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REGENERATION AND TRANSFORMATION OF BANANA CULTIVAR GRAND NAINE

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ABSTRACT

Genetic transformation of banana (*Musa sp*.) by particle bombardment has been achieved only in a few laboratories worldwide. In general, transformation frequencies are reported to be cultivar dependent. Thus, there is a need to adapt and optimize transformation protocols for a particular banana cultivar. Establishment of a highly efficient and widely used tissue culture system for banana will accelerate the application of transformation technology in breeding programmes. Standardization of growth regulator concentration for callus induction from male inflorescence buds, regeneration from callus and multiple shoot regeneration from *in-vitro* shoot cultures for the Grand Naine cultivar of banana was carried out. 2, 4-D (3.0 mgL^{-1}) produced friable white calli with higher per cent of calli (40 %) in MS media. This calli was suitable for biolistic transformation. Regeneration efficiency was high (36.11 %) in TDZ at 2.0 mgL^{-1} followed by BAP at 4.0 mgL^{-1} (34.10 %) in MS media. TDZ at 0.8 mgL^{-1} produced an average of 11 shoots per explant from the *in-vitro* shoot cultures. Multiple shoot induction was highest (81.60 %) with 0.8 mgL^{-1} TDZ. In this study, the Biolistic transformation method was followed and the effect of Target Cell Distance (TCD) on transformation frequency was investigated in Grand Naine *sp.* (AAA) banana cultivar. Efficiency of Biolistic transformation was found to be high at the Target Cell distance of 9 cm (stage 3) with 48.35 % *GUS* positive calli, while shoot tip cultures showed 45.88 % *GUS* positive cultures at the same distance.

KEYWORDS: Grand Naine, Male Inflorescence, Explant, Callus Induction, *in-vitro* Shoot Tip Culture, Genetic Transformation, Particle Bombardment, β-glucuronidase, Histochemical Assay

INTRODUCTION

Banana (*Musa sp.)* is one of the major staple fruit crops of tropical and sub tropical regions of the world. Banana is considered to have originated in South East Asia. Among the most important growing regions of the world, India is the largest producer of banana with an annual production of 26.21 million tonnes (FAOStat, 2008). Considering the nutritive value and its cheapest among all other fruits banana is called “Poor Man’s Apple”. Botanically Banana plants are large herbaceous monocots that belong to the *Musa* genus of the *Musaceae* family. Most cultivated bananas are seedless triploids (AAA), so they mainly propagated vegetatively by suckers. India has a rich genetic diversity of banana among all cultivars, Grand Naine a cultivar of Cavendish (AAA) has become one of the most popular varieties for commercial plantations. Grand Naine is said to be most suitable genotype for mass propagation through tissue culture method. The main constraints of banana cultivation in india are poor crop management, prevalence of virus complex such as bunchy top, banana streak and bract mosaic and fungal diseases like fusarium wilt, sigatoka leaf spot. Pest infestations like weevil, borers and nematodes have threatened the yield and quality of banana. In addition to this, sterility and polyploidy often hamper the breeding programmes for the development of superior banana varieties. In this context, Genetic transformation and *in-vitro* regeneration techniques have considered to provide the necessary tools for crop breeders to introduce value added traits into banana cultivars. In banana propagations, different

types of explants have been used. These include zygotic embryos, rhizome slices and leaf sheaths, proliferating Meristem cultures and immature male/female flowers. Among all these explants, Male inflorescence is used as a potential regenerable explant. Specifically, male inflorescence reduces contamination rate during micropropagation as compared to soil grown suckers. Therefore, the male inflorescence culture can help to increase the efficiency of micropropagation, as well as produce plantlets from the parts which could be lost during harvesting.

