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DEVELOPMENT OF ABNORMAL SOMATIC EMBRYOS IN BANANA CV. NANJANGUD RASBALE (SILK GP. RASTHALI, AAB)

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ABSTRACT

The traditional table choice elite variety of Karnataka, NanjanagudRasabale is in the verge of extinction due to the serious outbreak of panama wilt disease in the Nanjangudtaluk of Mysore district. Banana cultivar Rasthali (AAB) has been chosen to do *In vitro* studies on somatic embryogenesis. Embryogenic callus and somatic embryos were generated with male flower buds on MS medium supplemented with 2,4-D (18.10 µM), NAA (5.37 µM) and IAA (5.71 µM) with 3% sucrose and 0.2% gel rite. After 6–8 months well developed somatic embryos were sub cultured on to MS supplemented with NAA (1.07 µM), Zeatin (0.23 µM) ,2 ip (0.60 µM) and kinetin (0. 46 µM) for further maturation. Several morphological embryo variants have been observed which brought the germination frequency to the lesser extent. Histological and histochemical studies revealed that all the embryos making up the clump were developed asynchronously and had different patterns of development. Regardless of the stage of maturation, a large number of somatic embryos showed structural abnormalities some of which could be detected by histological observation. Different embryogenic structures were developed without passing through recognizable stages of zygotic embryogenesis. Localization of starch, total insoluble proteins and nucleic acids were observed in the somatic embryos. Evidence presented in this study points towards abnormalities which may cause somaclonal variations in the economically important plant like banana cv. Rasthali.

Keywords: Somatic Embryos, Rasthali, Histology, Abnormal, Male Flower Buds

No of Figures: 4 **No. of References: 21**

INTRODUCTION

Somatic embryogenesis **(Plate-1,1g and 1h)** is a process in which a bipolar structure, resembling a zygotic embryo develops from a somatic cell without vascular connection with the original tissue (Sara Von Arnold *et al*., 2002). Successful regeneration of banana plants from somatic embryogenesis is reported by many workers (Novak *et al*., 1989,Escalant*et al*.,1994). 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*., (1999). In this study similar to Strosse*et al*., (2005) different types of embryogenic response had been noticed and some of the embryo clumps showed fused vasculature, fused cotyledons and other embryos with shoot meristem at irregular places could be seen. According to padmanabhan*et al*., (1998) variety of somatic embryos develops in culture and varies greatly in their developmental patterns and their ability to convert to plantlets. Abnormal embryos with multiple shoot tips, many root initials and two cotyledons were also observed in this study (**Plate-4;4a ,4b and 4f).**

Therefore present histological study of the somatic embryos providesvaluableinsight into their developmental potentialities and help to identify the variants that can form plantlets ineconomically important plant like banana cv. Rasthali.Present studycan be a very useful tool to answer some of the problems associated with germination of somatic embryos, maturity and developmental aspects. It reveals the sequence of events which can lead to development of abnormal somatic embryos.

MATERIALS AND METHODS

The inflorescences of banana cultivar Rasthali (AAB) bearing male flowers was collected from Nanjungud near Mysore and was used as explants within 24 hours of its excision. The immature male flower clusters from position 0-16 were removed and inoculated on MA1 medium of INIBAP (2000) consisting of Murashige and Skoog medium (MS) supplemented with 18.10µM 2,4-dichlorophenoxy acetic acid (2,4-D), 5.71µM indole 3-acetic acid (IAA) , 5.37µM naphthalene acetic acid(NAA) and 4.09 µM d-Biotin, 3% sucrose and 0.2% gelrite for callus induction in dark at a temperature of 25 ± 2^0C and a relative humidity of 80 %. After 8-9 months, cultures producing embryogenic callus with somatic embryos were selected under a microscope and fixed for histological studies. Well developed somatic embryos **(Plate-1,1a,1b and 1c)** were sub cultured on to MS supplemented with NAA (1.07 µM), Zeatin (0.23 µM), 2-ip (0.60 µM) and kinetin (0. 46 µM) for further maturation. Plantlets were obtained when somatic embryos were cultured on MS with Morel vitamin (**Plate-1;1d,1e,1f),** IAA (11.42 µM)), and BAP (2.22 µM).

