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MICROPROPAGATION AND GENETIC TRANSFORMATION OF BANANA (*Musa paradisiaca* L. cv. Chinia)

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ABSTRACT
An attempt was made to develop callus mediated regeneration system and to check response towards *Agrobacterium* mediated transformation in the local variety of Banana (*Musa paradisiaca* L. cv. Chinia*)* cultivar *Chinia*. Callus induction was observed when male flower aggregates of 1 cm was used as explants and cultured in the MS media added with 2.5 mg/L 2, 4 D, 0.5 mg/L IAA, BAP 1mg/L and 0.5 gm/L of charcoal under dark condition at 25º C. MS medium fortified with 1.0 mg/l BAP with a fixed concentration of NAA (0.2 mg/l) and glutamine (100 mg/l) or with a fixed concentration of IAA (0.5 mg/l) and Caesin hydrolysate (500 mg/l), under 1500 lux intensity with 16/8 h light/dark cycle resulted in significant shooting. The combination of 1 mg/l of IBA and 2mg/l of NAA among three (0.5, 1.0 and 2.0 mg/l each) different concentration of two auxins viz., IBA and NAA in MS media resulted in distinct rooting. This local variety also showed response towards *Agrobacterium* mediated transformation with regeneration efficiency 1-2% when examined through GUS expression assay. The developed regeneration system and response towards *Agrobacterium* mediated response hold much scope for the genetic improvement and wide multiplication of this local cultivar of banana.

INTRODUCTION

Banana accounts for approximately 22 per cent of the world fresh fruit production and is ranked as the second most important fruit crop after mango and makes 39.8 per cent of India fruit production (Uzaribara, 2015). This paper incorporates methods of callus mediated regeneration and *Agrobacterium* mediated methods of genetic transformation in local variety of banana cv. *chinia*. Being highly delicious and widely grown, this cultivar needs efficient system of multiplication and transformation for its wide propagation and fortification respectively, like other cultivar of banana.

Tissue culture is the modern system for the multiplication and regeneration of the crop, particularly banana. Multiplication of banana through in-vitro/tissue culture technique has been reported by several workers using different explants sources and methods (Ahmed *et al*., 2014). For the standardization of the transformation of foreign genes in the banana usually callus is used for transformation (Ganapati *et al*., 2001; Smith *et al*., 2005; Sidha *et al*., 2007). Therefore, callus mediated tissue culture might be developed as preferred regeneration system, if regenerated from male flower of this local variety of banana. Since, this system is an established method for raising regenerable suspension cultures in case of few cultivars of banana only. Protocols have been established for the induction of callus using immature male flowers (Kulkarni *et al*., 2006). Immature male inflorescences have also been used to initiate cultures with the objective of developing a protocol for primary and secondary callus mediated embryogenesis (Khalil *et al*., 2002). Suitable protocol of callus mediated embryogenesis through male flower will not only accelerate the regeneration system of banana but will also help in the implementation of transformation approaches for their genetic improvement (Namuddu *et al*, 2013).

A standard transformation procedure using right candidate gene(s) may improve the banana genetically for abiotic and biotic stress tolerance, bio-fortification, production of vaccines and plant antibodies (Kumar *et al*., 2016). However, banana is known to show recalcitrance towards *in vitro* regeneration, perhaps due to long incubation time in the callus induction and difficulty in developing regenerable embryogenic callus. Therefore, despite the development of protocols of callus mediated regeneration in several commercial and elite cultivars, a large number of banana genotypes and various explants yet to be explored for embryogenic potential for the propagation and improvement though genetic transformation. Apical shoot containing meristem has been used as explants for the *Agrobacterium* mediated transformation of plantain (*Musa* spp.) cultivar Agbagba (Tripathi *et al*., 2005). *Agrobacterium* and microprojectile bombardment methods

have also been attempted in combination for the genetic transformation of various cultivars of banana (Rustagi *et al*., 2015). Following the success of regeneration and transformation in other cultivar of banana, study, was undertaken with objectives to develop callus mediated regeneration and *Agrobacterium* mediated transformation system in this local and popular cultivar of Banana cv. Chinia.

