for maintaining large number of embryogenic calli for a longer period. On delayed sub culturing embryogenic calli showed a tendency to become non-embryogenic. Maintenance of these calli in the dark was better as maintenance in light led to organogenesis.

Transformation of Rice Calli

Healthy embryogenic calli were infected with *Agrobacterium* culture for 20-25 min with intermittent gentle shaking. They were then co-cultivated on the callus induction medium for 48 h. After co-cultivation, the calli were transferred on to callus selection medium. Healthy, creamish transformed calli from the selection media was then transferred to the regeneration media containing growth regulators, sugar supplements and appropriate gelling agents.

Shoot Regeneration

To check embryogenecity of calli, embryogenic calli obtained from mature embryo was transferred to hormone free MS medium for plant regeneration. All calli required an initial culture period of 7-8 days in the dark. During this period globular embryo like structures developed from the friable callus (Fig. 1e-f). These globular structures on transfer to 16 / 8 hour light / dark period developed green pigments within one week and germinated to form shoots and roots within 10-15 days of transfer to light (Fig. 1g-h). Plant regeneration was significantly enhanced when MS medium was supplemented with BAP 1.0 mgL^{-1}, 2.5 mgL^{-1} kinetin and 0.2 mgL^{-1} NAA (Table 2). To standardize the shoot regeneration media for rice genotype Sonali, four different media combinations with different growth regulators and gelling agents were used. Granular creamish healthy calli collected after third selection were transferred to maintenance media (MS salts and vitamins supplemented with 30 gL^{-1} sucrose, 2.0 mg L^{-1} NAA, 0.5 mg L^{-1} Kinetin, 0.5 mg L^{-1} BAP, 200 mg L^{-1} Cefatoxime, pH : 5.8) for the better growth of calli. Actively growing calli was transferred to the four different shoot regeneration medium containing different hormonal combination and gelling agents (SRM 1, SRM 2, SRM 3, SRM 4- Table 2). The plant regeneration frequency was calculated by considering the number of shoots produced from each calli transformed (Islam, *et al.,* 2005). Among the shoot regeneration media (SRM) used, SRM3 showed highest frequency of plant regeneration (82.2%) followed by SRM4 (74.4%) and lowest in SRM1 (31.11%) (Fig. 3).

Although regeneration was observed in MS medium with only cytokinin source, presence of low concentration of auxin (NAA) and higher

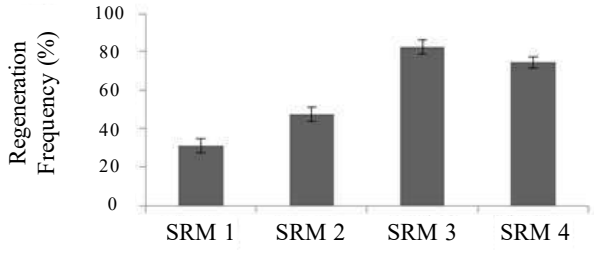

Fig. 3 : Regeneration frequency of the transformed calli from seed embryo of rice in different shoot regeneration media (SRM).

concentration of cytokinin (kinetin) in the regeneration medium improved plant regeneration from callus and similar observations were recorded in different crop species (Maddock 1985; Maggioni *et al.,* 1989 and Zapata *et al.,* 1999). Suitability of MS medium for plant regeneration in *indica* rice has been reported (Khanna and Raina, 1999).

Different type of the growth regulators and their concentrations, type of gelling agent as well as their concentration used in the regeneration medium influences the regeneration frequency. Different gelling agents such as clerigel, agar and agar, were used. The regeneration frequency for the tested cultivars, ranged between 30-48 per cent when agar was used. Importantly, significantly higher regeneration frequency (70-80%) was obtained when 0.3 per cent Clerigel was used as the gelling agent for regeneration of calli (Table 2).

Healthy shoots with defined stem were transferred to MS rooting medium (Fig. 4 i-j) and the rooted plants were taken out from the medium and allowed for hardening (Fig. 1k). The plantlets were established in such a way that the clump of plantlets originated from each callus was separated carefully and planted in several pots. The hardened plants were transferred to the soil in the mud pots and were grown green house and maintained at 30 °C with 80 per cent relative humidity.

There are 29 putative transformed lines generated through *in vitro Agrobacterium* mediated tissue culture approach. The plants were selected against the selectable agent hygromycin. The rooted plants were taken out from the medium and transferred to soil rite and hardened. Seventeen plants survived the hardening pressure that was transferred to the pots and established in green house. In the containment facility only 14 plants survived and attained maturity and set seeds.

Molecular Confirmation of Putative Transgenic Plants

Genomic DNA was isolated from the putative transformed lines and is subjected to PCR for confirming the integration of transgenes in the genome (Pooja and Sheshshayee, 2017). PCR was carried out using the selectable marker *hptII* gene specific primer and gene specific primers. Out of the 14 transformed plants, 12 plants showed amplification with *hptII* gene specific primers (Fig. 4).

Regeneration of the transformed calli is considered to be a major barrier in genetic transformation of rice. The success of any plant transformation depends on efficient and reproducible plant

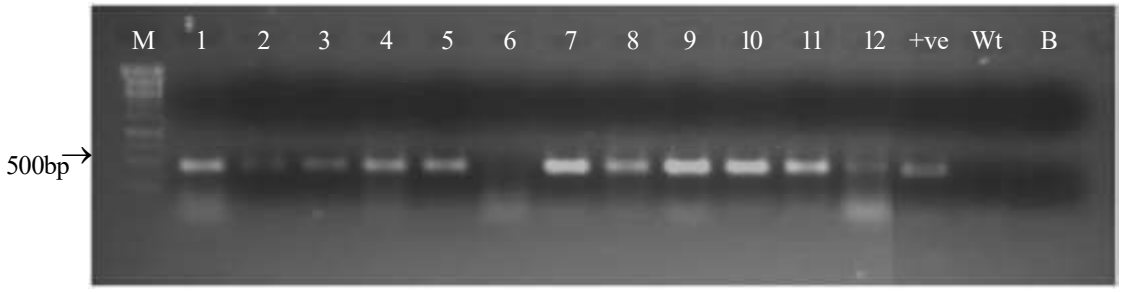

Fig. 4 : Molecular characterization of T_0 transgenic rice plants. DNA was extracted from T_0 transgenic plants and PCR analysis was done to confirm the integration of transgene using antibiotic resistant marker gene (*hptII)* specific primers showing amplicon size of 500bp.

regeneration system. We have standardized media composition for efficient callus induction and plant regeneration for *indica* rice genotype sonali by using mature seeds as explants. We have made a significant advance in enhancing both the transformation efficiency as well as regeneration frequency of *indica* rice genotype which will be of great help to rice studies. Our optimization of the kind and concentration of the gelling agent, proportion of growth regulators led to the formulation of a comprehensive and improved protocol. Which has the potential to serve as a simple, easy and efficient protocol for rice transformation paving the way for gene function studies on the model monocot plant-rice as well as for easier production of improved genotypes by genetic engineering.

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