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Studies on Histological Changes to Detect the Developmental Stages of Somatic Embryos of Banana cv. Neypoovan

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Abstract: The present study was designed to evaluate to evaluate the histological changes during the development of somatic embryos from embryogenic callus of male flower buds of banana cv. Neypoovan. The callus of male flower buds was taken at different stages of growth intervals to analyze the parameters for induction of embryogenesis. Embryogenic callus of banana cv. Neypoovan at successive stages of development were used for the present study. Histological sections were used for comparison of the histological changes occurring during the development of somatic embryos from embryogenic callus of male flower buds of banana cv. Neypoovan. Histological observation of embryogenic callus of Neypoovan, revealed the absence of apical meristem which may be the cause for failure to grow, even after subculturing. Formation of direct and indirect rhizogenesis was observed from nodular non-embryogenic callus of banana cv. Neypoovan

Keywords **- Banana cv. Neypoovan, Histological changes, Somatic embryos, Embryogenic callus**

I. INTRODUCTION

Polyploidy and sterility, characteristics of commercial cultivars of banana (*Musa spp*.,) are disadvantageous for the inclusion of banana cultivars in breeding programs.[1] Somatic embryogenesis associated with genetic manipulation techniques is of great interest for the production of new cultivars through plant transformation. Banana plants are threatened by many diseases and pests and because of the difficulties for conventional breeding there are high expectations on the use of biotechnology as a tool for breeding programs. However, an efficient somatic embryogenic protocol must be developed, with a high rate of embryo conversion.

Somatic embryogenesis is an in-vitro morphogenic response in which embryos are induced from somatic cells, with further regeneration of plants.[2] In general, the process follows three main stages: induction of embryogenic callus, development and maturation of somatic embryos, and conversion of somatic embryos into plants. The induction stage is considered of great importance for obtaining well-formed somatic embryos, contributing to the subsequent stages of development, i.e., maturation and conversion into plants. The choice of auxin is very important in the induction process and can affect the frequency and morphology of somatic embryos.[3]

Somatic embryogenesis of banana cultivars of different groups has been successfully achieved,[1,4,5] however, the conversion into plants is frequently low, thus limiting its association with genetic transformation techniques. The characterization of the different stages of the embryogenic process can help to detect possible limiting steps as well as to locate the embryogenic regions in the explant. This can assist in the definition of strategies for genetic manipulation of the material.

With these viewpoints, present study was undertaken to evaluate the histological changes during the development of somatic embryos from embryogenic callus of male flower buds of banana cv. Neypoovan.

II. MATERIALS AND METHODS

Materials

The callus of male flower buds and suckers were taken at different stages of growth intervals to analyze the parameters for induction of embryogenesis. Embryogenic and non-embryogenic callus of banana cv. Neypoovan at successive stages of development were used for the present study.

Histological Analysis

Histological analysis: Fixation and killing of the callus was done in FAA (formalin, acetic acid and ethyl alcohol in the proportion of 90:5:5 by volume) for a period of 24 to 48 hours. The fixed material was washed with 70% alcohol and dehydrated using different grades of alcohol such as 70%, 80%, 90% and absolute alcohol for a period of 24 hours in each treatment. They were further dehydrated using ethyl alcohol and n-butanol in the ratio of 3:1, 1;1, 1:3. Paraffin wax of 58-60oC melting point was opted for infiltration and further embedding samples. Thin sections of 10-15 µm thickness were taken with the help of a rotatory microtome. Deparaffinisation is a prerequisite for staining any slide. The slides were deparaffinised using xylene. For complete removal of xylene, the slides were passed through alcohol series like 100, 90, 70, 50% or passed through n-butanol. Such slides were subjected to histochemical staining either directly or after dehydration depending upon the need.

III. RESULTS

Histological sections were stained with the Haematoxylin, a nuclear stain and Orange-G or erythrosin a cytoplasmic stain. As the percentage of embryogenic calli was very less, embryogenic calli produced was scanty and took more time to form and to mature. After 3 months on induction medium male flower buds formed yellow nodular callus which produced embryogenic callus after 8 months. Neypoovan embryogenic callus was compact, had densely packed cells and nodular (Plate 1, Table 1). The peripheral cells showed meristematic activity and resulted in globular, heart shaped embryos (Plate 1, Fig. a-e). These embryos when subcultured onto MA3 media, gave rise to a plantlet without undergoing maturation, thus forming a plantlet with root pole and plumule which were distinguished by procambial strands (Plate 1, Fig. f, i, h). Histological section revealed that functional shoot apex had not developed even after 3 months on germination media i.e., MA4.