MATERIALS AND METHODS

Male buds of banana local cultivar Chinia were collected from the plants grown in the premises of the Department of Molecular Biology and Genetic Engineering, Bihar Agricultural University, Sabour.

Isolation and sterilization of explants

Very young male flower buds was isolated and used as explants to initiate the regeneration. Male flower buds were surface sterilized by cotton swab soaked in 70% ethanol for four minutes. To collect the young male flowers, 10/11 bracts were removed together with their male flower from the rachis. Then the male flower buds were again cleaned using cotton swabbed with ethanol. Young male flowers were isolated with the help of a pair of sterilized forceps in the laminar air flow hood and transferred to the sterilized Petri dish containing moistened filter paper to prevent desiccation of flowers.

Callus induction and plantlets regeneration

Male flower of the Banana was also used for the induction of callus using the standard protocol (Sidha *et al*., 2006). The male flower was allowed to grow on MS media supplemented with 18.10 μM 2, 4 D, 5.71 μM IAA and 4.09 μM NAA. It was repeated many times using different sizes of male flower as explants and under prescribed culture duration but no response were shown. But there was appearance of callus when very small flower of size 1cm and flower aggregates was used as explants in the MS media supplemented with 2.5 mg/L 2, 4 D, 0.5 mg/L IAA and BAP 1mg/L. 2,4-D is reported to be very useful for the induction of callus (Kauther et al., 2013). Therefore, it was included in the main composition. BAP was also added in lower concentration to modulate the effect of higher concentration of 2, 4-D. This resulted in the induction of callus but browning was the limiting factor. Therefore, different concentration of Charcoal 0.1 to 1 gm/L was optimized in the same media to overcome browning. 0.5 gm/L of charcoal in the same media resulted in the fresh callus after one month under dark condition at 25º C. For complete regeneration of plant, shooting and rooting was attempted following the protocol of Sultan *et al*., 2011 with slight modification. Obtained calli were cut into suitable pieces which ranged 2 - 4 for each callus depending on size and were transferred into fresh MS medium fortified with three different concentrations of BAP (0.5, 1.0 and 2.0 mg/l) together with a fixed concentration of NAA (0.2 mg/l) and glutamine (100 mg/l) or with a fixed concentration of IAA (0.5 mg/l) and Caesin hydrolysate (500 mg/l) and were kept under fluorescent illumination (at 1500 Lux intensity) with 16/8 h light/dark cycle. Shoots, thus obtained from calli in regeneration medium were multiplied by sub-culture up to a considerable number. Two auxins *viz*., IBA and NAA were used in three different concentrations (0.5, 1.0 and 2.0 mg/l each) in the MS medium to induce rooting and to optimise the optimum one.

***Agrobacterium* mediated genetic transformation and selection**

Apical shoot containing apical meristem has been used in the transformation successfully (Tripathi *et al*., 2005). An initial attempt was made to check response towards the *Agrobacterium* mediated transformation using apical shoot tip as explants and co cultivated with the *Agrobacterium* strain EHA 105 harboring pBI121 a GUS expression vector. The protocol of Tripathy *et al*., 2005 was followed with slight modification. The culture of *Agrobacterium* EHA 105 was done in YEP broth. Selection of the transformed Agrobacterium with pBI 121 was done on Kannamycin and Rifampicin supplemented Media. The culture of *Agrobacterium* EHA 105 harbouring pBI 121 was used to inoculate 25 ml of fresh medium and culture was grown at 28º C at 600 nm till O.D. reached at 0.8. The cells were harvested at 5000 g for 10 minutes at 4º C and re-suspended in 25 ml of antibiotic free regeneration medium supplemented with 100mM acetosyringone. The apical shoot tips were co-cultivated with culture of *Agrobacterium* strains for 30 minutes with gentle shaking and 100 mM acetosyringone was added during the co-cultivation. After 30 minutes the liquid culture was removed and shoots tips was blotted on tissue paper and co-cultured on regeneration media containing acetosyringone (100mM) for three days in dark. After three days transient expression of reporter gene in the explants was done through GUS histochemical assay and the explants were transferred to the regeneration media containing cefotaxime (500mg/l) and Kannamycin for seven days with 16 hour photoperiod.

