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Standardization of Embryogenic Callus induction protocol in Banana cv. Grand Naine

ORIGINAL ARTICLE

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Abstract

Commercial banana, being triploid has limited scope for genetic improvement though conventional breeding methods. For application of gene editing and transformation tools the availability of embryogenic callus is a vital factor. In this study we report standardization of protocol for induction of embryogenic Calli derived from anthers of immature male buds, inflorescence from the 16th whorl of flower and also invitro propagated rejuvenating buds. Although higher efficiency of callus induction was recorded with in-vitro propagated rejuvenating buds cultured in MS media with Picloram (2mg/L) and TDZ (3 Thidiazuron) (0.3mg/L) in MS media, but they also showed shoot growth within 10 -12 days. The N6 CHU media supplemented with NAA (0.5mg/L) was found most suitable to obtain embryogenic callii from anthers followed by the N6 CHU media supplemented with NAA (0.5mg/L) and 2,4-D (2mg/L) from whole inflorescence. The fragile embryogenic callus obtained from anthers and inflorescence were also subjected to microscopic studies that confirmed their haploid chromosome number.

Key Words

Embryogenic Callus, Micropropagation, Gene Editing, Anther Culture.

Abbreviations

2,4-D – 2,4 dicloro phenoxy acetic acid
MS- Murashige and Skoog
TDZ-N- Pheny l-1, 2, 3, -thidiazole-5 ylurea,

Introduction

Banana is a significant fruit crop of tropical regions, known for its high productivity and nutritional value. The Cavendish bananas, also known as "Grand Nain" means "Large Dwarf" is a triploid (AAA = 33

chromosomes). It shows parthenocarpy and produce seedless fruits leading to lesser genetic variation. Being clonally propagated, genetic improvement can only be achieved using in vitro techniques such as somaclonal variation or genetic modification. The availability of high-quality embryogenic calluses is crucial for the successful application of genetic transformation or genome editing methods. Callus cultures are suitable for transformation and play important roles in generating transgenic plants. Embryogenesis and shooting rely on callus induction. The callus may be compact or friable, where the compact calli that are typically green and sturdy are more suitable for organogenesis. The friable calluses are white to creamy yellow in colour, they fall apart easily and are more conducive to uptake foreign DNA. Callus, being an undifferentiated mass of tissues, has a higher capacity to receive the gene of interest and exhibits efficient organogenesis after infection.

In banana, *invitro* regeneration via cell suspension cultures (Dhed'a *et al.*;Cote *et al.* 1996 and somatic embryogenesis (Escalant *et al* 1994; Khalil *et al* 2002) have been used for genetic transformation via somaclonal variation and selection. Plant regeneration via *invitro* culture has been initiated from various sources of explants (Israeli *et al*. 1996; Ko *et al*. 1991) and have known to undergoes organogenesis without any intervening callus phase. Therefore, people have attempted cell suspension cultures or whole flower buds for the transformation. However, the cell suspension cultures show decline in productivity over prolonged subculture periods, sluggish growth, and low productivity of plant cells. Moreover, the cells may sustain damage under shear conditions. *In-planta* transformation, by floral dip method, referred as direct transformation was originally developed for Arabidopsis thaliana (Clough and Bent, 1998). However, in-planta transformation poses challenges, especially in the case of bananas, as the risk of contamination is significantly higher compared to tissue culture conditions. To overcome these limitations, induction of embryogenic calli was seen as an alternative approach. Therefore, the primary objective of this study was to standardize the protocol for inducing callus formation, that are conducive to take up the foreign DNA for application of gene editing and/or gene transfer tools.

During callus formation there is some degree of dedifferentiation both in morphology and metabolism resulting in the loss of the ability to photosynthesis. Callus cultures may be compact or friable. Compact callus shows densely aggregated cells while friable callus shows loosely associated cells. Friable callus becomes soft and breaks apart easily which is useful for suspension cultures formation and transformation with nucleic acids.