PLATE - 1

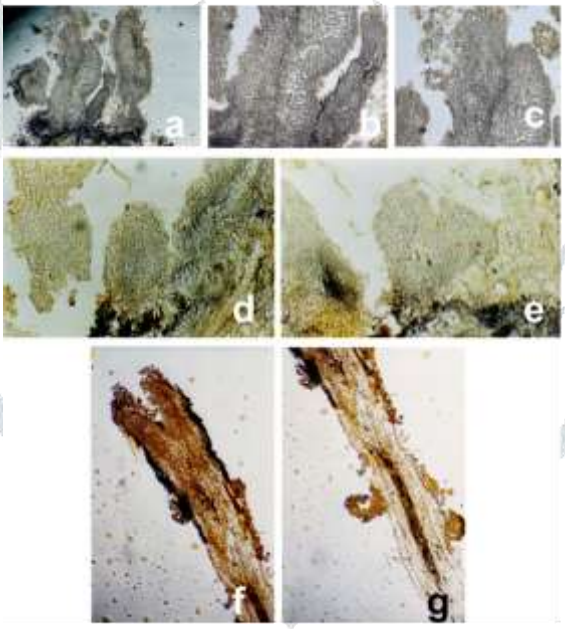

PLATE 1

Fig. a-h Sections of somatic embryos from embryogenic callus of immature flower buds banana cv. Neypoovan, stained with Haematoxylin and counterstained with orange G.

a. Peripherally formed somatic embryos showing different stages of growth.
b. Somatic embryo showing active division and expanding with provasculature in the centre.
c. Closer observation of the somatic embryo showing small meristematic cells.
d. Globular shaped somatic embryo on embryogenic callus.
e. Heart shaped embryo on embryogenic callus of banana cv. Neypoovan.
a. Germinated abnormal plantlet without proper shoot apical meristem.
g. Abnormally germinated plantlet showing well developed root with root hairs and vasculature.

Table 1: Embryogenic callus and non- embryogenic callus formation from MFB explants of Neypoovan clones

Musa Clone	Type of Explant	Type of Callus	No. of Response	Std Error
Ney Poovan [AB]	MFB	Non Embryogenic callus	297	1.04
		Embryogenic callus	03	0.09
		Somatic Embryos formed	02	0.07
		Germination of somatic Embryo	00	0.00

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

In Neypoovan cultivar, majority of the male flower buds cultured onto the different induction media resulted in formation of one common non-embryogenic callus i.e., yellow, compact (50%) nodular callus (Plate 3, Fig. e & f and Table 2). Irrespective of the type of the medium i.e., MS + 2,4-D (2 mg/l) + Zeatin (0.2 mg/l), MA1, MS + Picloram (2 mg/l) + Zeatin (0.8 mg/l) and on other combinations also, the majority of callus formed were yellowish green nodular callus. When this yellow non-embryogenic callus was analysed histologically, it showed formation of active meristematic centres originating at different zones of parenchymatous callus (Plate 4; Fig. c, d). In some of the yellow compact callus the meristematic zones were concentrically formed from the centre as small active cambial zones which progressively increased as the callus expanded and burst open the callus (Plate 4). These meristematic zones have smaller cells with dense cytoplasm, a prominent nucleus and circular in the beginning but as it matured it acquired different shapes like oval, spherical and elongated zones. The meristematic zones show middle procambial zones which act like primordia for organogenesis of root or shoot (Plate 2, Fig. d-i). These meristematic zones were different from embryogenic zones by being irregular in size, deeply buried in the callus, not having epidermis and usually forming roots when subcultured onto different media having BAP and other cytokinins (Plate 2, Fig. f). Sometimes these cambial zones fuse to form bigger meristematic zones (Plate 2, Fig. i).

Table 2: Types of Non- embryogenic callus formation from MFB explants of Neypoovan clones

Musa Clone	Type of Explant	Type of Callus	No. of Response	Std Error
Neyo-Poovan [AB]	MFB	Yellow nodulated callus	200	0.75
		White hard callus	30	0.24
		Necrotic explants	15	0.98
		Friable callus	35	0.23