GUS histochemical Assay

This assay was performed to check the expression of putative transgenic following the protocol of (Tripathi *et al*., 2005). The GUS histochemical assay for transient gene expression was performed after 3 days of co-cultivation. The explants were washed in 70% ethanol followed by incubation in fixation solution (0.3% v/v formaldehyde, 10 mM MES, pH 5.6, 0.3 M mannitol) for 30 to 45 min at room temperature. The explants were infiltrated for 2 min for proper fixation and washed with 50 mM phosphate buffer (pH 7.0). The fixed explants were incubated in the substrate solution (1 mM X-gluc, 50 mM sodium-phosphate (pH 7.0), 5 mM potassium ferricyanide, 5 mM ferrocyanide, 10 mM EDTA, 50 mM Ascorbic acid), at 37°C for 20 to 24 h. Chlorophyll was removed by immersing the explants in solution of methanol and glacial acetic acid (3:1) for 3 to 4 h followed by dehydration in a series of ethanol (50, 70, and 95%).

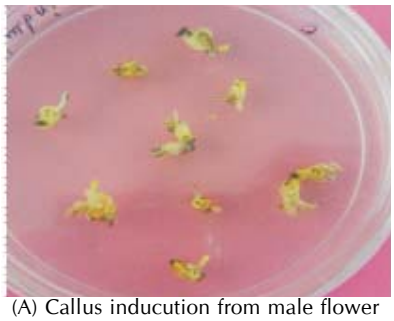
RESULTS AND DISCUSSION

Callus induction and shoot organogenesis

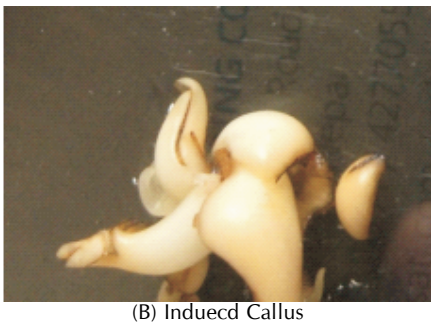
An attempt was made to develop callus mediated regeneration system and to check response towards *Agrobacterium* mediated transformation. It was found that like other cultivar of banana, Chinia is also responsive to the regeneration system, when initiated from the male flower (Fig. 1). Small flower aggregate of 1cm was found most responsive towards callus induction rather than larger and shorter. MS media supplemented with 2.5 mg/L 2, 4 D, 0.5 mg/L IAA, BAP 1mg/

Table 1: Optimum Media Composition for callus mediated regeneration of Banana from the male flower

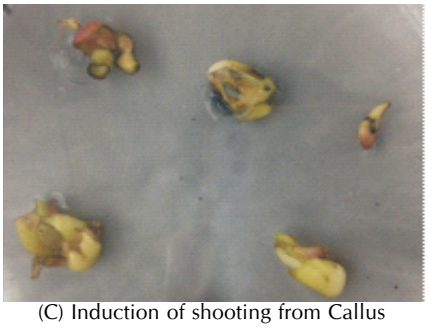
S.No.	Stages of culture	Conditions.
1	Callus generation	MS media supplemented with 2.5 mg/L 2, 4 D, 0.5 mg/L IAA, BAP 1mg/L and Charcoal 0.5gm/L
2	Shooting	MS media supplemented with 1.0 mg/l BAP with NAA (0.2 mg/l) and glutamine (100 mg/l) or with a fixed concentration of IAA (0.5 mg/l) and Caesin hydrolysate (500 mg/l)
3	Rooting	MS media with 1 mg/l of IBA and 2mg/l of NAA.