Materials and methods: The explants were obtained from the Grand Naine cultivar of banana grown in the the orchard of Indira Gandhi Krishi Vishwavidyalaya, IGKV, Raipur, Chhattisgarh. *Invitro* propagated rejuvenating buds, tender leaf tissues, root tissues, Immature unopened flower buds and Immature male buds (anthers) were used as explants for callus induction. As the buds are already *invitro* propagated and grown under aseptic conditions thus, there was no need to go for sterilization, just they were washed with autoclaved distilled water under laminar hood. Immature male flower buds were removed and made free of contaminants by using Tween-20 for a duration of 10 minutes. Subsequently, they were rinsed with autoclaved distilled water 4 - 5 times, followed by chemical sterilization using a 1% Bavistin solution for 5 minutes. After rinsing with autoclaved distilled water again for 4 to 5 times, the buds were treated with a 0.1% solution of Mercuric chloride ($HgCl_2$) for 2 minutes. Finally, after thorough rinsing with autoclaved distilled water in the laminar hood inoculation was done. The male flowers were cultured on agar gelled MS medium with full strength salts supplemented with different treatments (Table 2) and 3% sucrose. The N6 CHU media which is predominantly used for callus induction in rice was also used and the culture were incubated in dark for about 2-3 months for efficient callus formation.

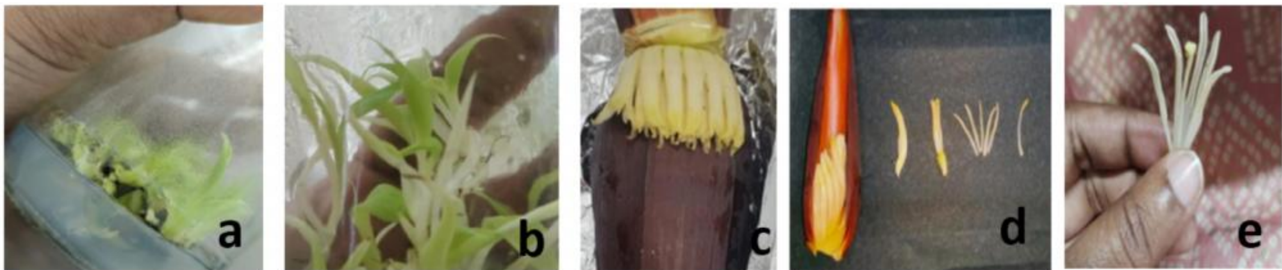

Fig. 1: Explants used for induction of callus a. In-vitro propagated rejuvenating buds b. In-vitro propagated tender leaf tissue c. mature flower bud d. Immature fingers e. anthers and gynoecium

Table 1: Treatments used for callus induction in banana with different explants.

SN	Explants	Basal media	Treatment	Hormone concentrations
1.	*In-vitro* propagated rejuvenating buds	MS	T1	2,4-D 2mg/L+ Kinetin 0.5 mg/L
			T2	2,4-D 0.5 mg/L
			T3	2,4-D 1.0 mg/L
			T4	2,4-D 2,2 mg/L
			T5	2,4-D 2.5 mg/L
			T6	2,4-D 2.0 mg/L +NAA 0.5mg/L
			T7	2,4-D 2mg/lt + TDZ 0.3mg/L
			T8	Picloram 2mg/lt + TDZ 0.3mg/L
		N6	T9	2,4-D 2.0mg/L
2.	*In-vitro* propagated Tender Leaf tissue	N6	T10	2,4-D 2.0 mg/L
3.	*In-vitro* propagated Root cells	N6	T11	2,4-D 2.0 mg/L
4.	Immature flower buds	N6	T12	2,4-D 2mg/L +NAA 0.5 mg/L
5.	Immature male Flower (Anthers)	N6	T13	NAA 0.5mg/L

Microscopic Study of Banana Callus

After the proliferation of callus from explants they were subjected to microscopical study to check the number of chromosomes, as in triploid (3X=33) or haploid (X=11). The cytological analysis was performed under microscope on squash preparations stained with acetocarmine staining agent (Murthy *et al,*.2000). Different samples of age 0 to 45 days were observed under microscope. Samples (1µl) were placed in center of each slides and one drop of acetocarmine was added on it, then kept for 5 minutes for staining. Samples were covered with cover slip and gently heated using spirit light for 10 sec to fix them. The slides were then observed under compound microscope in 10X and 40X magnifications.

