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Strategies for the Improvement of Micropropagation of Banana Through *In vitro* Culture

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Key words: Banana, Multiplication rate, Polarity, Plantlet Production, Micropropagation

Abstract

In banana micropropagation, the most critical limiting factor is the rate of shoot multiplication. Due to high demand for plantlets in a particular season for cultivation, different commercial tissue culture laboratories are always under pressure for space, sterile area, energy, etc., to cope with targeted production of plantlets on time. The current method of shoot tip culture has a lot of limitations, and many Laminar Air Flow (LAF) operators are involved in TC banana production. Advanced techniques are required to be developed to produce millions of banana plantlets at a low cost. Maintenance of a high and constant multiplication rate is key to the success of a commercial Tissue Culture banana laboratory. There are various reasons for the low and non-consistent multiplication rate of the banana. This has been observed that during the *in vitro* culture the growth of the developing shoots was better in the vertical position than in the horizontal position of the shoot meristem. It shows that a change in the polarity of the explant delayed the growth in the semi-solid nutrient medium.

Introduction

Banana is the most important crop for tissue culture plantlet production. Banana, *Musa* spp. of family Musaceae, is the most important staple fruit in the world, it is the fourth most important food crop after rice, wheat, and maize. It is a globally important fruit crop. According to recent data, the annual production of banana, including cooking banana and plantain, is about 170 million tons (Tripathi et al. 2023). Bananas are cultivated in more than 136 countries (FAO-STAT 2021). India is one of the top banana-producing nations in the world, contributing to 26.7% of the total world production. In India, banana contributes 37% of the total fruit production. Andhra Pradesh of India produced 5.83 million tons of banana in 2021-22, the highest for any state in country as per the first advance estimates of the Union Ministry of agriculture. Maharashtra took the second position with 4.62 million tones. Major banana producing states in India are

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Andhra Pradesh, Maharashtra, Gujarat, Tamil Nadu, Karnataka, Uttar Pradesh, Bihar, West Bengal, Assam, Chhattisgarh, Kerala, Odisha and Madhya Pradesh.

Annual requirement of sucker/planting material in India is 3436 million (Table 1), which shows there is huge potential to produce TC (tissue culture) - raised planting material to meet the current demands of farmers.

About 20% of the total cultivated banana is under TC-raised plants of the Grand Nain / G9 cultivar. The installed production capacity in India to produce TC raised planting material is 900 million plantlets per annum, and the actual supply is 650 million per annum. The Jain Tissue Culture laboratory is the largest banana TC lab in the country with a production capacity of 85 million plants per annum, followed by Cadila, Ahmedabad, 80 million, Rise and Shine Biotech, in Pune, Maharashtra, 30 million, and many smaller ones producing 1 to 20 million.

Table 1. Year-wise area and production of banana in India (Meenakshi and Monikanda 2022). Plantlet requirement is calculated from this data by keeping the average plantation (1200 approx.) per acre.

Year	Area' 000Ha	Production '000MT	Productivity in MT/ha	Productivity in MT/Acre	Productivity in Kg/Acre	Average Plantation/ Acre	Average Plantation/ Ha	Plant Required in Million
2010-11	830.00	29780.00	35.88	14.35	14351.81	1196.0	2990.0	2481.67
2011-12	796.50	28455.10	35.73	14.29	14290.07	1190.8	2977.1	2371.26
2012-13	776.00	26509.10	34.16	13.66	13664.48	1138.7	2846.8	2209.09
2013-14	802.60	29724.60	37.04	14.81	14814.15	1234.5	3086.3	2477.05
2014-15	821.80	29221.40	35.56	14.22	14223.12	1185.3	2963.1	2435.12
2015-16	841.20	29134.80	34.63	13.85	13853.92	1154.5	2886.2	2427.90
2016-17	860.00	30477.20	35.44	14.18	14175.44	1181.3	2953.2	2539.77
2017-18	883.80	30807.50	34.86	13.94	13943.20	1161.9	2904.8	2567.29
2018-19	866.00	30460.00	35.17	14.07	14069.28	1172.4	2931.1	2538.33
2019-20	897.00	32957.00	36.74	14.70	14696.54	1224.7	3061.8	2746.42
2020-21	920.00	33733.00	36.67	14.67	14666.52	1222.2	3055.5	2811.08
2021-22	959.00	35131.00	36.63	14.65	14653.18	1221.1	3052.7	3436.00

Source: Season and Crop Report -2020-2021. Department of Economics and Statistics, Chennai.