The process of genetic transformation entails several steps, the most important being DNA delivery method, efficient selection for transformants and regeneration of transformants. Currently, a number of procedures exist for the genetic transformation of plant cells. These range from exploitation of the natural gene transfer system of *Agrobacterium* (Hooykaas *et al*., 1992), to the chemical treatment of isolated protoplasts by Polyethylene glycol (Potrykus *et al*., 1985) and the physical procedures of DNA introduction, including Electroporation of Protoplasts (Rhodes *et al*., 1988) and Tissues (D'Halluin *et al*., 1992), Microinjection (Neuhaus *et al*., 1987) and Silicon Carbide Fiber mediated transformation (Kaeppler *et al*., 1990). The first report on banana transformation was given by Sagi *et al*. (1994), who transformed protoplasts of cv. Bluggoe by Electroporation and frequency of DNA introduced as detected by transient expression of the *uid A* gene and he was also reported efficient method for direct gene transfer via particle bombardment of embryogenic cell suspension in cooking banana cultivar Bluggoe and plantains.

Particle bombardment or gene gun method of transformation is one of the promising physical gene transfer technique for transformation of monocots like banana. It offers several advantages like ready applicability to a variety of biological systems, a wide range of cells and tissues, and has the ability to effectively overcome physical barriers to gene transfer, such as the cell wall. This technique was first described as a method of gene transfer into plants by Klein *et al*. (1987) and was initially named as biolistics by its inventor Sanford (1988). The transgenic nature of the plants can be confirmed by transgene expression assays, molecular analysis, and inheritance of the introduced gene in subsequent generations. Therefore, selectable marker genes such as antibiotic resistance genes, herbicide resistance gene and visible reporter genes such as β-glucuronidase (GUS), luciferase (LUC) and green fluorescent protein (GFP) are co-transformed with the gene of interest for transient expression studies as easy indicators of the integration of a transgene. Among reporter genes, β-glucuronidase (GUS) is said to be one of the most widely used reporter gene in transgenic plant research.

The present study includes induction of callus derived from male inflorescence, shoot regeneration from callus and multiple shoot induction from *in-vitro* shoot cultures of banana *cv*. Grand Naine. The plasmid (p^{ABC}) construct, containing *GUS* gene was transferred to the callus and shoot tip cultures of banana by particle bombardment method. The effect of Target Cell Distance (TCD) on transformation frequency was investigated. The GUS histochemical assay was performed to confirm integration and expression of the transgene (*GUS* gene) in the Grand Naine cultivar.

MATERIALS AND METHODS

Location

The present investigations on “Regeneration and Transformation of Banana cultivar Grand Naine” were carried out during the years 2009-10 at the Plant Tissue Culture Laboratory, Department of Plant Biotechnology, University of Agricultural Sciences, GKVK Campus, Bangalore-560065.

Chemicals and Glassware's

All the chemicals (Salts of Macro and Micro elements), plant growth regulators (2,4-D, BAP and TDZ) and vitamins, amino acids, sucrose, Agar, myo-inositol used in the present investigations were of analytical grade and were procured from standard chemical manufacturing companies, Himedia, co., Mumbai and Merck Ltd., Mumbai. Petri plates, flasks, beakers etc., of Borosil made, were procured from M/S. Borosil India Ltd, Bombay. Apart from this the culture bottles (Jar) used were obtained from the Local market.

Cleaning and Sterilization of Glassware's

The required laboratory glassware was soaked in detergent solution (Teepol 0.1%) overnight and were thoroughly washed in running tap water and rinsed twice in double distilled water (DDW). Then washed glasswares were autoclaved at 121^0C at 15 lbs pressure for 15 minutes. Clean and dry glassware were used throughout the research work.

Preparation of Tissue Culture Media

The culture medium used for the study was Murashige and Skoog, (1962) Medium. This medium was supplemented with different growth regulators at varying concentrations.

Preparation of MS Stock Solutions

The different groups of stock solution (macro, micro, Fe-EDTA and vitamins) were prepared initially by dissolving the analytical grade chemicals in required quantities in volumetric flasks using double distilled water. Iron-EDTA stocks were prepared and heated for a few minutes until it turn golden yellow and was then stored in a brown bottle in refrigerator for further use.

