Standardization Of Regeneration Protocol Through Somatic Embryogenesis in Banana, CVS. Rasthali

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Abstract*— The present study was designed with the main aim to standardization of regeneration protocol through somatic embryogenesis in banana, cvs. Rasthali (AAB). The male flower buds and suckers of banana cvs. Rasthali were collected periodically from Hesaraghatta and Nanjangud. The explants were cultured within 24 hours of collection. Experiments were conducted using Murashige and Skoog's medium (MS) with different plant growth regulators and MS medium was found to be the best for establishment of various cultures because of high concentrations of mineral salts. Growth regulators viz., auxins, cytokinins, gibberellins and abscisic acid were used in tissue culture experiments. Various experiments were carried out in order to standardize the media composition and type of growth regulators to obtain highest frequency of plantlets through germination of somatic embryos. A statistical analysis of the response of the various explants was done using GraphPad Prism Software. $P<0.05$ was considered statistically significant. The results revealed that Banana cvs. Rasthali (AAB) are seriously under the threat of extinction due to its susceptibility to fusarium wilt. A regeneration protocol using somatic embryogenesis was developed in this study, using immature flower buds. This reliable regeneration method is a prerequisite for molecular manipulation to obtain disease resistance bananas. Immature flower buds cultured in-vitro resulted in embryogenic callus formation in this study in banana cvs. Rasthali (AAB). Secondary somatic embryo formation was commonly observed on the primary embryos of banana cv. Rasthali. In summary, banana cv. Rasthali the cvs. exhibited differential response to embryo development depending on their genotype. The study has shown immature flower buds were better choice of explants to induce embryogenic callus than shoot tip explants in banana cv. Rasthali. Nodular yellowish green callus was the most common non-embryogenic callus observed in this study.

Index Terms*— Banana, cv. Rasthali, Somatic embryogenesis, Regeneration protocol, Callus induction.

I. INTRODUCTION

Bananas and plantains belonging to the genus Musa of Musaceae family, are perennial herbaceous monocots. Musa is a major crop in the tropical and sub-tropical regions of the world, with an approximate production of 70 million tones per annum. India is rich in genetically diverse varieties of banana, cultivated over an area of 802.6 (000ha), with production of 297.24 lakh tones, contributing 33.4 per cent of the global production share [1]. Owing to female sterility, parthenocarpy and polyploidy, production of new banana cultivars is difficult and time-consuming. Genetic manipulation is a promising technique for introducing desired traits in banana. In Karnataka, Hill banana (AAB), Monthan (ABB), Nanjangud Rasabale (AAB, Rasthali), Ney Poovan (Elakkibale, AB), Dwarf Cavendish (AAA), Robusta (AAA), Poovan (AAB), Jawari bale (AAB), Karibale (ABB), Grand Naine (AAA) are the most important banana cvs. grown. The cultivation of banana in Karnataka is in four divisions – Bangalore division, Belgaum division, Gulbarga division and Mysore division. Bangalore division produces highest of 4,87,466 tonnes followed by Mysore, Belgaum, Gulbarga. Micropropagation has played a key role in plantain and banana improvement programs worldwide [2]. Application of micropropagation has greatly improved *Musa*

germplasm for faster, uniform production and breeding.

Somatic embryogenesis is defined as a process in which a bipolar structure, resembling a zygotic embryo develops from a somatic cell without vascular connection with the original tissue [3]. Somatic embryogenesis occurs through a series of stages characteristic of zygotic embryogenesis. Somatic embryos are used for studying regulation of embryo development, mass micropropagation and the development of cellular tools for genetic improvement like genetic transformation and protoplast fusion. In some cases, somatic embryogenesis is favored over other methods of vegetative propagation because of possibility to scale up the propagation by using bioreactors [4]. In addition, embryogenic cultures can be cryopreserved which make it to establish gene banks [5]. Somatic embryos morphologically resemble zygotic embryos; however, they develop via different pathway. During the course of evolution many plant species have evolved different strategies for asexual embryogenesis including somatic embryogenesis to overcome various environmental and genetic factors that prevent fertilization. Somatic embryogenesis occurs to a limited extent under natural conditions, e.g., within ovules in Paeonia [6]. Therefore, in the present study was designed with the main purpose to standardize the regeneration protocol for somatic embryos were taken for the Rasthali cvs.

II. MATERIALS AND METHODS

The male flower buds and suckers of banana Rasthali cvs. were collected periodically from Hesaraghatta and Nanjangud. The explants were cultured within 24 hours of collection (Plate 1).

Plate 1: Photographs of the study material, banana Rasthali, cvs.

Figures - Field grown plants of a. banana cv. Rasthali (AAB), b. banana cv. Grand Naine (AAA) and c. banana cv. Neypoovan (AB).

- Preparation of Culture Medium

Improved technology and application of *in-vitro* methods can be attributed to a better understanding of nutritional requirements of cultured cells and tissues. Experiments were conducted using Murashige and Skoog's medium (MS) with different plant growth regulators and MS medium was found to be the best for establishment of various cultures because of high concentrations of mineral salts. (Table 1) [7].

- Growth Regulators

In the present study growth regulators *viz.*, auxins, cytokinins, gibberellins and abscisic acid were used in tissue culture experiments. Growth hormone stock solutions were prepared by dissolving 50 mg of pure chemicals in 2-5 ml of NaOH or 1 HCl or ethanol or water and gradually diluted to 50 ml with distilled water and was refrigerated. They were used depending upon the requirement of the experiments. Most of the inorganic salts and chemicals used were from Hi

media. Plant growth regulators were of highest purity and purchased from Sigma Inc., St. Louis, MO, USA. In general chemicals of analytical reagents (Analar), sterilized distilled water and bacteriological grade agar or Gelrite were used to prepare solid media.

- Surface sterilization of plant material

Male flower buds of banana cvs. Rasthali were collected one to ten weeks after flowering. In non-sterile conditions, the size of the male buds was reduced until the immature flowers were of 0.8 cm x 2 cm. The reduced bud is kept in non-dehydrating conditions until sterilization in a container having water (similarly field grown suckers were collected and reduced to 3 cm and kept in water). Surface of the plant parts usually carry a wide range of microbial contaminants. Therefore, surface disinfections have to be done to minimize the chances of contamination. The explants were washed with 1% (v/v) soap solution for 5 minutes and then washed in running water. The male flower buds were again cleaned with (0.1%) cetrimide solution for 5 minutes and washed with sterilized distilled water for 3-5 times. Later flower buds were transferred to 70% alcohol for 1 minute and rinsed with sterilized double distilled water. The trimmed suckers were also treated with 0.1% (v/v) cetrimide for 30 minutes and rinsed with sterile distilled water. Suckers were further surface sterilized with 0.1% mercuric chloride and 70% alcohol followed by thorough washing with sterilized distilled water, to remove all traces of sterilant. Surface sterilization of explants were carried under the laminar airflow.