Production of banana through the traditional method has various drawbacks, including low multiplication rates, non-uniform growth, low productivity, and the spread of various banana pests and diseases. *In vitro* micropropagation techniques are used to overcome such limitations. In TC banana production, the rate-limiting stage is the rate of multiplication. The enormous demand for plantlets during a specific growing season puts pressure on various commercial TC labs to meet deadlines for targeted production. The existing shoot tip culture process has many drawbacks. So, it is important to develop a technique by which a large number of plantlets (more than 10 million) can be produced at low cost.

Maintenance of a high and constant multiplication rate is key to success of a commercial TC banana laboratory. There are various reasons of low and non-consistent multiplication rate of banana. Diploids like Matti, Anaikomban and Senna Chenkadali produce more buds than commercial cultivars. Among commercial cultivars, the number of buds produced during subculture is high in Cavendish (Robusta, Grand Naine-AAA genome) group, followed by Plantain, (Nendran-AAB genome) and Monthan (ABB genome) types.

The objective of this investigation is to increase and maintain the multiplication rate of TC banana in subculture cycles of commercial production by using novel and conventional strategies to maintain the vigor and vitality of mother stock. The method of shoot tip culture to develop plantlets is cumbersome and needs more time and space due to the low multiplication rate. To get rid of that, it is highly necessary to grow the banana from a single cell either directly or through an embryoid. Remakantan et al. (2014) reported direct regeneration of embryos from shoot tips from 4-week-old cultures as explants, but the technology has not yet been reproduced. Our strategy is to increase the potential of shoot tip meristem to get a higher multiplication rate (MR) and initiate the cell suspension culture from the same calli/proliferating meristematic tissue and get an embryogenic cell line. Various explants viz Inflorescence, Scalp, Male and Female Flower, Shoot tip etc. (Table 2) were tried to get the embryogenic cell, however their potency to be converted in to suspension cell culture (SCC), Proembryogenic cell suspension (PECS) and Pre-embryo (PE), varies from cultivar to cultivar.

The process of embryoid production takes 36 months (Wang et al. 2022) whereas through conventional shoot tip culture plantlets are release for plantation maximum within 9 to 11 months. Tripathi et al. (2015) reported regeneration efficiency of ECS about 20,000-50,000 plantlets per ml of settled cell volume (SCV), depending on variety whereas Strosse et al. 2004 reported that 1 ml of settled cells of a highly re-generable cell suspension can yield more than 100,000 plants, cell cultures are most suitable for mass clonal propagation but only restricted to development of transgenic line (Tripathi et al. 2008, 2012 and 2015). Field evaluation of ECS-derived banana plants showed no negative effects on the vegetative and yield parameters of the plants regenerated from ECS remarkably no phenotypic types were observed in the field trial (Kumaravel et al. 2017), but only a small number of plants, a total of 300 plants of cv. Grand Nain (100 plants each from Sucker, Shoot tip culture and ECS derived) were planted in commercial plantations located at Kamagoundanpatti, Theni, Tamilnadu (10°03′57.5″ N 77°30′30.0″ E) by National research center for banana (NRCB).

Review of literature of shoot tip culture in Table 3 shows that many protocol of plantlet regeneration is reported, but none of the protocols talk about how many multiplications cycles we can go to get a clonally stable plantlet.

Table 2. Different explants used to develop somatic embryo (SE) in different cultivars of banana, the efficiency of SE is highlighted in the remarks.