Figure 2: Callus before preparing for microscopical study of cells

Results and Discussion

Obtaining good quality calluses is critical in the genetic improvement of clonally propagated crops like banana (Rajput et al., 2022; Justine et al.,2023). The success of the gene transformation experiments is largely determined by the number of callus bombarded or transformed. To achieve successful transformation targeting as many calli as possible is usually recommended. Although, micropropagation of banana has been standardized and commercially used widely since long using different explants, such as shoot tips (Kulkarni *et al*. 2006; Roels *et al*. 2005), immature male flowers (Jalil et al. 2003;Khalil *et al*. 2002), young female flowers (Grapin *et al*.2000), protoplasts (Matsumoto *et al*. 2002), anthers (Assani *et al.* 2003), and rhizome (Li *et al*. 2006) but lesser studies have been reported on obtaining embryogenic callus. In this study we used *in-vitro* propagated rejuvenating buds, tender leaf tissue and root cells. immature flower buds and immature male flower from orchard of banana as explants for callus formation. Different treatments of growth regulators were used to grow the explants (Table 2) viz. MS (Murashige-Skoog) Media with 2,4-D 2mg/L in combination with Kinetin 0.5mg/L (T1), 2,4-D (1.0,1.5,2.2,2.5)mg/L (T2-T5), 2,4-D 2.0mg/L in the combination with NAA 0.5 mg/L (T6), 2,4-D 2.0mg/L with TDZ 0.3mg/L (T7) ,combination of Picloram 2.0mg/L with TDZ 0.3mg/L (T8 Table.1). N6 CHU media with 2,4-D 2mg/L (T9,T10,T11), 2,4-D 2mg/lt with NAA 0.5mg/L and alone with NAA 0.5mg/L (T13). In order to evaluate and compare the effectiveness of various treatments, the number of plates inoculated is determined by using a consistent measure of 100 plates for each treatment. The results of the inoculated explants under different treatments are shown in Table 2.

Table 2: Response of the explants under 13 treatments for callus formation after 21days to 3 months of incubation

S.No.	Treatment	Plates with Callus Initiation
01.	T-1	0
02.	T-2	0
03.	T-3	0
04.	T-4	0
05.	T-5	65
06.	T-6	0
07.	T-7	80
08.	T-8	98
09.	T-9	15
10.	T-10	0
11.	T-11	0
12.	T-12	90
13.	T-13	92

The inoculation of 25 days old *invitro* propagated rejuvenating buds under the treatment of T1(MS-2,4-D 2mg/lt+ Kinetin 0.5 mg/L), T2(MS- 2,4-D 0.5mg/L), T3(MS- 2,4-D 1.0 mg/L) and T4(MS- 2,4-D 2.2 mg/L) showed no callus induction after 21 days of incubation. The explants turned black in T2, T3, T4 but they depicted organogenesis with high rate of shoot growth in T3 followed by T2 (Fig 3 a, b, c, d). Thus, all the treatments (T1,T2,T3,T4) were found to be unfavorable for callus induction. The treatment T5(MS-2,4-D 2.5mg/L) with higher concentrations of 2,4-D showed callus induction 65% of plates, after 21 days of incubation. The treatment T6 where *invitro* propagated rejuvenating buds were inoculated into MS media containing 2,4-D at 2.0 mg/L along with NAA 0.5mg/L was also showed organogenesis instead of callus formation (Fig 3f). The treatment T7 had TDZ (Thidiazuron), which is known to exhibit unique property of mimicking both auxin and cytokinin effect on growth and differentiation of tissue cultured plants. The use of TDZ along with 2,4-D in our experiment was also found suitable for callus induction in invitro propagated rejuvenating buds of banana, with 80% of plates with callus induction (Fig. 3g). However the combination of

Picloram (a synthetic auxin) about 2.0mg/lt and TDZ at 0.3mg/L concentration was found to be more suitable for callus induction (Fig. 3h) which showed highest, 98% of callus induction with invitro propagated rejuvenating buds as explants in banana.

The N6 CHU media helps in the initiation, growth and differentiation of callus. The treatment T9 (N6 CHU- 2mg/L 2,4-D) showed that the callus initiation from rejuvenating buds about 15% (Fig. 3i).The treatment T10 and T11 (MS-2,4-D 2.0 mg/L) where invitro propagated tender leaf tissues and root cells were used as explants respectively showed negative results for callus formation(Fig. 3j and 3k). The treatment where immature flower buds were used as explant in the (T12) N6 CHU media with 2,4-D at 2mg/lt along with 0.5mg/L NAA, 90% of plates showed callus formation after 45 days of inoculation under dark (Fig. 3l). The inoculation of the immature un-opened male buds (anthers) from inner most bract of banana inflorescence in the N6 CHU media with NAA at 0.5mg/L (T13) showed more positive results for callus formation 60 days after inoculation (Fig. 3m). Based on these results it can be concluded that the treatments T5,T7,T8 (where MS media was used) and T9,T12,T13 (where N6 CHU media was used) showed sufficient callus initiation. Among these 6 treatments, T8 was found to be favorable for callus formation followed by T13 and T12.