- *Regeneration Studies*

Various experiments were carried out in order to standardize the media composition and type of growth regulators to obtain highest frequency of plantlets through germination of somatic embryos.

- Statistical Analysis

A statistical analysis of the response of the various explants was done using a simple statistical tool. The percentage responses of the explants in different media combinations were calculated using the following formula:

$$\text{Percentage response} = \frac{\text{No. of explants showing response (n)}}{\text{Total no. of explants cultures (N)}} \times 100$$

where,

n = swelling / callusing / callogenesis /rhizogenesis or somatic embryogenesis

N = total no. of explants cultured per experiment

The data were tabulated in the tabular columns separately for different explants at different concentration levels. Standard deviation (SD) and standard error (SE) was found using following statistical formula.

$$SD = \sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}$$

Where, X= individual observation

$\bar{X}$= mean of the experiments

$$SE = \frac{SD}{\sqrt{n}}$$

All data were represented as mean SE changes in all the parameters which were analyzed by two-way ANOVA. Significant 'F' ratios between groups means were further subjected to least significant differences (LSD) probability (P) values < 0.05 were considered significant using GraphPad Prism Software [8].

III. RESULTS

Callus Induction and Plant Regeneration from Male Flower Buds of banana cv. Rasthali (AAB)

The primordia of male flower buds were cultured on various induction media for the induction of embryogenic callus and incubated in dark at 25±2ºC. The explants showed curling around 3-4 weeks and 50% of them became necrotic after 5 weeks. However, the remainder formed callus of different types depending on the position of male flower primordia. Larger hands of flower primordia continue to grow for some time and formed the callus later. Usually, medium sized hands and very few smaller hands formed yellow compact callus after 3 months of

inoculation on MS + 2,4-D (4 mg/l) + NAA (1 mg/l) + IAA (1 mg/l) [MA1] (Plate 2, Fig. d). A positive embryogenic white fragile, translucent, hydric callus appeared on the yellow callus of 4 to 8 month old culture (Plate 2 and 3). This white friable callus started to form embryogenic cell clusters at the periphery of the callus. Sometimes individual embryos appeared directly from the necrotic explant without showing any white friable callus (Plate 3, Fig. e). Regular morphological observations revealed changes in colour and appearance of callus and the time at which embryogenic structures emerged. The earliest appearance of embryogenic callus on male flower buds on induction media was 3-4 months. Delicate, transparent, loosely bound embryos with different sizes were seen at the opposite side of the explant facing the media (Plate 3, Fig. g). After two months of origin of embryogenic callus, distinct white, definite non-transparent somatic embryos appeared on white callus. At this stage the embryogenic callus and individually developed embryos were subcultured on to MA3, *i.e.*, MS + 2-ip (0.2 mg/l) + Kinetin (0.1 mg/l) + Zeatin (0.05 mg/l) + NAA (0.2 mg/l) for 2 to 3 months for further maturation of somatic embryos under light conditions. On MA1 medium, the explants showed different types of calluses which comprised of yellow nodular, white hard, white hairy, loose cellular mass along with white translucent embryogenic callus. The percentage of embryogenic callus formation (Table 1) is very less (6.6%) compared to non-embryogenic callus in case of banana cultivar Rasthali (Table 2).

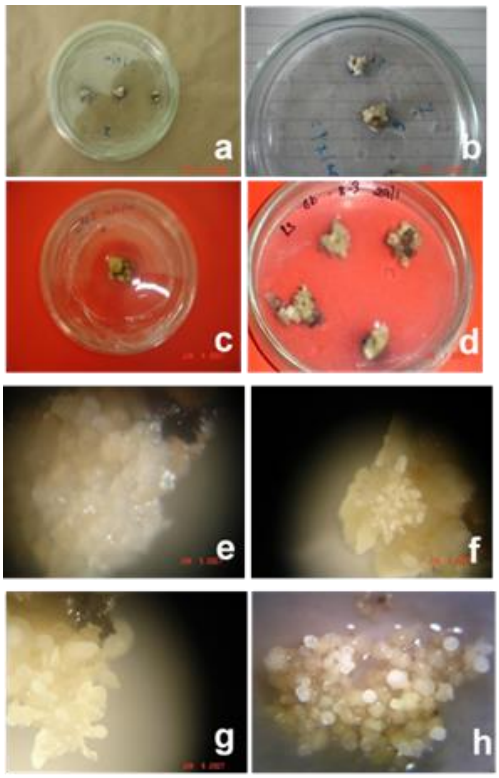

Plate 2: Embryogenic callus induction from immature male flower bud explants of banana cv. Rasthali on MAI medium (MS+2, 4-D (4mg/L) + IAA (1mg/L) + NAA (1mg/mL).

Figure a. Immature male flower primordia (1,2,3 position) showing initial formation of callus after 4 months.

a. Embryogenic callus formation (4,5 position) from male flower buds on MAI.
b. yellow primary callus showing white embryogenic callus from immature flower buds (5^{th} position) on MAI.
c. Non-embryogenic yellow callus along with white embryogenic callus formed from immature flower (6,7,8,9) buds.
d. Ideal embryogenic white, translucent, fragile callus formation from immature male buds is the best type of callus to induce for suspension cultures.
e. Embryogenic callus showing individual embryos at the initial stages of development was the common type of embryogenic callus observed.
f. Embryogenic callus showing peripheral differentiation of fragile, transparent, hydric type of proembryos.

g. Globular stage of proembryos on embryogenic callus of immature male flower buds of banana cv. Rasthali.

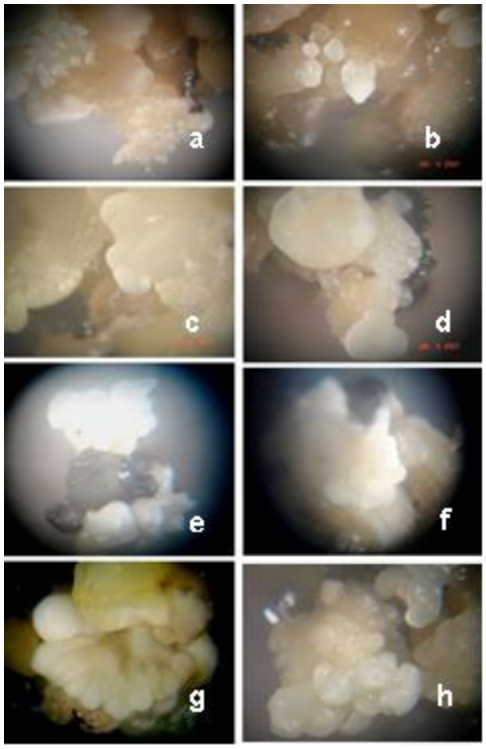

Plate 3: Somatic embryos at different stages of development from embryogenic callus of immature flower primordia of banana cv. Rasthali on MSI medium (MS+2,4-D (4 mg/l) + IAA (1mg/l) + NAA (1 mg/l).