Histological studies:

Somatic embryos were fixed 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. They were further dehydrated using ethyl alcohol and n-butanol and the material was then embedded in paraffin wax of 58-60^{o}C melting point by cold and hot infiltrations.Each embedded somatic embryo was fixed to the rider of Erma Rotatory Microtome and thin sections of

12µm thickness were taken. The slides were deparaffinised using xylene and rehydrated using ethanol series and stained with per-iodic acid schiffs, toluidine blue, mercuric bromophenol blue and haematoxylin. They were dehydrated subsequently using ethyl alcohol, mounted in DPX and observed under microscope.

Micrometry:

The micrometry observations were recorded using an occulometer and were taken at different stages of development of callus and during photomicrographs.

Photomicrographs:

Photomicrographs of the sections were taken with Olympus microscope using photo micrographic equipment and Kodak positive film of 400 ASA.

RESULTS AND DISCUSSION

Medium sized hands and very few smaller hands formed yellow compact callus after 3 months of inoculation on MS + 2,4-D (18.10 µM) + NAA (5.37 µM) + IAA (5.71 µM) [MA1]. A positive embryogenic white fragile, translucent, hydric callus appeared on the yellow callus of 4-8 months old culture (**Plate-1;1a and 1b)**and is similar to the one reported by Escalant *et al*. (1994), Grapin*et al*., (1996). The embryogenic callus possessed surface lobes where growth seemed to be more active and constituted the friable units **(Plate-2;2a,2c and 2d**). These centres of irregular proliferation became localized and produced swellings which cut off different sized somatic proembryos on the embryogenic callus. These zones subsequently differentiated to globular, oval, circular shaped proembryos. Similar type of indirect embryogenesis has been reported by authors in banana cvs. (Ganapathi*et al*., 1999, MeenakshiSidha*et al*., 2007).

Histological observations indicated that the group of neighbouring cells (**Plate-2;2b and 2f)** participated to produce an embryo and the resulting embryos are largely fixed to the callus, implying probable multiple cell origin. In banana somatic embryo studies, Lee et al. (1997) has reported multicellular origin of somatic embryos. These proembryos appear to be translucent globules on the embryogenic callus and formed in large numbers. As the embryo started expanding, different forms of embryos like heart shaped, cylindrical to different shapes of embryos could be seen **(Plate-2;2e and 2h**). Many embryogenic structures developed without passing through recognizable stages of primary somatic embryogenesis. In the same area of embryogenic callus one could see asynchronous development of proembryo, heart shaped, and oval shaped embryos (**plate-3;3a,3b,3c and 3d**) which is a very common observation made by number of authors in many plants like Sandalwood (RavishankarRai*et al*., 2002), Coffee (Rafel Fernandez *et al*., 2005), Gentiana (Anna Mikula*et al*., 2004).

One of the main malformation observed in this study is the abnormally formed embryo with different shapes and sizes without proper shoot apical meristem (**Plate-3,3d;plate-4;4d and 4e**). Padmanabhan*et al*., (1998) observed similar result in *Ipomoea batatus*.

The other common abnormality observed in the study was the fusion of embryos, fusion of cotyledons, vascular distortion in fused embryos (**Plate-3;3a,3e,Plate-4;4c and 4g**) which further caused multiple

shoots and rooting in a different zone. Similar fused embryos have been reported by number of authors in many plants. Maheshwaran and Williams, 1985 reported fusion of embryos in Trifolium where cotyledon showed fasciations. In the present study it was observed (**Plate-3;3c and 3f**) that two embryoids of the same origin at different stages of development like one already having shoot apical meristem but other fused embryo still at globular stage which was also seen in ginseng cultures (Eva Cellarova*et al*., 1992). Abnormal multiple shoot formation (**Plate-4;4b)**in somatic embryo have been seen in present study similar to *Papaversomniferum* (Oveckа*et al*.,1997).