PLATE - 2

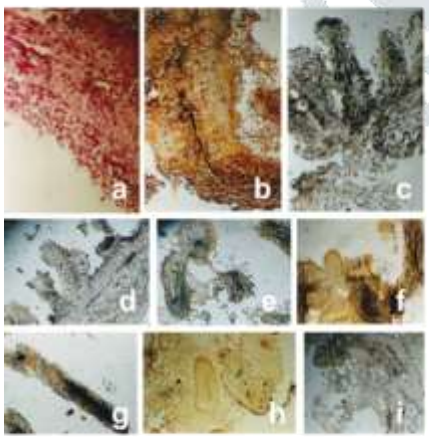

PLATE - 3

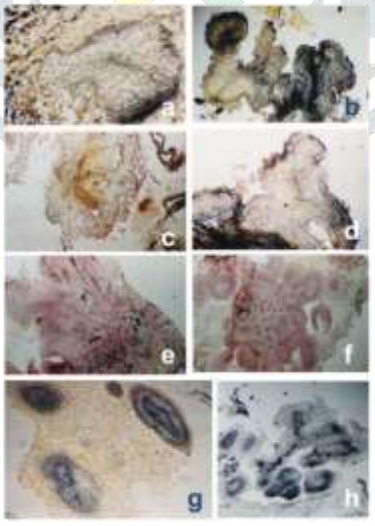

PLATE - 4

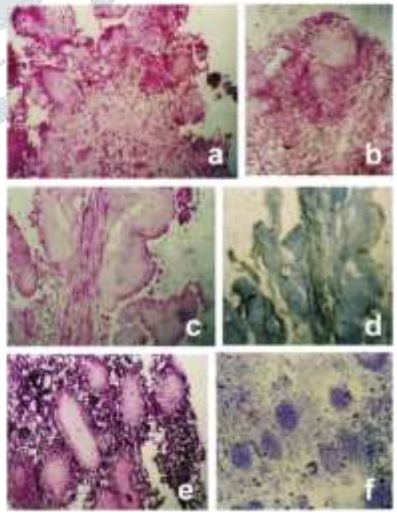

PLATE 2

Fig. a-i Sections showing organogenesis from non-embryogenic callus of immature flower buds and shoot tip explant of banana cv. Neypoovan.

a. Protocorm longitudinal section showing well developed shoot region with vasculature.
b. Protocorm shoot tissues showing middle vasculature and outer parenchymatous cortical region.
c. Profuse indirect rhizogenesis from non-embryogenic callus of shoot tip explants.
d. Non-embryogenic callus showing indirect rhizogenesis at different stages of growth from MFB explants.
e. Random indirect rhizogenesis from callus of MFB of Neypoovan.
f. Stunted shoot primordia with rhizogenesis from non-embryogenic callus of MFB.
g. Root detached from non-embryogenic callus of MFB.
h. Yellow callus showing central root formation at different places of callus from MFB.

i. Rhizogenesis showing 2 root formations at time from non-embryogenic callus of banana Neypoovan.

PLATE 3

Fig. a-h Sections showing non-embryogenic callus and organogenesis from banana cv. Neypoovan.

e. Meristematic centres originated from centre bursting the callus cells to expand nodulated callus of male flowers buds of Neypoovan.

f. Dispersal of meristemoids originated in the centre from vasculature cells, of a non-embryogenic callus spread over the callus in a sequential way, in MFB of Neypoovan.

h. Nodular non-embryogenic callus of Neypoovan showing irregular distribution of meristemoids of MFB.

PLATE 4

Fig. a-f Sections showing non-embryogenic callus stained with PAS, MBB, TB to localize macromolecules in banana cv. Neypoovan.

c. Nodulated non-embryogenic callus of MFB of Neypoovan showing irregular intense localization of total insoluble polysaccharides.

d. Nodulated non-embryogenic callus of MFB of Neypoovan showing irregular localization of insoluble proteins.

e. Undifferentiated nodular callus of MFB of Neypoovan showing non-uniformal localization of insoluble poly-saccharides.

Formation of direct rhizogenesis was observed when these calluses were subcultured onto MS + BAP (0.5 mg/l) + IAA (2 mg/l) (Plate 2, Fig. i). Indirect rhizogenesis was also observed when these yellow compact nodular calluses were subcultured onto ½ MS + glutamine (80 mg/l) (Plate 2). Irrespective of the type media majority of yellow compact nodular callus formed roots only (Plate 3, Table 3). Occasionally white fragile callus obtained from the male flower buds happened to be non-embryogenic when analyzed histologically. They possessed irregularly distributed meristematic zone like yellow compact callus. In one of the plates protocorm like structures were observed in the plate having MS + 2,4-D (2 mg/l) + Zeatin (0.2 mg/l). On subculture to media having BAP, they grew further forming shoot tips (Plate 2, Fig. a & b) but failed to grow further. Indirect organogenesis of shoot and root together was seen when yellow compact callus was subcultured repeatedly on MS with BAP (Plate 2, Fig. f).