Figure a. yellow callus showing 2 clusters of embryogenic calluses from immature male buds.

a. Proembryos at different stages of (globular heart shaped) growth / development.
b. Single embryo with expanded region showing cotyledonary notch in the centre.
c. Unusual shaped somatic embryos on embryogenic callus of banana cv. Rasthali.
d. Direct formation of somatic embryos on necrotic region of MFB.
e. Fused somatic embryos cluster showing asynchronous growth.
f. White, compact, matured somatic embryo cluster on embryogenic callus.
g. Somatic embryo cluster showing cotyledonary notch in the centre.

Table 1: Different *in vitro* phases involved, time needed during the establishment of male flower bud derived embryogenic callus and resulting plant material

Sl. No.	In vitro phase	Musa clone	Resulting plant material	Duration (months)
1	Preparation of embryogenic competent callus	Rasthali	Embryogenic callus	4 - 8
2	Maturation of embryogenic callus	Rasthali	Globular, heart shaped and matured embryo with cotyledonary slit	2 - 5
3	Regeneration	Rasthali	Rooted plantlets of test tube size	3 – 10

Grand total: Rasthali = 23 months (nearly 2 years)

Table 2: Embryogenic callus and non-embryogenic callus formation from MFB explants of Musa clones

Musa Clone	Type of Explant	Type of Callus	No. of Response	SE
Rasthali [AAB]	MFB	Non-Embryogenic callus	280	0.76
		Embryogenic callus	20	0.19
		Somatic Embryos formed	20	0.19
		Germination of somatic Embryo	20	0.19

The data is based on the evaluation of 20 male cones of 15 explants from each. Callus types were significant in two-way ANOVA.

Different developmental stages of somatic embryos like globular, heart shaped, to cylindrical stages along with other forms of shapes could be seen on

embryogenic callus (Plate 3, Fig. c, d, g, h, j). These embryos on maturation media attained maturity by expanding themselves to pear shaped, elongated cone shape, other shapes and also by forming definite root meristem and shoot meristem with cotyledonary slit (Plate 4, Fig. a, Plate 3, Fig. g, h). But development and maturation of embryos were not synchronous. Plate 4 shows the secondary somatic embryo formation on the primary somatic embryos. Initially transparent, fragile secondary buds were seen on the primary somatic embryos (Plate 5, Fig. a-e). Once the embryos formed cotyledonary notch, they were further subculture onto germination media MA4 having MS + BAP (0.5 mg/l) + IAA (2 mg/l) with morel vitamins. Plantlets were formed from matured embryos after 2-3 months on germination media (Plate 5, Fig. b-h, Table 3). They were further separated and subcultured to MA4 for further growth development of the plantlets. Later they were transferred to tubes and into soil rite (Plate 6, Figs. c-f). The male flower primordia inoculated onto MS + 2,4-D (2 mg/l) + Zeatin (0.2 mg/l) also formed embryogenic callus but the percentage was less (Table 4). Different forms of non-embryogenic callus formation were more in this induction media (Table 7, 8). On media having MS + BAP (2 mg/l) + IAA (1 mg/l) the male primordia formed greenish nodular non-embryogenic callus (Table 4). Gamborgs media with 2,4-D (2 mg/l) + Zeatin (0.2 mg/l) resulted in vitrification of explants to form watery non-embryogenic callus (Plate 7, Fig. a-c, Table 6). Using two-way ANOVA statistical data were analyzed for different types of callus and media, it showed ‘p’ value in Bonferroni post-hoc tests.

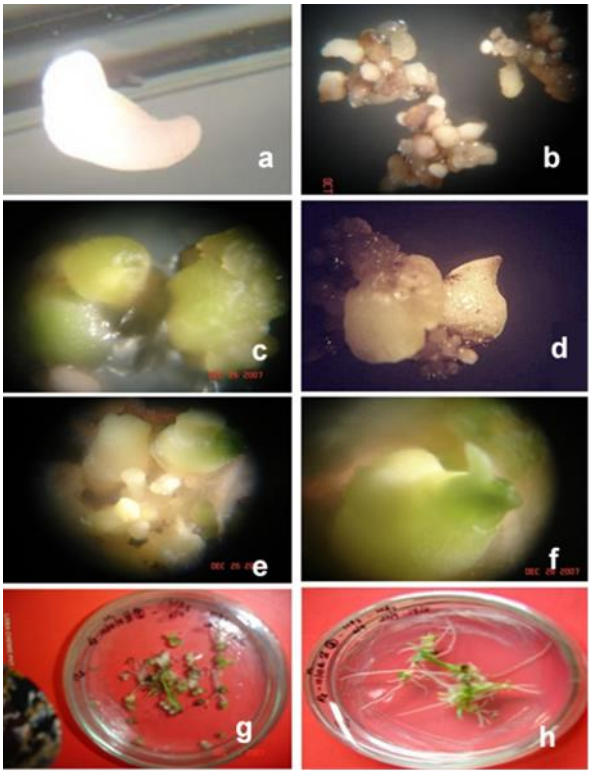

Plate 4: Maturation and regeneration of somatic embryos from immature male buds of banana cv. Rasthali on 1. Maturation media (MS+Kin (0.1 mg/l) + 2-ip (0.2 mg/l) + Zeatin (01 mg/l). 2. Germination media (MS+BAP (0.5 mg/l) + IAA (2 mg/l)

Figure a. Matured cylindrical somatic embryo with cotyledonary notch on MA3 medium.

b. Plumule development from somatic embryos indicating first sign of germination on MA4 medium.

c. Germinated plumule part of somatic embryo acquired green colour during further growth on MA4.

d. Completely developed plumule along with few degenerating somatic embryos on MA4 medium.

e. Cluster of regenerating embryos at different (asynchronous) stages of development on MA4 medium.

f. Single germinated embryo showing greenish plumular region with a leaf primordium.

g. Complete plantlet formation with the vigorous growth of root and shoot on MA4 medium.

h. Regeneration of whole plants with elongated root formation along with shoots in banana cv. Rasthali