Explant	Method	Variety	Remarks	Reference
Protoplast	Somatic embryogenesis	Grand Nain (*Musa* spp., Cavendish sub-group AAA)	43%	Assani et al. 2001
Protoplast	Somatic embryogenesis	*Musa* spp., cv. Bluggoe (AAA)	10-50%	Megia et al. 1993
Protoplast	Somatic embryogenesis	Banana (*Musa* spp. AAA).	10-14%.	Panis et al. 1993
Cultured buds of female flowers	Somatic embryogenesis	AAB False Horn Plantains, 'Curraré' and 'Curraré Enano'	21.42%	Grapin et al. 2000
Immature male flower buds	Somatic embryogenesis	Dwarf Brazilian (*Musa* spp. AAB group)	90%	Khalil et al. 2002
Inflorescence (Young male flower)	Somatic embryogenesis	*Musa* (cv. Dwarf Cavendish, AAA)	12.5%	Perez-Hernandez and Rosell-Garcıa 2008
Proliferative multiple buds	Embryogenic cell suspension (ECS) with *Agrobacterium*-mediated transformation	Plantain cv. "Gonja manjaya (*Musa* spp AAB)	56.8%	Tripathi et al. 2012
Multiple buds	ECS	Cavendish Williams (AAA)	50%	Tripathi et al. 2015
Multiple buds	ECS	Gros Michel (AAA)	44%	Tripathi et al. 2015
Immature male flower	ECS	Sukali Ndiizi (AAB)	6.6%	Tripathi et al. 2015
Immature male flower	ECS	Cavendish Williams (AAA)	0%	Tripathi et al. 2015
Immature male flower	ECS	Gros Michel (AAA)	0%	Tripathi et al. 2015
Multiple buds	ECS	Mpologoma (AAA-EAHB)	0%	Tripathi et al. 2015
Immature male flowers	ECS	Ngombe (AAA-EAHB)	0%	Tripathi et al. 2015
Scalp	Somatic embryogenesis	Calcutta 4 (AA)	0%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Cavendish GN FHIA (AAA)	0-2.9%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Cavendish GN JD (AAA)	0-4.2%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Cavendish Gran Enano (AAA)	0-11.7%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Cavendish Williams BSJ (AAA)	0%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Cavendish Williams JD (AAA)	0-22.2%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Highland Ingarama (E-AAA)	0%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Highland Mbwazirume (E-AAA)	0%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Highland Nyamwihogora (E-AAA)	0%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Plantain Agbagba (AAB)	0-0.5%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Plantain Obino I Ewai (AAB)	0-2%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Plantain Orishele (AAB)	0-5.8%	Strosse et al. 2004
Scalp	Somatic embryogenesis	Cooking bababa Burro Cemsa (ABB)	0%	Strosse et al. 2004

As per the guideline developed by the Department of Biotechnology (DBT) - Tissue Culture Raised Plants (TCP), Govt. of India, the recommendation is to go up to 8 multiplication cycles with low genetic fidelity. In practice, the recommendation is not practical for a commercial banana tissue culture laboratory.

Table 3. Number of multishoots obtained in banana by different workers using mostly the shoot tip as explant in different varieties. Only a few workers have mentioned about number of subcultures and subculture time.

Explant	Variety	Multiple shoots No.	No. of subcultures	Subculture time	Reference
Shoot tip	*Musa* spp. cv. Basrai	124	5	4 W	Muhammad et al. 2004
Wounded Shoot tip	Ensete ventricosum	75		14 W	Birmeta and Welander 2004
Shoot tip	Banana cv. BARI Banana-I	6		30 days	Al Amin et al. 2009
Shoot tip	Banana cv. Agishwar	5.9			Rahman et al. 2013
Shoot tip	Banana cv. Grand Nain	10.66			Ahmed et al. 2014
Shoot tip	Banana cv. Matti	8.66	3	20 days	Lohidas and Sujin 2015
Shoot tip	Ensete (Ethiopian banana)	23		11.66 days	Gezahegn and Mekbib 2016
Shoot tip	Poyo and Giant Cavendish	6.0 and 4.5	10 and 8	8 & 10 Days	Kelta et al. 2018
Shoot tip	Banana cv. Poovan	8	4	3 W	Sivakumar and Visalakshi 2021
Shoot tip Scalp	*Musa* sp. cv. Tanduk	40.63	2	4 W	Elhory et al. 2009