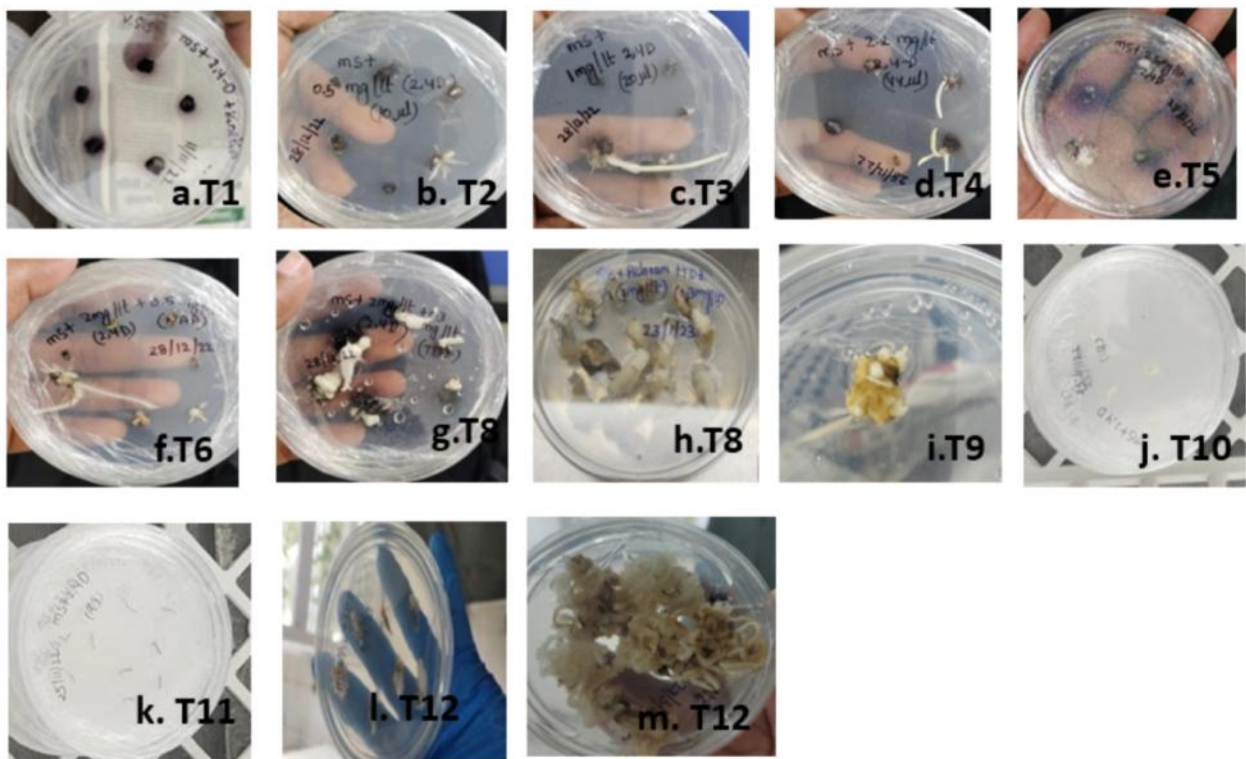

Fig.3: Callus induction in each treatment (T1 to T13) observed in different explants as after 21 days incubation of *in-vitro* propagated rejuvenating buds, after 3 months incubation of inflorescence and immature male flowers

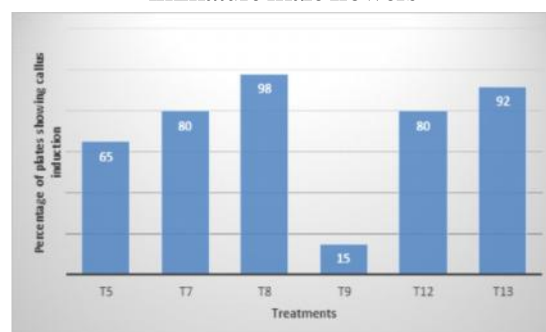

Fig. 4: Response of different treatments showing positive results for callus induction (%)