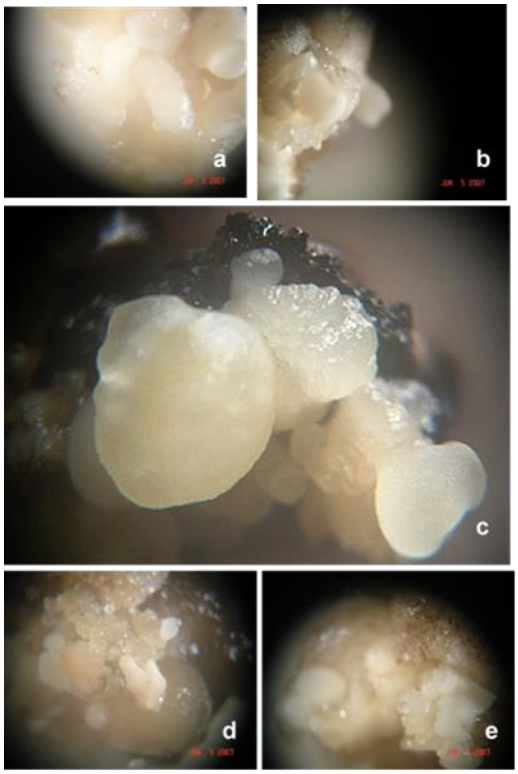

Plate 5: Formation of secondary somatic embryos on primary somatic embryos from immature male flower buds of banana cultivar Rasthali (AAB)

Figure a.Tip of primary somatic embryo showing small bud of secondary somatic embryo
b. Secondary somatic embryo cluster differentiating in cluster from primary embryos.
c. A budding secondary somatic embryo on matured primary somatic embryo.
d. Younger globular primary proembryo already forming secondary embryo on its tip.

Table 3: Media tried for maturation and germination of somatic embryos from male flower buds of banana cv. Rasthali

Sl. No.	Maturation Media	Response	Frequency of Response	SE
1	MS + 2-ip (0.60 µM) + NAA (1.07 µM) + Zeatin (0.23 µM) + Kinetin (0.46 µM)	Matured into complete somatic embryo	70	0.198
2	Germination Media MS + IAA (11.42 µM) + BAP (2.22 µM)	Developed into plantlets	30	0.114

The data is based on evaluation of 20 MFB of 15 explants from each. Maturation and germination of white embryogenic callus were highly significant in Two-way ANOVA.

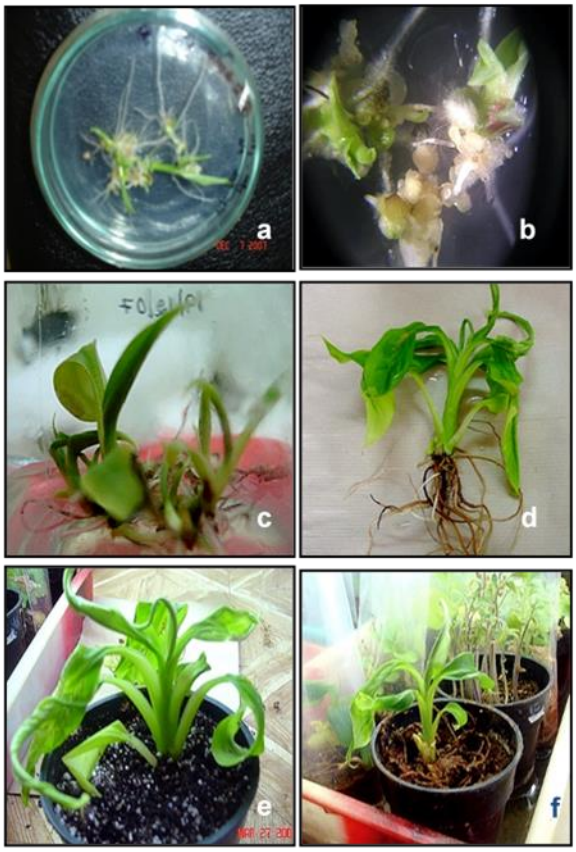

Plate 6: Transfer of regenerated plants from somatic embryos to different conditions in banana cv. Rasthali (MA4 – MS + BAP (0.5 mg/l) + IAA 2 mg/l).

Figure a. Proliferating roots grow faster during regeneration than shoots on MA4 medium.
b. Germinated plantlets showing numerous hairy roots, plumule, cotyledon, leaves.
c. Regenerated plantlet transferred to bottle having MA4 for further growth of the plant.
d. Complete regenerated plant obtained from somatic embryo showing well grown roots and shoot having many leaves.
e. Regenerated plant transferred to plastic pot containing autoclaved soil rite.
f. Acclimatization of regenerated plants to the laboratory conditions.

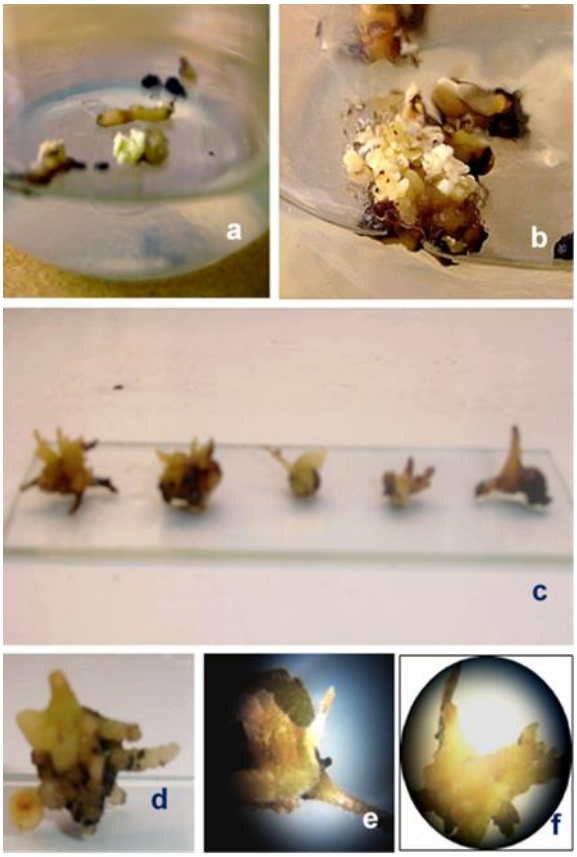

Plate 7: Callus cultures of male flower buds of banana cv. Rasthali showing different types of morphogenetic responses.