The number of initials needed to start with to give a targeted supply of TC banana plantlets varies as per culture bottles keeping capacity in the growth room, whereas the rate of multiplication achieved by a commercial tissue culture lab is crucial for production planning. Table 4 shows the number of initiations needed in an absolute ideal condition for MR 2, 3 and 4, respectively. The number of initiations decreases with an increase in multiplication rate. Table 5 shows how much banana initiation is needed with different multiplication rates to get the production of 1.0 million to 100.00 million plantlets with turnover of 1.5 to 150 cr respectively.

Due to such a high economic perspective, many new small labs are emerging in every parts of the country every year. This is a very pertinent question how much maximum multiplication can we go for and why not more than that? Ministry of Agriculture has vide Gazette of India Notification dated 10th March 2006 notified that "In exercise of the powers conferred under section 8 of the seeds Act, 1966 (54 of 1966), the Central Government hereby authorizes Department of Biotechnology, Ministry of Science and Technology, Government of India to act as Certification Agency for the purpose for certification of the tissue culture-raised propagules up to laboratory level and to regulate its genetic fidelity as prescribed by them".

Accordingly, the National Certification System for Tissue Culture Raised Plants (NCS-TCP) has been developed for the first time, not only in the Country but also globally, where currently no such organized structure exists for certification of Tissue Culture planting material. According to NCS-TCP: (i) all samples of banana varieties being initiated should be tested in an accredited laboratory and be free of viruses (Banana Bunchy Top Virus, Cucumber Mosaic Virus, Banana Bract Mosaic Virus, Banana Streak Virus) and other endophytic or epiphytic bacteria and fungi. (ii) The basic material for sub-multiplication needs to be obtained afresh from the nodal organization as soon as the maximum permitted number of passages (as confirmed by DNA fingerprinting) of

Table 4. The number of initiations of banana needed in ideal conditions for multi-rate (MR) 2, 3, and 4 is shown for a subculture interval of 4 weeks.

		1	2	3	4	5	6	7	8	9	
Multi Rate (MR)	1st W	4th W	8th W	12th W	16th W	20th W	24th W	28th W	32 W	36th W	Remarks
2	1										20,000 initial culture stock will be needed to get 10 million plantlet after 9 months
		2									
			4								
				8							
					16						
						32					
							64				
								128			
									256		
										512	
3	1										510 initial culture stock will be needed to get 10 million plantlet after 9 months
		3									
			9								
				27							
					81						
						243					
							729				
								2187			
									6561		
										19683	
4	1										40 initial culture stock will be needed to get 10 million plantlet after 9 months
		4									
			16								
				64							
					256						
						1024					
							4096				
								16384			
									65536		
										262144	

shoot multiplication with old cultures has been completed. iii) *In vitro* multiplication of an imported variety or a non-notified variety can be taken up by the industry exclusively for export purposes. Such varieties, however, should be introduced following the approved guidelines of Government of India.

Table 5. The number of initiation of bananas needed for a small, medium, or large production lab for targeted production of 1.0 to 100.0 million for different multi-rate (4 to 2).

Required No. of Plants	No. of Initiations for MR 4	No. of Initiations for MR 3	No. of Initiations for MR 2	Turn Over (in Cr) INR
1.0 Million	100	500	6250	1.5
10 Million (1.0 cr)	1000	5000	62500	15.0
50 Million (5.0 cr)	5000	25000	312500	75.0
100 Million (10.0 cr)	10000	50000	625000	150.0

There is a difference of opinion among researchers and production labs on the number of passages that could be regarded as "safe" for shoot multiplication in banana concerning clonal uniformity of plants. In tissue culture, it is well known that the lower the number of subcultures, the lower will be the chances of somaclonal variation. However, it must also be realized that if the number of passages is far too small, then the entire production process becomes economically unviable. Therefore, efforts should be made to optimise the shoot multiplication process and extend the number of passages only till the clonal uniformity of the progenies is maintained. This may be achieved through (a) strict monitoring of the shoot multiplication process, ensuring that adventitious shoots are not multiplied, and (b) confirming the clonal fidelity of tissue-cultured plants using molecular markers in different passages. Currently, there is no data on clonal uniformity for several multiplication subcultures; however, in banana, under no circumstances should shoots be subcultured for more than 8 passages (NCS-TCP, Fourth Revision, April 16, 2019).