Source of Explants

The initial plant materials used in this present study were immature male inflorescences buds and *in-vitro* shoot tip culture explants of Banana *cv*. Grand Naine. Explant material were collected from Department of Horticulture, UAS, GKVK, Bangalore.

Preparation of Explant

The study was carried out using field grown one month old banana immature male inflorescence of cultivar Grand Naine. A total of 16-20 segments were obtained from a single male flower. Inflorescence buds were sterilized by washing with 1% (v/v) detergent solution for 5 min and the buds taken from 8^{th} to 16^{th} position of meristematic dome was shortened to 6-8 cm in length by removing the enveloping bracts and these explants were sterilized in 70% ethanol for about 2-3 min and 0.1% (w/v) mercuric chloride for 2 min followed by three rinses in sterile water for 5 min each rinse. In a sterile laminar hood, the male buds were further reduced to 2-3 cm in length for culture and the tiny bracts along with minute hands of male flower bud were removed aseptically without damaging the apical dome and the male flower buds were inoculated on MS medium supplemented with different concentration of 2,4-D growth regulators for callus induction (Plate 1).

Standardization of the Growth Regulator Concentration for Callus Induction

Immature male flower explants were isolated and cultured in Petri dishes containing MS medium with $1mgL^{-1}$ biotin, $100mgL^{-1}$ L-glutamine and different concentrations of 2,4-D 0.5, 1.5, 2.0, 3.0, 4.0 mgL^{-1} for callus induction along

with no 2,4-D as control. All the aseptic manipulations like surface sterilization, preparation, inoculation of explants and their subculture were carried out in a laminar air flow chamber. The prepared cultures were incubated in the dark in incubation room at a temperature of 25^0 C, relative humidity of 60-80 %.

***In-vitro* Regeneration of Shoots from Male Flower Bud Induced Callus**

The study was carried out to know the optimum concentrations of TDZ and BAP cytokinins for regeneration of shoots from male flower bud induced callus. 3-4 cycles sub-cultured callus was transferred on to MS media containing TDZ 0.0, 0.4, 0.6, 0.8, 2.0 mgL^{-1} and BAP 2.0, 3.0, 4.0 mgL^{-1} treatments along with no cytokinins as control. Plates are incubated in 16 hrs of light and 8 hrs of dark at 27^0 C.

Multiple Shoots Induction from i*n vitro* Shoot Cultures

The study was carried out to know the optimum concentrations of TDZ and BAP on multiple shoot regeneration from *in-vitro* shoot explants of Banana cv. Grand Naine. Grand Naine *in-vitro* shoot cultures borrowed from Department of Genetics and Plant breeding, UAS, GKVK, Bangalore. These *in-vitro* shoot cultures were transferred to the below mentioned multiple shoot induction media containing TDZ 0.2, 0.4, 0.6, 0.8, 2.0 mgL^{-1} and BAP 2.0, 3.0, 4.0 mgL^{-1} treatments along with no cytokinins as control.

TRANSFORMATION OF BANANA WITH *GUS* GENE

Plasmid Constructs

The Plasmid construct p^{ABC} containing GUS gene (Figure 1) is kind gift of Dr. Neal steward from University of Tennessee, USA.

Biolistic/Gene Gun Method of Transformation

PDS He-1000 Biolistic Gene gun (Bio-Rad) was used for transformation. For transformation of explants (calli/ shoot tips); 2-3 week subcultured loosely attached friable calli (1-3 mm in diameter) were transferred to standardized callus induction medium for 4 days pre-culture and incubated in dark at 25^0 C. Pre-cultured calli were transferred to osmotic medium (MS basal medium with 30 gL^{-1} of Mannitol) four hours before transformation. Osmotic treated calli plate positions were optimized to study the effect of different Target Cell Distance (TCD) 6, 9, 4 cm from the macrocarrier assembly (Figure 2). Sterilization of Macro-carriers, stopping screen, rupture discs (1100 psi) were soaked in 70% ethanol for 15 min and dried on laminar hood on a sterile blotting paper. Consumables such as 5 µL of plasmid DNA (1 µg mL^{-1}), 50 µL of 2.5M $CaCl_2$, 20 µL 0.1M Spermidine are prepared. The preparation of plasmid DNA, precipitation of plasmid DNA onto tungsten particles, and particle bombardment were carried out as described previously by Raghvendra, G., (2007).