Figure a. Shoot like outgrowth from non-embryogenic callus on MS+BAP (4 mg/l).
b. Proliferating white callus of immature flowers buds on MS+2,4-D (2 mg/l) + Zeatin (0.2 mg/l).
c. Profuse rhizogenesis (common type of organogenesis) from different type of non-embryogenic callus of Rasthali.
d. Indirect rhizogenesis from all the directions of non-embryogenic callus on MS + BAP (2 mg/l) + IAA (1 mg/l).
e. Friable callus with hairy roots on MS + 2,4-D (4 mg/l) + IAA (1 mg/l) + NAA (1 mg/l).
f. non-embryogenic callus showing suppressed shoot like outgrowth on MS + BAP (2 mg/l) + IAA (1 mg/l).

Table 4: Culture media used for induction of embryogenic callus from immature male flower buds of banana cv. Rasthali

Sl. No.	Media Composition	Type of Callus	No. of Response	SE
1	MS + 2,4-D (18.10 µM) + NAA (5.37 µM) + IAA (5.71 µM)	Not responding	150	0.42
		Yellow nodular	130	0.36
		White embryogenic	20	0.162
2	MS + 2,4-D (9.05 µM) + Zeatin (0.91 µM)	Not responding	117	0.283
		Yellow nodular	180	0.217
		White embryogenic	3	0.0819
3	MS + BAP (8.88 µM) + IAA (5.71 µM)	Not responding	120	0.22
		Yellow nodular	180	0.22
		White embryogenic	0	0
4	Gamborgs + 2,4-D (9.05 µM) + Zeatin (0.91 µM)	Not responding	50	0.15
		White, hydric vitrified	250	0.36
		White embryogenic	0	0

Table 5: Types of Non- embryogenic callus formation from MFB explants of Musa clones

Musa Clone	Type of Explant	Type of Callus	No. of Response	SE
Rasthali [AAB]	MFB	Yellow nodulated callus	150	0.320
		White hard callus	25	0.203
		Necrotic explants	25	0.1426
		Friable callus	50	0.245

Neyo-Poovan [AB]	MFB	Yellow nodulated callus	200	0.746
		White hard callus	30	0.245
		Necrotic explants	15	0.98
		Friable callus	35	0.2278

The data is based on the evaluation of 20 male cones of 15 explants from each. Interaction and non-embryogenic callus were significant in two-way ANOVA.

Table 6: Types of calluses formed when Musa clones were cultured onto different types of media like MS and Gamborgs having hormones IAA (5.37 µM), NAA (5.37 µM), 2,4-D (18.10 µM)

Musa Clone	Explant	Type of Culture medium	Type of callus	Remarks
Rasthali	MFB Shoot tip	Gamborgs Medium	Watery callus	Not suitable for embryogenic induction

The data is based on the evaluation of 20 male flower buds of 15 explants from each. Highly significant Statistical data were seen in Not-responding, Yellow nodular and White embryogenic callus of Rasthali in two-way ANOVA.

Callus induction from shoot tips of banana cultivar Rasthali

Thin transverse sections of shoot tips of 5-10 mm in thickness were cultured on different induction media for the callus induction (Plate 8, Fig. a, b, Table 7). Irrespective of the media used 70% of the explants inoculated became necrotic unchanged after 3 months of inoculation. On MA1 medium shoot tips produced yellow nodular callus and only (0.2%) produced embryogenic white callus (Plate 9, Figs. g, h; Table 7). This white callus when subcultured onto media having MS + BAP (2 mg/l) + IAA (0.5 mg/l) produced greenish friable callus with root like structures (Plate 10, Figs. e, f). Shoot tips on MS + 2,4-D (2 mg/l), Zeatin (0.2 mg/l) produced heterogeneous callus with yellow nodular compact callus along with white friable callus (Table 8). On MS with BAP (2 mg/l) and IAA (0.2 mg/l) the shoot tips produced greenish non-embryogenic nodular callus. Bulged explants with callusing were seen on MS media having picloram (2 mg/l) + Zeatin (0.2 mg/l) (Plate 10, Figs. b, h). But these calluses when subculture to MS media having BAP and IAA produced only roots. Gamborgs media induced watery non-embryogenic callus (Table 9). Various media combinations tried were given in Tables 10, 11. Statistical data showed significant in types of calluses and interaction.

Table 7: Embryogenic callus and non-embryogenic callus formation from ST explants of Musa clones

Musa Clone	Type of Explant	Type of Callus	No. of Response	SE
Rasthali [AAB]	ST	Non-Embryogenic callus	99	0.622
		Embryogenic callus	01	0.099
		Somatic Embryos formed	00	0.0
		Germination of somatic Embryo	00	0.0

The data is based on the evaluation of 10 shoot tips of 10 explants cultured on MA1 medium. Callus types were significant in two-way ANOVA.

Table 8: Types of Non- embryogenic callus formation from ST explants of Musa clones

Musa Clone	Type of Explant	Type of Callus	No. of Response	SE
Rasthali [AAB]	ST	Yellow nodulated callus	20	0.333
		White hard callus	10	0.210
		Necrotic explants	50	0.557
		Friable callus	10	0.258

The data is based on the evaluation of 10 shoot tips of 10 explants cultured on MA1 medium. Non embryogenic callus of Shoot tips was significant in two-way ANOVA.

Table 9: Types of calluses formed when Musa clones were cultured onto different types of media like MS and Gamborgs having hormones IAA (5.37 µM), NAA (5.37 µM), 2,4-D (18.10 µM)

Musa Clone	Explant	Type of Culture medium	Type of callus	Remarks
Rasthali	MFB Shoot tip	Gamborgs Medium	Watery callus	Not suitable for embryogenic induction

Table 10: Culture media used for induction of embryogenic calli from shoot tip of banana cv. Rasthali

Sl. No.	Media Composition	Type of Response	Frequency of Response	SE
1	MS+ 2,4-D (18.10 µM) + NAA (5.37 µM) + IAA (5.71 µM)	Not responding	50	0.258
		Yellow nodular	46	0.221
		White friable embryogenic	4	0.163
2	MS+2,4-D (9.05 µM) + Zeatin (0.91 µM)	Not responding	50	0.33
		Yellow nodular	50	0.36
		White friable embryogenic	Nil	0
3	MS + BAP (8.88 µM) + IAA (1.14 µM)	Not responding	60	0.258
		Greenish yellow nodular callus	40	0.21
		White embryogenic callus	Nil	0
4	MS+ Picloram (8.28 µM) + Zeatin (4.56 µM)	Not responding	70	0.258
		Yellow nodular with bulged explants	30	0.36
		White embryogenic callus	Nil	0
5	MS+ Picloram (8.28 µM) + TDZ (0.90 µM)	Not responding	70	0.36
		Yellow nodular	20	0.21
		Dark brownish callus	10	0.258
		White embryogenic callus	Nil	0
6	Gamborgs + 2,4-D (9.05 µM) + Zeatin (0.91 µM)	Yellow nodular	20	0.21
		Watery callus	80	0.365
		White embryogenic callus	Nil	0

Ten shoot tips with 10 explants from each. Interaction and callus types were highly significant in Two-way ANOVA.