We have developed the strategies to invigorate the initiation/multiplication stock for its vitality, a key for quality stock for production. Therefore, the objectives of this study includes 1. To increase and maintain the multiplication rate of TC banana in subculture cycles of commercial production by using different concentrations of hormones and by changing the polarity of the explant (shoot tip). 2. To develop ways to get the desired cells from the shoot meristem for a cell suspension culture from the same calli/proliferating meristematic tissue, and get an embryogenic cell line.

Materials and Methods

The maintenance of the multiplication rate of banana in micropropagation is of great concern since there is a loss of vitality in successive subcultures. The development of techniques by which the vitality of mother stock can be restored for better and healthier proliferation and multiplication is very necessary for commercial banana tissue culture. The experimental materials used were in process multiplication stock culture, sucker and shoot tips.

The method of shoot tip culture to develop plantlets is also cumbersome and needs more time due to the low multiplication rate. To get rid of that, it is highly necessary to grow the banana from a single cell either directly or through an embryoid. Cell line is the descendant of a single initial cell, there is no report on banana cell line; however, there are many reports of suspension cell culture to get embryoid. To get the highest meristematic potential containing cells, for screening the potential cell for suspension culture / cell line needs to be well recognized in the shoot apical meristem of banana.

The following two strategies were employed to achieve regeneration.

i. By changing the polarity of the explant:

Cut the base of multi shoot vertically and inoculate apical meristem and lateral meristem vertically and rotate other part of explant at 90° and inoculate in MS media containing 11.1 µM BAP and incubate it at 27°C temperature, 16 : 8-hrs light (3000 lux): dark condition as demonstrated in the Fig. 1.

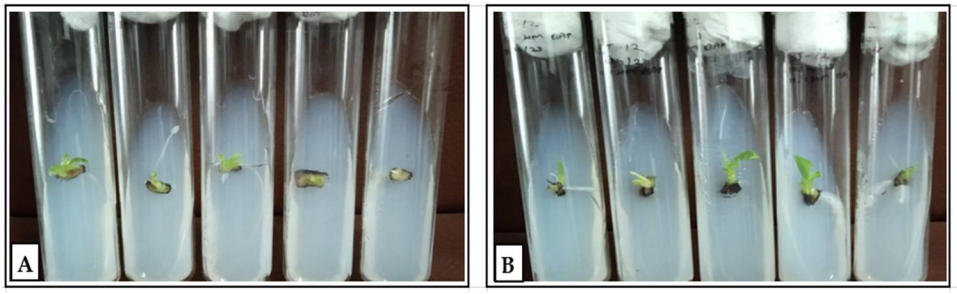

Fig. 1. Inoculation of multi shoots of banana in (A) Horizontal position of proliferated shoot tip, (B) Vertical position of proliferated shoot tip.

ii. Development and maintenance of a cell culture/cell line for Embryoid Production

Cut the 3 slices (upper, middle and lower) from the base of multi shoot culture/Sword Suckers Apical meristem as in Fig. 2c. Inoculate 50 % slice to the semisolid and liquid MS basal media plus 11.1 µM BAP (scalp induction media) and incubate at 27°C temperature, 16 : 8-h light (3000 lux): dark condition. The remaining 50% was kept in dark conditions. Cut the proliferating base of multishoot culture horizontally and vertically and inoculate it into liquid MS basal media plus 11.1 µM BAP and incubate at 27°C temperature on a rotary shaker at 80 rpm and 16:8-h light (3000 lux): dark condition. Incubate another set of inoculated flasks at 27°C in the dark.