***GUS* Histochemical Assay: (Jefferson, 1987)**

Transferred calli and *in-vitro* shoot tip explants was taken in 1.5 ml microcentrifuge; freshly prepared incubation medium (1mg of X-gluc, 1.87 ml of phosphate buffer, 10 µL of 0.1M Potassium ferrocynide, 10 µL of 0.1M Potassium ferricynide and 10 µL of 10% w/v Triton – X-100) was added to it till the whole calli/shoot tips in the tube is immersed. The samples were incubated at 37^0 C for overnight. The samples with *GUS* expression were examined under phase contrast light microscope. The callus was observed for blue colour formation.

EXPERIMENTAL RESULTS

Callus Induction

The surface sterilized male inflorescence buds explant was placed on MS agar medium containing 5 different concentrations of 2,4-D for callus induction and these cultures were incubated in dark for 30 days. It took 26 days to induce callus. The colour of the callus observed was pale white to white in all the treatments. Calli were compact in T_1 to T_3 whereas friable calli were found in T_4 and T_5. Percent callus induction ranged from 10 % in T_1 (0.5 mgL^{-1}) to 40 % in T_4 (3.0 mgL^{-1} 2, 4-D) on MS media. The treatment T_4 (40 %) induced highest per cent of callus and it is significantly differed from other treatments (Plate 2). The data obtained from the above study is presented in Table 1.

Regeneration of Shoots from Callus

One month old calli were placed on MS media containing different concentrations of TDZ and BAP for shoot induction separately. Treatments with 0.2, 0.4, 0.6, 0.8, 2.0 mgL^{-1} of TDZ and 2, 3 and 4 mgL^{-1} BAP gave multiple shoots. 2.0 mg L^{-1} TDZ took least number of days (20.11 days) for shoot initiation which is significantly different from other treatments (Table 2). Whereas in case 4.0 mg L^{-1} BAP took least number of days (20.16 days) for shoot initiation which is significantly differed from other treatments (Table 2). Percent calli initiating shoots ranges from 20 % to 36.11 %. TDZ 2.0 mgL^{-1} induced highest shoots with 36.11 % which is significantly differed from other treatments (Table 2). 4.0 mgL^{-1} BAP induced highest shoot with 34.10 % which is significantly differed from other treatments (Table 2 and Plate 3).

Multiple Shoots Regeneration from *in-vitro* Shoot Culture

Multiple shoot regeneration from *in vitro* shoot cultures was achieved by using different concentration of TDZ and BAP. Highest per cent of multiple shoot induction was observed in 0.8 mgL^{-1} TDZ (81.60 %) which was significantly differed from other treatments. Significantly higher number of shoots per explants was produced by 0.8 mgL^{-1} TDZ which produced an average of 10.67 shoots per explant compared to other treatments (Table 3 and Plate 4).

Standardization of Biolistic Transformation of Banana Using *GUS* Gene

In this study, callus and shoot tips cultures were used for Biolistic transformation. Study was carried out to optimize target cell distance (TCD) of biolistic PDS 1000/He driven particle driven system. Three stages 2^{th}, 3^{rd} and 4^{th} with target cell distance of 6, 9 and12 cm respectively were studied. p^{ABC} plasmid containing *GUS* gene was used for transformation. GUS histochemical assay was carried out, a day after transformation (Table 4). The results indicating the blue colouration of the calli/shoot tip cultures confirmed the expression of *GUS* gene. Whereas, control callus tissues and shoot tip cultures showed no *GUS* expression. In case of both the explants, the Biolistic transformation at the 3^{rd} stage with target cell distance of 9 cm (T_2) gave highest *GUS* expression. Calli shown 48.35 % *GUS* positive and shoot tip cultures shown 45.88 % *GUS* positive. More intensive blue colouration was seen at T_2 and was significantly different from other treatments (Table 4).