Table 11: Culture media used for subculturing the callus to induce further growth of embryogenic callus from shoot tips of banana cultivar Rasthali

Sl. No.	Media Composition	Type of Callus Subcultured	Response
1	MS+2-ip (0.60 µM) + Kinetin (0.46 µM) + Zeatin (0.23 µM) + NAA (1.07 µM)	White embryogenic	Become necrotic
		Yellow / white nodular	Continue to grow further
2	MS + BAP (2.22 µM) + IAA (11.42 µM)	White embryogenic	Converted to green callus
		Yellow / white nodular	Continue to grow further
3	MS+BAP (8.88 µM) + IAA (2.85 µM)	White embryogenic	Expansion
		Yellow / white nodular	Green friable callus
4	MS+BAP (8.88 µM) + Adenine sulphate (271.45 µM)	Yellow / white nodular callus	Green nodular callus with rooting
5	MS+BAP (8.88 µM)	Yellow / white	Callus continue to

	+ IAA (5.71 µM) + NAA (5.37 µM)	nodular callus	grow with roots
6	MS+ Sucrose 40 gms + BAP (4.44 µM)	Yellow / white nodular callus	Continue to grow
7	MS+BAP (17.76 µM) + Glutamine (547 µM)	Yellow /white nodular callus	Greenish callus with rooting
8	MS + Maltose (110 µM) + Glutamine (680 µM+ ABA (7.57 µM)	Yellow / white nodular callus	Continue to grow further
9	MS + GA3 (2.89 µM)	Yellow / white nodular callus	Continue to grow further
10	MS + Kinetin (4.65 µM) + Glutamine (547 µM)	Yellow / white nodular callus	Heterogenous callus with yellow nodules

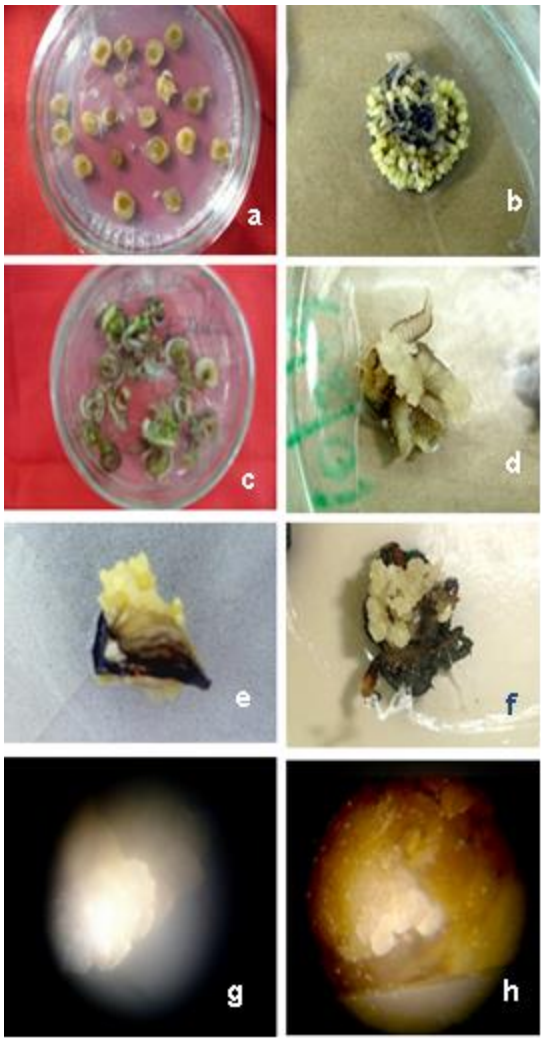

Plate 8: Field detection of morphological soma clonal variants in commercially *in vitro* propagated banana cv. Grand Naine at various forms.

Figure a.True-to-type in vitro propagated banana plant with healthy looking bunch.
b. Normal healthy bunch showing proper hands and fingers and correct orientation of fingers.
c. Dwarf off-type variant among normal banana plants.
d. Extra ordinary dwarf without bunch.
e. III Ratoon crop showing dwarf off-type *in vitro* grown banana plant.
f. Non-flowering extraordinary dwarf off-type in Parvathia's farm.
g. Bunch variant showing small, abnormally looking, with less hands and fingers inside dwarf off-type banana.
h. Bunch variant facing sky with less hands and fingers.

Plate 9: Embryogenic callus induction from shoot tip explants of banana cultivar Rasthali on MAI medium (MS + 2,4-D (4 mg/l) + IAA (1 mg/l) + NAA (1 mg/l).

Figure a. Thin transfer sections of shoot tip explants cultured onto MAI.
b. Greenish yellow nodular non-embryogenic callus formation after 4 months of culture on shoot tip explants on MAI medium.
c. Fragile greenish non-embryogenic callus on MS + 2,4-D (2 mg/l) + Zeatin (0.2 mg/l).
d. White, transparent, friable embryogenic callus on shoot tip explants on MAI medium.
e. Yellow non-embryogenic callus of shoot tip on MAI medium.
f. Necrotic shoot tip explant showing embryogenic callus after 6 months of culture on MAI medium.
g. Peripheral differentiation of initial stages of proembryos on embryogenic callus on MAI medium.
h. Transparent, white, fragile embryogenic callus from shoot tip explants on MAI medium.

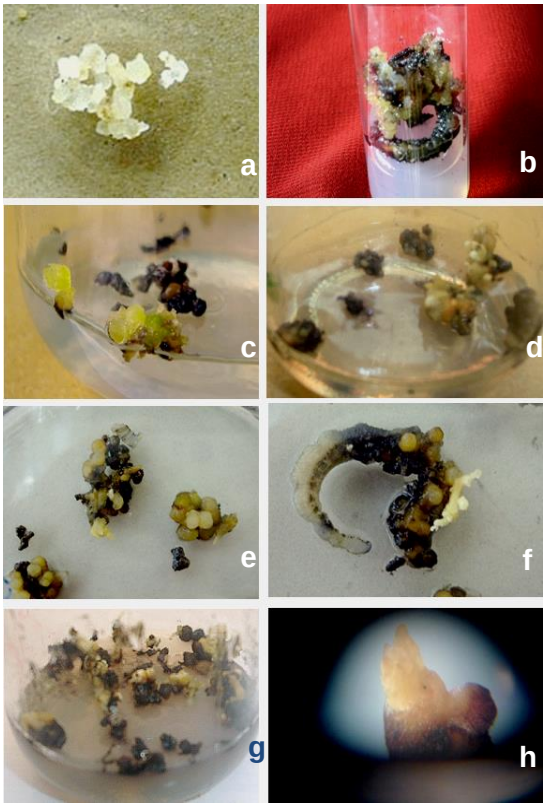

Plate 10: Embryogenic and non-embryogenic callus formation from shoot tip explants of banana cv. Rasthali