(A) Callus inducution from male flower of Banana



(B) Induecd Callus



(C) Induction of shooting from Callus

(D) Shooting from Callus

(E) Developed Shoot

(F) Plantlet

Figure 1: Callus induction and plantlets regeneration from explant (male flower)

L and Charcoal 0.5gm/L was appropriate to induce callus. 2, 4-D is supposed to be an important regulator for the callus induction and therefore, played decisive role. This has been evidenced from the study in other cultivar carried by Sidha *et al*., 2007; Khalil and Elbanna, 2004. Optimal concentration of 2, 4-D on callus induction of seven banana cultivars has also been investigated in other study. It was revealed that banana needs higher concentration of 2, 4-D as in case of Chinia (Karintanyakit *et al*., 2014). Among different concentration of BAP, MS medium fortified with 1.0 mg/l with a fixed concentration of NAA (0.2 mg/l) and glutamine (100 mg/l) or with a fixed concentration of IAA (0.5 mg/l) and Caesin hydrolysate (500 mg/l), under 1500 lux intensity with 16/8 h light/dark cycle resulted in significant shooting. BAP has also been used in the shooting of other cultivar of Banana (Meenakshi *et al*., 2011; Kauther *et al*., 2013). It seems that BAP is an important cytokinin which regulates the growth and differentiation while shooting in banana. The only combination of 1 mg/l of IBA and 2mg/l of NAA among three (0.5, 1.0 and 2.0 mg/l each) different concentration of two auxins *viz*., IBA and NAA in MS media resulted in rooting distinctly. NAA has also shown good response in the rooting of many variety of Banana i.e. Grand Naine, Amritasagar, Sabri (Hossain *et al*., 2016). But the combination of IBA and IAA has shown profound effect on the rooting of other variety of banana cv. Grand Naine (Miilion *et al*., 2015). This could be envisaged due to homology in the signalling pathway of both regulators IAA and NAA and their synergistic interaction with IBA.

The media containing cefotaxime and Kannamycin was used to select putatively transformed plants and GUS expression assay was carried out to screen at primary level. The shoots from regenerated plants were examined by GUS histochemical assay for stable expression of the reporter gene. Blue coloration was observed in the transformed apical shoots (Fig. 2). Only 1-2% shoots developed from suckers showed regeneration.

After transformation, regeneration efficiency observed was 1-2% and up to shoot stage only. This could be due to complicated transformational pathway of *Agrobacterium* in the case of banana. The transformation in banana is also cultivar to cultivar dependent. The process of transformation and regeneration needs to be fine tuned yet, for its reproducibility. However good results have come out when particle bombardment method has been adopted (Kido *et al*., 2005). The regeneration and *Agrobacterium* mediated transformation in this local variety of Banana is encouraging. This finding has given hope that like other variety of Banana and other crop, *Chinia* may also be improved by transforming