DISCUSSIONS

During this study, male inflorescence induced callus and shoot tips were transformed with plasmid (p^{ABC}), containing *GUS* reporter gene using Gene Gun, and transient expression was verified by *GUS* histochemical assay.

Callus Induction

In MS media, the treatment T_4 (3.0 mgL^{-1} 2,4-D) induced highest per cent of callus (40%) followed by T_5 (4.0 mgL^{-1} 2,4-D) induced 35% callus. Calli induced in T_4 and T_5 were white and friable which is considered suitable for both transformation and regeneration. It seems that 2,4-D plays an important role in callus induction. These results were consistent with the findings of Azlin (2008) who obtained 53.9 % of embryogenic callus in the media containing 2 mgL^{-1} 2,4-D and Ganapathi *et al*. (2001) obtained 42% callus in MS medium supplemented with 2 mgL^{-1} 2, 4-D and 0.2 mgL^{-1} zeatin.

Regeneration of Shoots from Callus

Highest shoot regeneration (36.11 %) was obtained in 2.0 mgL^{-1} TDZ followed by 34.10 % shoot induction in 4.0 mgL^{-1} BAP. Shoot regeneration was found significantly greater in TDZ as compared to BAP. It is found that TDZ was more effective at lower concentrations as compared to higher concentrations of BAP. This is Consistent with studies of Arinaitwe *et al*., (2000); Hamide and Pekmezci, 2004. The better performance of TDZ at 2.0 mgL^{-1} in shoot induction as reported in this work is contrary to Sreeramanan *et al*. (2008) and Shirani *et al*. (2009).

Induction of Multiple Shoots from *in-vitro* Shoot Cultures

Except control and 0.2 mgL^{-1} TDZ, Significantly higher number of shoots per explants was produced by 0.8 mgL^{-1} TDZ (81.60 %) was observed in 0.8mgL^{-1} TDZ and 3.0 mgL^{-1} BAP. Both were significantly differing from the other treatments. 0.8 mgL^{-1} TDZ found most suitable concentration for multiple shoot production. Banana plants regenerated from this system were normal and could be regenerated in short time. These results were consistent with the results obtained by Sin-Wan Lee (2001).

Biolistic Transformation and GUS Histochemical Assay

Target cell distance of 9 cm produced higher transformation efficiency. It was observed that 48.35% of the transformed selective calli and 45.88% shoot tips were found *GUS* positive at the target cell distance of 9 cm with uniform distribution of tungsten particles to the tissues. Target cell distance of 6 cm has caused damage to the tissues. While higher target cell distance 12 cm has caused uneven distribution of DNA carrying tungsten particles. So, these two TCD have lowered the transformation efficiency. These results were consistent with the findings of Sreeramanan *et al*. (2005).

CONCLUSIONS

In vitro regeneration and genetic transformation techniques have provided necessary tools for the improvement of banana. By further standardizing the protocols for efficient transformation methods, one can introduce genes for bunchy top virus disease resistance, panama wilt resistance etc.

Table 1: Effect of Different Concentrations of 2,4 on Physical Characteristics of the Callus and per Cent Callus Induction in Banana cv. Grand Naine

Treatments	2,4-D (mgL^{-1})	Extent of Callus Production	Colour of the Callus	Type of Callus	No. of days taken for callus induction	No. of Explants producing Callus	% Callus induction
T_0	0.0	-	-	-	-	-	-
T_1	0.5	+	Pale white	Compact	26	2.00	10.00
T_2	1.5	++	Pale white	Compact	26	3.66	18.30
T_3	2.0	++	White	Compact	26	6.67	33.35
T_4	3.0	++++	White	Friable	26	8.00	40.00
T_5	4.0	+++	White	Friable	26	7.00	35.00
CV%						7.0176	6.9825
SEm						0.1598	0.795133
CD @1%						** 0.6466	** 3.2160