After the production of a white fleshy mass-like structure, press it with the help of a sterile blade to release the cells. Then transfer it into liquid MS basal media plus 11.1 µM BAP and incubate at 27°C on a rotary shaker at 80 rpm under a 16: 8-hrs light (3000 lux): dark condition. This way generates the cell suspension and screens it by dilution plating to get the progeny of a single cell for embryoid production.

Multiplication of banana shoot formation by changing the polarity of the explant. The scalp induction media was used to test the ability of different regions of the shoot meristem for their potential cell line/culture development. The active meristematic zone in the shoot meristem is in the middle region. The upper and lower regions of the explant did not show any regeneration. Cell differentiation was also observed in the middle part of the sucker in scalp induction media in semi-solid nutrient media (Fig. 2)

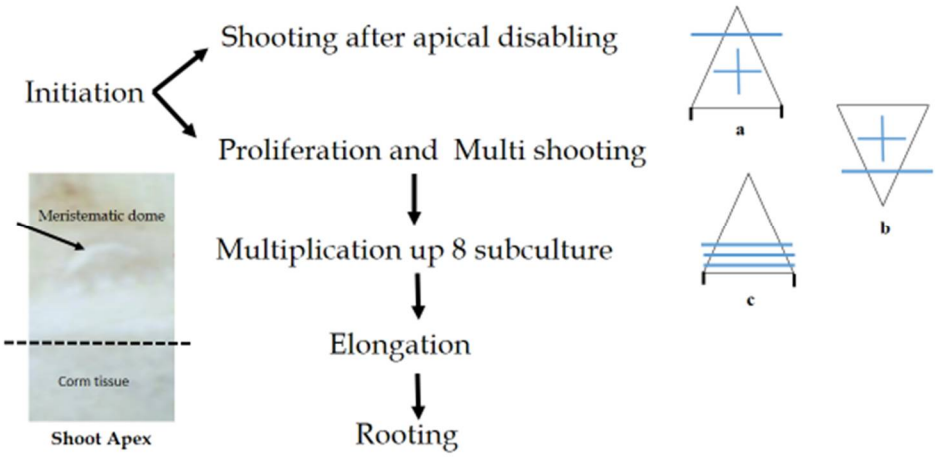

Fig. 2. (a) Shows the standard method of shoot tip initiation by making an apical cut (decapping) followed by a small horizontal and vertical incision (b) Same as 'a' but during inoculation for culture, polarity is changed (c) To increase the surface area of explant decapping of shoot tip was done little deeper and then two horizontal cut was made to get three inoculations of shoot tip (upper, middle and lower).

Results and Discussion

Natural genetic variation occurs more slowly and can occur between hundreds and thousands of years when compared to the induction of *in vitro* variation. Therefore, some genetic alterations observed in the field may come from micropropagated plants in which the use of PGRs and frequent subcultures occur (Park 2023). The occurrence of somaclonal variation in micropropagated plants has been studied for many years, and these variations occur in diverse cultures subjected to *in vitro* cultivation. Somaclones can be identified in a greenhouse, in the field and *in vitro* by observing changes in plant traits, such as leaf color, texture, etiolation and other phenotypic changes. Epigenetic changes are responsible for phenotypic changes observed in somaclones, and these changes, such as loss of DNA methylation, may be reversible (Imran et al. 2022). *This indicates that clonal variation / genetic fidelity can be reversed or restored by using innovative strategies.* Shoot tip culture of banana was initiated on MS media (Fig. 3), proliferated shoot was sub cultured by changing the polarity (Fig. 1A and B).

Fig. 3. Initiation of banana in semi-solid MS media.