Figure a. Embryogenic callus formation on white friable callus of shoot tips on MAI medium.
b. Shoot tip explants showing nodular callus on MS+2,4-D (2 mg/l) + Zeatin (0.2 mg/l).
c. Greenish friable callus from shoot tip explants on MS+BAP (2 mg/l) + IAA (0.2 mg/l).
d. Proliferation of nodular non-embryogenic callus on subculture to same medium *i.e.*, MS + 2,4-D (2 mg/l) + Zeatin (0.2 mg/l).
e. Indirect rhizogenesis from callus of shoot tip explants on MS+2,4-D (4 mg/l) + IAA (1 mg/l) + NAA (1 mg/l).
f. Abnormal indirect rhizogenesis on MS+picloram (2 mg/l) + Zeatin (1 mg/l).
g. Profuse callusing along with necrosis of nodular non-embryogenic callus on MS+Picloram (2 mg/l) + TDZ (0.2 mg/l).
h. Stunted shoot growth on MS+BAP (2 mg/l) + IAA (0.2 mg/l).

IV. DISCUSSION

Micropropagation is an efficient method for rapid propagation of disease free banana and plantains, for the introduction of new cvs. and for the conservation of germplasm. The commercial multiplication of a large number of bananas cvs. represents one of the major success stories of utilizing tissue culture technology profitably. Micropropagation has now become a multibillion-dollar industry practiced all over the world. Of the various method used to micropropagate plants, somatic embryogenesis has become the principal method of multiplication [9]. Somatic embryogenic cultures provide suitable and convenient target tissues for genetic transformation. Therefore, in this study *in-vitro* somatic embryogenesis was initiated to obtain disease resistance banana plant against panama wilt which is in progress in the laboratory. Confirmation of bipolar nature of somatic embryos were done with histological and histochemical studies in the present work. Long term benefit of meristem micropropagation however lie in the production of clonally uniform plants. Concept of morphological and genetical uniformity among micropropagated plants was proven wrong by several convincing reports of soma clonal variation at the morphological and molecular levels. The economic consequences of soma clonal variation can be enormous in fruit crops like banana. Hence field evaluation and isolation of molecular markers for

soma clonal variants were undertaken to assess the magnitude of the problem.

Callus Induction and Plant Regeneration from Male Flower Buds of Banana cv. Rasthali (AAB)

Developing culture systems with reliable regeneration efficiency from important varieties of banana is a prerequisite for realizing the potential of cellular and molecular tools of crop improvement [10]. Towards this goal studies were made to develop protocols for somatic embryogenesis and plant regeneration from two different banana cvs. (Rasthali AAB), which has a unique taste and thin peel, Neypoovan (AB) which has a sweet taste. These two cvs. are highly susceptible to fusarium wilt and Rasthali is in the verge of extinction because of the fungal epidemic. Hence developing *in vitro* protocols may help to obtain disease resistant clones. In the present study, young immature male flower buds were used to induce embryogenic callus of Rasthali similar to the one reported by Ganapathi et al. [11], Escalant et al. [12], Grapin et al. [13] demonstrated the good embryogenic potential of floral tissues and Khalil et al. (2002) [14] used male flower buds to initiate secondary embryogenesis in banana cv. Bluggoe. Immature inflorescences have been used as explants for other monocots like *Cocos nucifera* [15], *Oryza sativa* [16]. Somatic embryos of banana have been grown on a range of media from relatively dilute white medium [17], Schenk and Hilderbrandt [18] and Murashige and Skoog [7]. Ganapathi et al. [19] reported that the embryogenic callus of banana cv. Rasthali in MS medium (66.5%) was better than white's medium (41.6%). Embryogenic callus derived from shoot tip explants of banana cultivar Rasthali was 42% compared to SH medium (39.5%) [19]. In our study along with MS medium, Gamborgs medium were tried to induce somatic embryogenesis [20]. MS medium induced the growth of the embryogenic callus whereas Gamborgs medium induced non-embryogenic watery callus from the male flower buds of banana cv. Rasthali. Watery callus on Gamborgs medium may be because of high concentrated formulation than MS medium. According to Reinert et al. 1977, the key element of MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate required for embryo initiation and maturation [21].

Navarro et al. explained the importance of auxins in the embryogenesis of banana cultivar Grand Naine. [22] He concluded that exogenous supply of auxins during indirect somatic embryogenesis is very important for both de and re differentiation. 2,4-D is the most commonly used effective auxin being used in 57.1% of successful embryogenic cultures [23]. Meenakshi Sidha et al. used different auxins to induce embryogenesis in 5 different banana cvs. and of all the auxins, 2,4,5-trichloropropionic acid 2,4,5-trichlorophenoxy acetic acid and 2,4,5-trichlorophenoxy acetic acid gave best result towards initiation of embryos [24]. In our study MAI, having three auxins *i.e.*, 2,4-D, NAA, IAA has shown good response towards embryo formation than MS having 2,4-D and zeatin. Similar induction media has been reported by number of authors in banana embryogenesis [12, 25]. INIBAP technical guidelines has given MAI media to be the suitable one for induction of embryogenic callus in banana cvs. [25]. Nataliji barbalis et al. observed that prolonged exposure to auxins inhibits continued development of globular embryos in Brassica [26].

During the induction phase, 50% of the explants formed yellow nodulated callus and the few of the male flower primordia became necrotic and only 6.6% of explants showed embryogenic callus in our study. Michelle Hollou et al, has also noted 60% of calluses were non embryogenic and 40% calluses showed embryogenic characteristics [27]. According to Schoofs et al. embryogenic callus formation depends on the type of explant, culture conditions and more importantly the genotype [28]. Till now they were unsuccessful to obtain embryogenic callus from Calcutta-4 and ingarama banana cvs. Ganapathi et al. 1999 observed that Rasthali was found to be highly responsive than 4 cvs. (*i.e.,* Shreemanti, Basrai, Lokhandi, Trikoni) [11]. Even in our report Rasthali was best compare to Neypoovan in terms of embryogenic callus formation and produced embryogenic callus earlier than the Neypoovan.