Table 3: Culture media used to subculture callus obtained on induction medium to induce further growth of the embryogenic callus from male flower buds of banana cv. Neypoovan

S. No.	Media Composition	Type of callus sub-cultured	Response
01.	M.S.+2-ip (0.60 µM) + Kin (0.46 µM) + Zeatin (0.23 µM) + NAA (1.07 µM) (MA3)	White embryogenic	Expansion of the embryos
		Yellow nodular	Further growth of yellow nodular becoming necrotic later
02.	M.S.+BAP (2.22 µM) + IAA (11.42 µM) (MA4)	Matured embryos	Formed plantlet without plumule
		White embryogenic	Formation of roots
		Yellow nodular	Further growth of nodular with roots.
03.	M.S. + BAP (8.88 µM) + IAA (2.85 µM)	White embryogenic	Converted into rooting green callus
		Yellow nodular	Further growth of yellow nodular with formation of roots in same
04.	M.S.+BAP (8.88 µM) + Adenine sulphate (271.45 µM)	Yellow nodular	Rooting
		White nodular	Converted to yellow Nodular
05.	M.S.+BAP (8.88 µM) + IAA (5.71 µM) + NAA (5.37 µM)	Yellow nodular	Rooting and further becoming of yellow nodular
		White compact	Formed hard nodules
06.	M.S.+Sucrose 40 gms + BAP (4.44 µM)	Yellow nodular	Further growth of callus
		White compact	Becomes yellow nodular
07.	M.S.+BAP (17.76 µM) + Glutamine (547 µM)	Yellow nodular	Continue to grow
		White compact	Converting into yellow Nodular
08.	M.S.+Maltose (110 µM) + Glutamine (680 µM+ ABA (7.57 µM)	Yellow nodular	Continue to grow
		White compact	Remains same
09.	M.S.+GA3 (5.6 µM)	Yellow nodular	Continue to grow
		White compact	Forms heterogenous callus
10.	M.S+Kinetin (4.65 µM+ Glutamine (547 µM)	Yellow nodular	Expansion of nodular callus
		White compact	Heterogenous callus

IV. DISCUSSION

Histological investigations of embryogenic callus of Neypoovan showed peripheral meristematic zone which was smaller, denser with darkly stained nucleus similar to the reports of Navarro *et al* in banana cultivar *Musa acuminata*.[6] These meristematic zones formed globular and heart shaped somatic embryos having definite epidermis and provasculature. Some of them on germination media formed embryo with proper root pole, procambial strand and poorly developed shoot meristem. Lee *et al* also observed similar vacuolated shoot meristem in rhizome derived somatic embryos of Grand Naine.[7] Jarret *et al* also noticed lack of meristem in somatic embryos of Musa cultivar.[8] The rhizoids (in L.S.) which originated from somatic embryos had lots of root hairs,

middle parenchymatous cortex and outer thin layer of epidermis with continuous provasculature in the centre, similar to the adventitious roots reported by Eva Cellarova *et al* in ginseng.[9]

Histological observation revealed the similarity of non-morphogenetic callus of banana cv. Neypoovan (AB). The formation of yellow nodular compact callus was similar which started to appear from 6th week onwards on the induction media. Generally, all non-morphogenetic callus has elongated cells which are not organized compare to the embryogenic callus where cells are smaller and organized according to Rodriguez *et al.*[10] Nodular callus originated usually from multiplication of perivascular cells in flower buds and in the shoot tips which forms rings of meristematic zones which continued their division led to the growth of callus. Each nodule consists of compact small meristematic zones surrounded by parenchymatous region of the callus, in accordance with the reports of Wan *et al* in *Medicago sativa*,[11] Samaj *et al* in *Papver somniferum.*[12]

Some of the older meristematic nodules formed protracheal elements in the centre and growth of these nodular callus reached towards periphery of the callus bursting open the parenchymatous callus tissue. The exposed meristematic zone gave rise to roots as seen in Oil Palm.[13] Such organogenesis from nodular structures were reported by number of authors. Seibioka *et al* observed in barley, nodulated callus forming organogenesis rather than embryogenesis.[14] In *Nicotiana tobacum*, formation of shoots through nodular callus of hypocotyl explant were reported by Attfield *et al*.[15]

In this study sometimes continuous formation of nodular zones in the parenchymatous callus have been observed which is similar to the report by Mikula *et al*., Fortes *et al*. in Humulus organogenesis.[16] Many of these nodular calluses during subculture on to various media gave rise to roots as seen in many other plants like in oil palm,[13] and in gingseng.[9] Sometimes direct rhizogenesis occurred where the roots originated from the vascular zone of the callus and many a time roots arose independently away from vasculature of the callus. These type roots formation has also been reported in *Brasica napus* by Halina Slesak *et al.*[17] Similar shoot primordia with roots have also been observed in oil palm.[13] Similar organogenesis from non-embryogenic nodulated callus has been reported by some other researchers in Kiwi fruit, and in *Ipomea batatas*.[18]

V. CONCLUSION

The histological studies on somatic embryo of Neypoovan showed the absence of apical meristem which may be the cause for failure to grow, even after subculturing. Formation of direct and indirect rhizogenesis was observed from nodular non-embryogenic callus banana cv. Neypoovan.