The above graph represents that the treatments where callus induction and growth was observed in different explants as in-vitro regenerated buds after 21 days of inoculation, male flower and inflorescence after 3 months of inoculation. To compare the relative success, rate the number of plats inoculated was brought to referral value of 100 plates per treatment (Table 2). Among them only 6 treatments (T5,T7,T8,T9,T12,T13) gave the positive resposne for callus induction. Among these 5 treatments, T8 (MS-Picloram 2mg/lt + TDZ 0.3mg/lt) showed highest efficiency of callus formation (Fig.3h) that is 98%, which was followed by T13 (N6 CHU-0.5 mg/L NAA) (Fig.3m) and T12(N6 CHU-2,4-D 2mg/L+ NAA(0.5mg/L)(Fig 3 l) with 92% and 80% of plates showed good callus growth.

Microscopic Study of Callus to Confirm Ploidy Level

Since embryogenic callus derived cell suspension culture (embryonic cell suspension-ECS) are known to be critical for high efficiency transformation in banana (Rustagi et al., 2019) and are considered more competent for stable inheritance of the edited or transgenes it is critical to establish the ploidy level of the cells in the callus. Earlier studies reported on somatic embryogenesis and development of ECS in bananas were found to be prone to somaclonal variations although they showed high regeneration potential (Cronauer-Mitra and Krikorian, 1988; Teision 1989; Kavitha et al., 2021). Therefore, it's imperative to check the cells obtained from these calluses for change in the ploidy level. The microscopic examination of the cells in the callus were classified as somatic or gametic callus based on the chromosome number i.e., 33 or 11. The somatic callus are developed from the parent plant through vegetative propagation in an *invitro* setting, were found to have 33 chromosomes. While the Gametic callus that were derived from the anther were found to have chromosome number, n=11. It indicated that these calluses were developed from pollen cells in the anther.

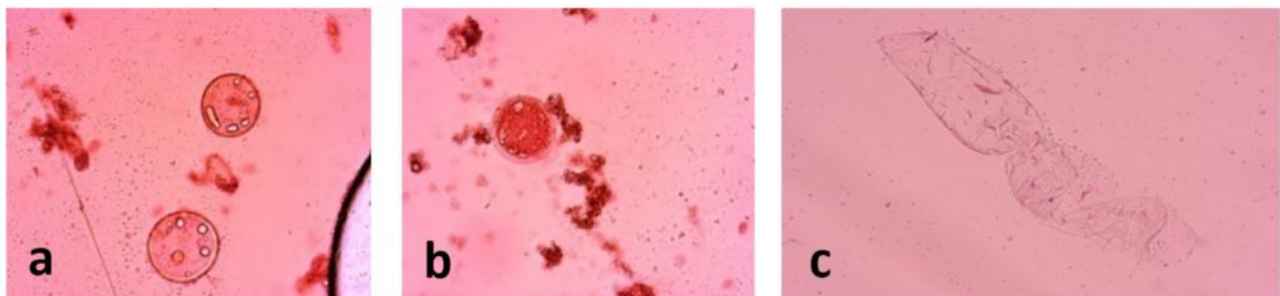

Fig. 4: Cells observed under microscopes after staining with acetocarmine (a) and (b) cell from somatic embryogenic callus and (c) cells from the gametic callus

The major advantage of *invitro* haploid calluses or cells is to obtain homozygous diploid or polyploid plants in less time as compared to the approaches were transgenes segregate and then selected for homozygous lines. Haploids find practical application in accelerated breeding programs as they have potential to achieve homozygosity for the transformed or edited genes in a single generation.

Conclusion

Bananas are widely cultivated tropical fruits, but their genetic improvement is hindered by sterility. The availability of embryogenic callus is a crucial prerequisite for efficient transformation and genetic modification. Therefore, developing a protocol for the efficient production of embryogenic callus is essential for the application of gene editing or genetic modification tools. Obtaining embryogenic calluses from bananas is challenging due to their specific growth characteristics. We report the standardized protocol for inducing callus formation in bananas using male anthers and whole flower as explants. The protocol was evaluated for different hormones and their combinations. The results indicated that the highest formation of callus obtained from *in-vitro* propagated rejuvenating buds under Picloram at 2mg/lt and TDZ at 0.3 mg/L, although they were somatic. The embryogenic callus were obtained from immature male flower with application of 0.5mg/lt NAA in N6 CHU media, which have potential use for genetic transformation or gene editing in banana.

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