Change in polarity shows a delayed response of shoot development in the horizontal condition than the vertical condition. This indicates that cells and cell organelles have taken time in reorienting themselves to resume the normal growth and differentiation. Our approach is to make use of this reorientation mechanism in restoring the clonal fidelity. The vascular tissues of plants can often regenerate around a wound (Jacobs 1952, Sachs 1981). If the orientation of the vascular cells along the transporting files is taken as an expression of polarity, this regeneration must mean that polarity can be reoriented. Embryogenic cell suspension (ECS) culture was developed from protoplast (Megia et al. 1993), Cultured buds of female flowers (Grapin et al. 2000), Immature male flower buds (Khalil et al. 2002), Inflorescence (Perez-Hernandez and Rosell-Garcıa 2008), Proliferative multiple buds (Tripathi et al. 2012), and immature male flowers and multiple buds (Tripathi et al. 2015). All these ECS were developed from a group of cells and not from a single cell initial, we are trying to develop ECS from a single cell initial. We have used proliferating base of multishoot and different portion of apical shoot from sucker as explant to do the induction of meristemetic mass to screen the cell. Our observation shows that till 30 days after inoculation (Figs 4, 5, 6 and 7) there were no signs of callusing or tissue growth either in 16 hrs photoperiod or in dark conditions. Growth of the tissue was observed in scalp induction media after 30 days of inoculation in middle portion of apical shoot from sucker (Fig. 8) in dark conditions. We are using this tissue mass, originating from the middle region of the apical shoot, for cell line development.

Fig. 4. Induction in banana, in scalp induction media by inoculating the upper, middle, and lower portions from the base of the multi - shoot as explant in liquid media and dark conditions.

Fig. 5. Induction in banana, in scalp induction media by inoculating the upper, middle, and lower portion from the base of multi - shoot as explant in liquid media and in 16-hrs of light and 8-hr dark photoperiod.

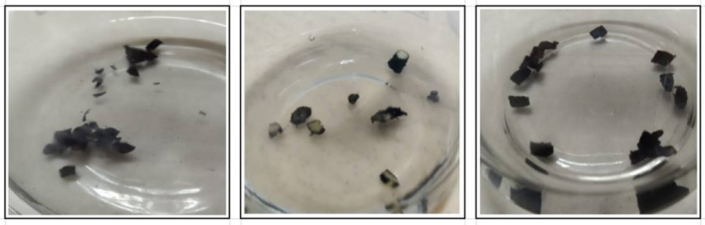

Fig. 6. Induction in banana, in scalp induction media inoculating upper, middle and lower portion of sucker as explant in liquid media and dark condition.

Fig. 7. Induction in banana in scalp induction media by inoculating upper, middle and lower portion *of sucker* as explant in liquid media at 16 hr. light and 8 hr. dark photoperiod.

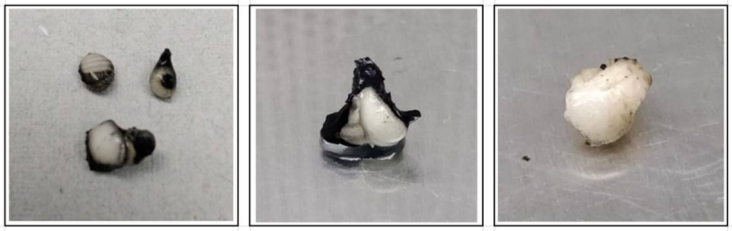

Fig. 8. Growth of tissue in scalp induction media after 30 days of inoculation.

From the above result of the initiation of banana for multishoot formation by changing the polarity of explant, growth of the shoot is better in the vertical position of the explant than the horizontal position of the explant. In the scalp induction media, growth of the tissue was observed only in middle part of sucker and meristematic base in liquid media in dark condition. It shows that meristematic regions are in the middle region. Upper and lower region of explant did not show any result however slight initiation was observed in the middle part of sucker in semi-solid condition of scalp

induction media too. Due to ever-increasing domestic as well as export demand every year, it is highly important to rethink the strategies to develop a robust protocol with an economically good multiplication rate and to take higher multiplication cycle. Moreover, this investigation shows that polarity can play a major role in increasing shoot meristems' vigor, multiplication rate, and clonal stability. Sub-apical region of the shoot meristem is the potential zone for cell line/culture development.

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