REFERENCES

1. Novak FJ, Afza R, Van Duren M, Perea-Dallos M, Conger BV, Xiaolang T. Somatic embryogenesis and plant regeneration in suspension cultures of dessert (AA and AAA) and cooking (ABB) bananas (*Musa spp*.). Bio/Technology. 1989;7(2):154-9.
2. Williams EG, Maheswaran G. Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Annals of Botany. 1986;57(4):443-62.
3. Rodriguez AP, Wetzstein HY. A morphological and histological comparison of the initiation and development of pecan (*Carya illinoinensis*) somatic embryogenic cultures induced with naphthaleneacetic acid or 2, 4-dichlorophenoxyacetic acid. Protoplasma. 1998;204(1-2):71-83.
4. Cote FX, Domergue R, Monmarson S, Schwendiman J, Teisson C, Escalant JV. Embryogenic cell suspensions from the male flower of Musa AAA cv. Grand nain. Physiologia Plantarum. 1996;97(2):285-90.
5. Grapin A, Schwendiman J, Teisson C. Somatic embryogenesis in plantain banana. In Vitro-Plant. 1996;32(2):66-71.
6. Navarro C, Escobedo RM, Mayo A. In vitro plant regeneration from embryogenic cultures of a diploid and a triploid, Cavendish banana. Plant Cell, Tissue and Organ Culture. 1997;51(1):17-25.
7. Lee KS, Zapata-Arias FJ, Brunner H, Afza R. Histology of somatic embryo initiation and organogenesis from rhizome explants of *Musa spp*. Plant Cell, Tissue and Organ Culture. 1997;51(1):1-8.
8. Jarret RL, Fisher JB, Litz RE. Organ formation in Musa tissue cultures. Journal of plant physiology. 1985 Nov 1;121(2):123-30.
9. Cellarova E, Rychlová M, Vranova E. Histological characterization of in vitro regenerated structures of *Panax ginseng*. Plant cell, tissue and organ culture. 1992;30(3):165-70.
10. Rodríguez S, Mondéjar C, Ramos ME, Díaz E, Maribona R, Ancheta O. Sugarcane somatic embryogenesis: a scanning electron microscopy study. Tissue and cell. 1996;28(2):149-54.
11. Wan YS, Sorensen EL, Liang GH. The effects of kinetin on callus characters in alfalfa (*Medicago sativa* L.). Euphytica. 1988;39(3):249-54.
12. Samaj J, Bobák M, Erdelský K. Histological-anatomical studies of the structure of the organogenic callus in *Papaver somniferum* L. Biologia plantarum. 1990;32(1):14-8.
13. Schwendiman J, Pannetier C, Michaux-Ferrière N. Histology of somatic embryogenesis from leaf explants of the oil palm Elaeis guineensis. Annals of Botany. 1988;62(1):43-52.
14. Oka S, Saito N, Kawaguchi H. Histological observations on initiation and morphogenesis in immature and mature embryo derived callus of barley (*Hordeum vulgare* L.). Annals of Botany. 1995;76(5):487-92.
15. Attfield EM, Evans PK. Developmental pattern of root and shoot organogenesis in cultured leaf explants of Nicotiana tabacum cv. Xanthi nc. Journal of experimental botany. 1991;42(1):51-7.
16. Mikula A, Tykarska TE, Zielinska M, Kuras MI, Rybczynski JJ. Ultrastructural changes in zygotic embryos of *Gentiana punctata* L. during callus formation and somatic embryogenesis. Acta Biológica Cracoviensia Series Botánica. 2004; 46:109-20.
17. Ślesak H, Popielarska-Konieczna M, Góralski G. Morphological and histological aspects of 2, 4-D effects on rape explants (*Brassica napus* L. cv. Kana) cultured in vitro. Acta Biologica Cracoviensia. Series Botanica. 2005;47(1).
18. Mukherjee A, Debata BK, Mukherjee PS, Malik SK. Morphohistobiochemical characteristics of embryogenic and nonembryogenic callus cultures of sweet potato (*Ipomoea batatas* L.). Cytobios. 2001;106(412):113-24.