Legend: Total number of explants = 20 in each treatment.
- \- No callus
- \+ Poor callus growth
- ++ Moderate growth
- +++ Good callus
- ++++ Very good growth

Table 2: Effect of TDZ and BAP on Shoot Regeneration from Male Inflorescence Induced Callus of Banana cv. Grand Naine

Treatment with TDZ (mgL^{-1})	No. of days taken for shoot initiation	% Calli producing shoots
0	-	-
0.2	28.00	20.00
0.4	25.37	21.11
0.6	23.21	22.16
0.8	21.37	25.51
2.0	20.11	36.11
Treatment with BAP (mgL^{-1})	**No. of days taken for shoot initiation**	**% Calli producing shoots**
0	-	-
2.0	27.00	23.11
3.0	23.37	26.05
4.0	20.16	34.10
CV%	0.90301	0.9046
SEm	0.094668	0.1044
CD @1%	** 0.370941	** 0.4094

Total number of Calli = 20 in each treatments.

Table 3: Effect of TDZ and BAP on Multiple Shoot Regeneration from *in-vitro* Shoot Cultures of Banana *cv*. Grand Naine

Treatment with TDZ (mgL^{-1})	No. of Explants producing Multiple shoots	% Multiple shoot production	No. of Shoots per Explant
0	-	-	-
0.2	0.00	0.00	1.00
0.4	13.00	65.00	5.67
0.6	14.20	71.00	6.66
0.8	16.32	81.60	10.67
2.0	15.18	75.90	8.00
Treatment with BAP (mgL^{-1})	**No. of Explants producing Multiple shoots**	**% Multiple shoot production**	**No. of Shoots per Explant**
0	-	-	-
2.0	15.20	76.00	6.67
3.0	16.33	81.60	8.00
4.0	15.30	76.50	7.00
CV%	22.420	0.810471	61.14486
SEm	1.2670	0.237597	1.576060
CD @1%	** 4.9667	** 0.93098	** 6.175530

Total number. of Explants = 20 in each treatments.

Table 4: Effect of Target Cell Distances on Transformation of Banana Callus and Shoot Tip Cultures with GUS Gene by Biolistic Gun

Treatments	Stages	Target cell distance (cm)	No. of callus taken	No. of Shoot tips taken	GUS Histochemical assay			
					No. of GUS +ve calli	% of GUS +ve calli	No. of GUS +ve shoots	% of GUS +ve shoots
T0	0	-	20	8	-	-	-	-
T1	2	6	20	8	6.51	32.55	2.51	31.37
T2	3	9	20	8	9.67	48.35	3.67	45.88
T3	4	12	20	8	4.67	23.35	2.00	25.00
CV%					0.42358	0.42358	7.70850	7.70850
SEm					0.0147196	0.07359	0.10509	1.31365
CD@1%					** 0.06765	** 0.33825	** 0.483001	** 6.03751

Legend: T0 = Control (non transformed), T1= 6 cm TCD, T2= 9 cm TCD, T3= 12 cm TCD.

Figure 1: Gene Map of Plasmid (p^{ABC}) Containing *GUS* Gene

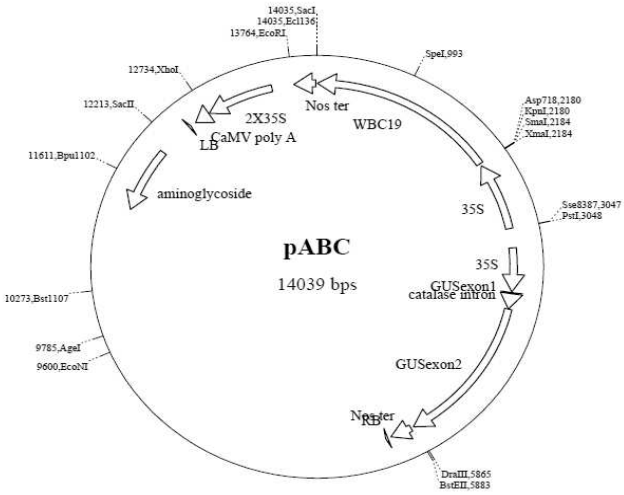

Figure 2: Gene Gun (PDS-1000/He. Bio-Rad)