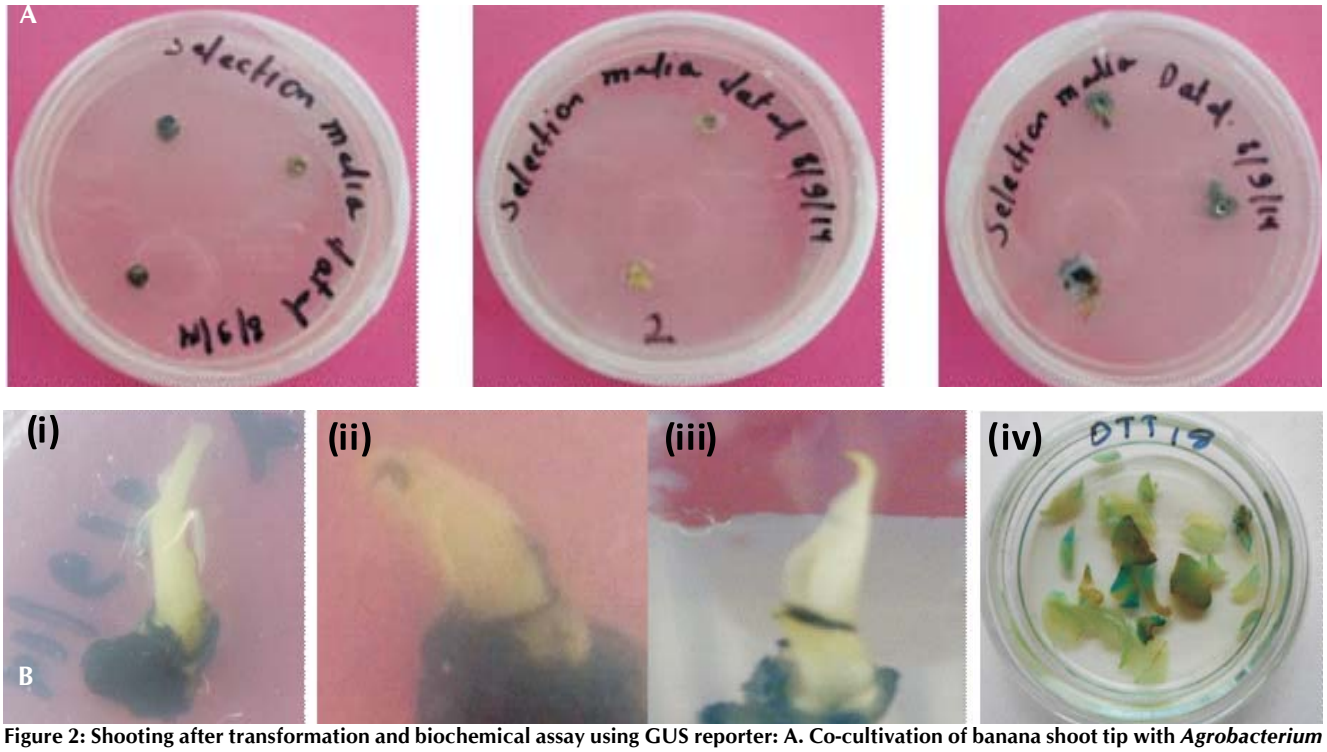


Figure 2: Shooting after transformation and biochemical assay using GUS reporter: A. Co-cultivation of banana shoot tip with *Agrobacterium tumefacience;*B. Putatively transformed banana on the selection media containing Cefotaxime and kanamycin (i-iii) and GUS histochemical assay using leaf tissue of putatively transformed banana (iv).

gene (s) of desired trait with the help of molecular biological exercise. Moreover this may be brought effectively, if both methods should be applied in integration.

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REFERENCES

Ahmed, S., Sharma, A., Bhushan, B., Wali, V. K., Bakshi, P. and singh A. K. 2014. Studies on hardening and acclimitization of micropropagated plantlets of banana cv. Grand naine. *The Biosca.* **9(3):** 965-967.

Ganapathi, T. R., Higgs, N. S., Palint-Kurti, P. J., Arntzen, C. J., May, G. D. and Van Eck, J. M. 2001. *Agrobacteriu*m-mediated transformation of embryogenic cell suspensions of the banana cultivar Rast-hali (AAB). *Plant Cell Rep*. **20:** 157-162.

Hossaina, M.A., Rubelb, M.H., Nasiruddinc, K.M. and Evamonia, F.Z. 2016. Influence of BAP and NAA on in vitro plantlet regeneration of local and exotic banana cultivars. *Journal of Bioscience and Agriculture Research*. **6(2):** 553-564.

Karintanyakit, P., Suvittawat, K., Chinachit, W., Silayoi, B. and Saratultad, P. 2014. The impact of genome and 2,4-d on callus induction from immature male flowers of seven banana cultivars. *Acta Hoti. DOI* 10.17660/ActaHortic.2014.1024.33.

Kauther S. A., Anas A. E., Sayeda O. E. and Hind, E. M. 2013. Embryogenesis and Plantlet Regeneration via Immature Male FlowerCulture of Banana (Musasp.)cv. Grand Nain. *Journal of Forest Products and Industries*. **2(3):** 48-52.