The embryogenic callus formed after 4-7 months of induction was white, translucent and lobed and delicate embryos could be seen on their periphery. This type of indirect embryogenesis has been reported by authors in banana cvs. [11, 12, 27]. In this study similar to Strosse et al. different types of embryogenic response had been noticed [28]. Formation of compact embryogenic complexes were more compared to individual embryos and ideal callus

having large surface area full of embryogenic determined cells. The embryos passed through different developmental stages and some of the embryos were having unusual shapes. This abnormality has also been reported by Dhed'A. et al. in banana cultivar bluggoe [29]. Asynchronous development could be seen as some of the embryos completely matured and some were still in globular form in the induction media similar to other reports of embryogenesis [24,30].

In the present study we have observed the formation secondary somatic embryogenesis on the primary embryos if the primary embryos were left in the induction media little longer (7-8 months). Transparent, fragile, secondary buds emerge either on cotyledonary region or on top of globular primary somatic embryos. Dhed'A et al. reported secondary embryogenesis from the suspension cultures of scalp derived embryos in banana cv. Bluggoe [29]. Escalant et al. reported that in temporary immersion system, primary globular embryos multiplied by adventitious budding directly through epidermis and resulted in doubling of embryogenic potential and germination, in 5 banana cvs. [12].

In the present investigation, embryo induction, maturation and germination of somatic embryos had been done on solid culture media similar to Ganapathi et al. in Rasthali. But in this study, maturation media having (MA3) kinetin, zeatin, 2-ip was used [11]. Ganapathi et al. have used proliferating media having BAP and IAA [11]. Germination media (MA4) having BAP and IAA was used in this experiment to germinate mature embryos. Ganapathi et al. have used ½ MS without hormones for germination purpose. Ontogeny of primary and secondary embryos along with histochemical works have been done for the first time by present studies, to show the exact developmental behaviour of embryogenic callus. Present histological studies also showed abnormalities in somatic embryos and possible reason for low germination could be answered and redefined for better germination rate of somatic embryos of banana cv. Rasthali.

It was observed in the present study that the embryos formed on induction medium would never germinate if they were not treated with cytokinins hormone. According to Dhed'A et al. it is comparatively easier to obtain somatic embryos but their development into plumule, which is the main morphogenetic event is an important and complex step [29]. Cytokinins are important in fostering somatic embryo maturation [31] and especially in cotyledon development. Combinations of zeatin, kinetin and 2-ip has been used in this study to obtain cylindrical to cone shaped matured embryos having shoot apical meristems root meristems along with cotyledon and provasculature. Dhed'A. et al. reported that zeatin was better compared to BAP for maturation of somatic embryos in banana cv. Bluggoe [29]. Similarly, Novak et al. also reported that zeatin containing medium was an essential step in banana embryogenesis [32].

The percentage of germination observed was 6.6% in the present study. Many authors have doubled the frequency of germination through suspension cultures of embryogenic callus of banana like Dhed'A et al. reported 12% germination in banana cv. bluggoe [29], Cote et al. reported 3-20% germination in banana cv. Grand Naine. [33]. Grapin et al. observed 10-40% germination in banana cv. Sombre plantain [13]. Although the plant conversion rates were low in present study, further refinement is going in the laboratory to enhance embryo to plant conversion frequency through suspension cultures and to produce disease resistant plant using genetic engineering techniques.

Callus induction from shoot tip explants of banana cvs. Rasthali (AAB)

This study showed that shoot tip explants induced less embryogenic response compared to male flower buds. Strosse et al. discussed the competence of various explants and stated the low embryogenic capacity of rhizome and shoot tip explants [28].

Ganapathi et al. used suspension culture to produce somatic embryos from embryogenic callus of *in vitro* shoot tip explants of banana cv. Rasthali [19]. From suspensions they could obtain only 200 regenerants per 0.5 ml packed cell volume making the system less efficient according to Strosse et al. [25]. In the present study shoot tips of Rasthali of soil grown plants showed very less embryogenic response compared to *in vitro* explants as reported by Novak et al. who observed less embryogenic capacity of soil explants compare to *in vitro* [32]. This study has shown majority of cultured explants forming nodular on non-embryogenic protuberances or became necrotic

similar to the observation made by Novak et al. who used rhizome explants to induce somatic embryogenesis in triploid and diploid banana cvs. [32]. According to Strosse et al. the protocol given by Novak group could not be repeated elsewhere. In this study only the shoot tips of Rasthali cultivar produced white embryogenic callus on MA1. [28]. When embryogenic callus of shoot tip of Rasthali subcultured onto the solid media it formed roots only similar to observations made by Lee et al. in Grand Naine, Fillipi et al. 1997, Naincao cvs. [34, 35]. Above authors have used rhizome explants to induce embryogenic callus and obtained somatic embryos which did not have apical meristem but formed roots on germination media.

Venkatachalam et al. reported shoots cultured in liquid medium produce cormlet from leaf explants of Rasthali. They reported that plants grown on Agar medium never formed any cormlet even after transferring to liquid medium whereas shoots developed on liquid medium produced cormlet. According to them physical forms of medium imparts specific effect on the multiplication of shoots. Similar observation could be justifiable in our study as compared to embryogenic callus obtained by Ganapathi et al. in-liquid medium [19]. We were able to obtain embryogenic callus but the germination was very poor because of influence of physical form of the medium. Even Novak et al. have reported embryogenic callus of rhizome explants developed only roots on solid medium but on liquid medium showed 1.5% of normal germination [32].

Srinivas et al. have also used liquid medium along with ABA treatment to enhance germination efficiency of shoot tip derived somatic embryos of banana cv. Rasthali [36]. Type of explant and its genotype along with the physical form of the medium whether solid or liquid medium influences the production of embryogenic callus, maturation and germination of somatic embryos according to Lee et al., Strosse et al., Venkatachalam et al. [28, 34, 37]. According to Lee et al, development of efficient plant regeneration from somatic embryos of bananas can be achieved either by manipulating media or by selecting different explants other than soil grown rhizome or shoot tip [34].

CONCLUSION

Banana cvs. Rasthali (AAB) are seriously under the threat of extinction due to its susceptibility to fusarium wilt. A regeneration protocol using somatic embryogenesis was developed in this study, using immature flower buds. This reliable regeneration method is a prerequisite for molecular manipulation to obtain disease resistance bananas. Immature flower buds cultured *in-vitro* resulted in embryogenic callus formation in this study in banana cvs. Rasthali (AAB). Secondary somatic embryo formation was commonly observed on the primary embryos of banana cv. Rasthali. In summary, banana cv. Rasthali the cvs. exhibited differential response to embryo development depending on their genotype. The study has shown immature flower buds were better choice of explants to induce embryogenic callus than shoot tip explants in banana cv. Rasthali. Nodular yellowish green callus was the most common non-embryogenic callus observed in this study.

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