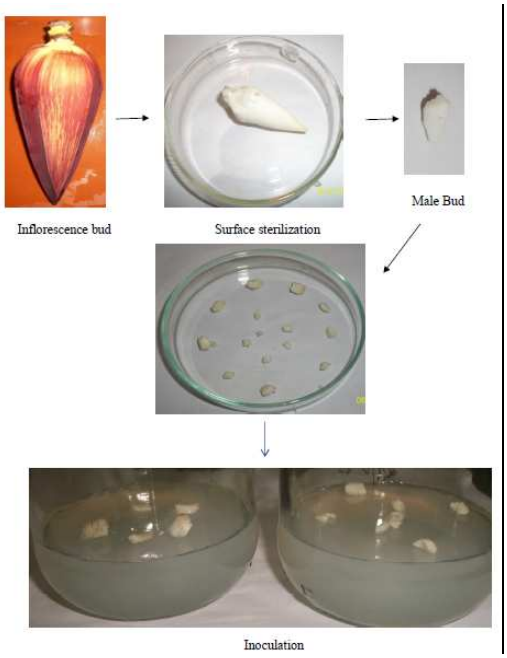

Plate 1: Preparation of Banana Male Flower Bud

Plate 2: Callus Obtained from Male Inflorescence Explants for Callus Induction

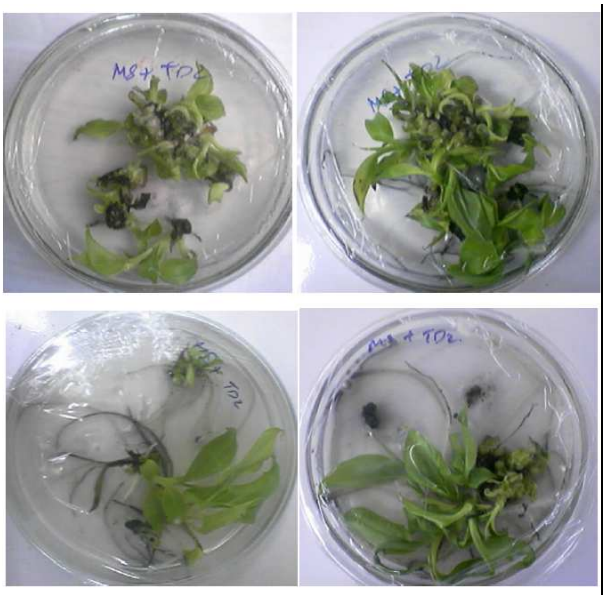

Plate 3: Regeneration of Shoots from Callus

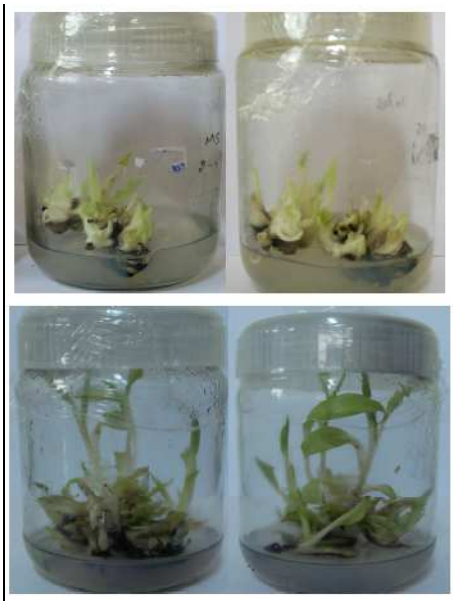

Plate 4: Multiple Shoot Regeneration Shoot Cultures

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