Khalil, S. M., Cheah, K. T., Perez, E. A., Gaskill, D. A. and Hu, J. S. 2002. Regen-eration of banana (*Musa* spp. AAB cv. "Dwarf Brazilian) via secondary somatic embryogenesis. Plant Cell Rep. **20:** 1128-1134.

Khalil, S.M. and Elbanna, A.A.M. 2004. Highly efficient somatic embryogenesis and plant regeneration via suspension cultures of banana (Musa spp.). *Arab J. Biotech*. **7:** 99-110.

Kido, L.M., Kido, E.A., Falco, M.C., Filho, M.C. and Figueira, A.V.O. et al. 2005. Somatic embryogenesis and the effect of particle bombardment on banana Maçã regeneration. *Pesquisa Agropecuária Brasileira*. **40:** 1081-1086.

Kulkarni, V.M., Suprasanna, P. and Bapat, V.A. 2006. Plant regeneration through multiple shoot formation and somatic embryogenesis in a commercially important and endangered In-dian banana cv. Rajeli. *Curr. Sci.* **90:** 842-846.

Kumar, T., Singh, R.S., Pal, A.K. and Kesari, R. 2016. Bio-technological advancement in banana (*musa* spp.) with perspective on value addition-a review, *Progressive Research*, **11(2):** 139-149.

Meenakshi, S., Shinde, B.N., and Supersanna, P. 2011. Somatic embryogenesis from immature male flower and molecular analysis of regenerated plants in Banana "Lal Kela"AAA. *Journal of fruit and ornamental Plant Research* volume **19(2):** 15-30.

Miilion, P. M., Varsha, G. K., Bhagyashri, H. N. 2015. Effect of IAA and IBA on In vitro Rooting of Banana (Musa paradisiaca) Cv.Grand Naine. *International Journal of Science and Research*. **4(5):** 959-962.

Namuddu, A., Kiggundu, A., Mukasa, S.B., Kurnet, K., Karamura, E. and Tushemereirwe, W. 2013. *Agrobacterium* mediated transformation of banana (*Musa* sp.) cv. SukaliNdiizi (ABB) with a modified *Carica papaya* cystatin (*CpCYS*) gene. *African Journal of*

Biotechnology. **12:** 1811-1819.

Rustagi, A., Jain, S., Kumar, D., Shekhar, S., Jain, M., Bhat, V. and Sarin, N. B. 2015. High Efficiency Transformation of Banana [Musa acuminate L. cv. Matti (AA)] for Enhanced Tolerance to Salt and Drought Stress Through Overexpression of a Peanut Salinity-Induced Pathogenesis-Related Class 10 Prot. *Mol Biotechnol*. **57:** 27–35.

Sidha, Meenakshi, Suprasanna, P., Bapat, V.A., Kulkarni, U. G. and Shinde, B.N. 2007. Developing Somatic Embryogenic Culture System and Plant Regeneration in Banana Issue No. 285. pp 153-161.

Smith, M.K., Hamill, S.D., Becker, D.K., Dale, J.L. 2005. *Musa* spp. banana and plantain. In: Litz R.E. (ed.), Bio-technology of Fruit and Nut crops. CAB International Wallingford, UK, pp. 366-391.

Sultan, M.T., Khan, M.H., Hakim, M.L., Mamun, A.N.K., Morshed, M.A., Islam, M.A. Islam, M.R. and Islam, M.R. 2011. In vitro plant regeneration from male flowers of banana. *International Journal of Biosciences*. **1(1):** 1-11.

Tripathi, L., Tripathi, J.N. and Hughes, J. 2005. Agrobacterium-mediated transformation of plantain (Musa spp.) cultivar Agbagba. *African Journal of Biotechnology*. **4:** 1378-1383.

Uzaribara, E., Nachegowda, V., Ansar, H., Sathyanarayana, B. N. and Amreen, T.A. 2015. in vitro propagation of red banana (*Musa acuminata*). *The Bioscan*. **10(1):** 125-129.

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