According to Maheshwaran and Williams (1985), polarity expressed after first division of zygote also exists when a group of cells cooperate to form somatic embryo but this implies the coordination and sequencing of several process. Poor regulation of these sequences causing polarization defect in these units of multicellular origin is the cause for abnormalities along with the problem of maturation. Banana being a monocot, lateral formation of shoot tip is expected but in the present work many embryos showed central shoot meristem similar to dicot embryos. Another reason for abnormal somatic embryos is the close proximity of embryoid initiation sites appeared to produce frequent fusion or fasciations of embryos (Maheshwaran and Williams, 1985).

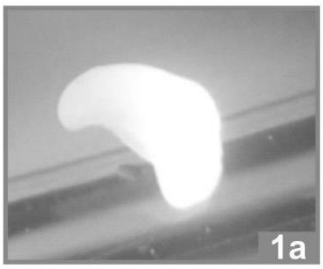
Many authors have linked the abnormality seen in embryos to the exogenous supply of various hormones. Dhed'A*et al*., (1991) reported the abnormality in somatic embryos of banana cultivar bluggoe due to the high cytokinin level in the medium. According to them, high concentration of zeatin and BAP in the maturation medium caused abnormal embryos and 2-3% of abnormal plantlets were formed as compared with 14% of normal plantlets. In the present study, the delayed culture period in auxin rich media (NAA, IAA, 2,4-D) would have caused abnormal embryos with different shapes, fused cotyledons. In these study embryos showing precocious vacuolation in the shoot meristem could be seen as in case of Alfalfa somatic embryos (Fuji *et al*., 1990).According to Arnold (2002) in long term culture periods, embryogenic potential of callus decreases and eventually lost and sometimes forms abnormal embryos.

The studies on histochemical changes during somatic embryogenesis in Rasthali showed the presence of higher amounts of biomolecular substances such as insoluble polysaccharides(**Plate-2;2g**), proteins (**Plate2;2d,2e;Plate3;3c and 3f**) and nucleic acids **(Plate2-2b, Plate3; 3a ; Plate 4-4d**) during different stages of embryo formation. This is necessary for the germination of somatic embryo into a plantlet. The presence of bio moleculesin the unicellular / multicells which forms somatic embryo has been reported in plants like cork oak (El Maataui et al., 1990), Michaux-Ferriere*et al*., (1992) in *Heveabrasiliensis*.

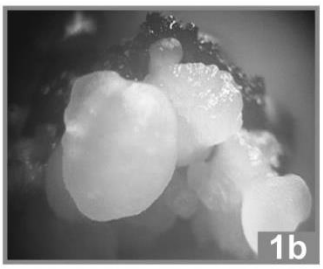
CONCLUSION

Present histological studies 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 also affects the conformity of the clone produced which is a fundamental criterion in vegetative propagation. Our studies points towards abnormalities which may cause somaclonal variation in the economically important plants like banana cv. Rasthali.

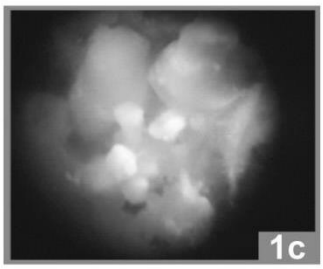
Somatic Embryogenesis
Plate - 1



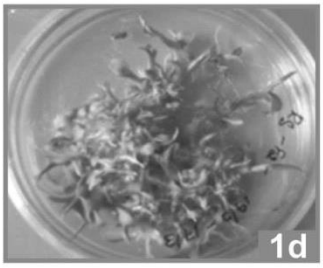
Single embryo with expanded region showing cotyledonary notch in the centre.



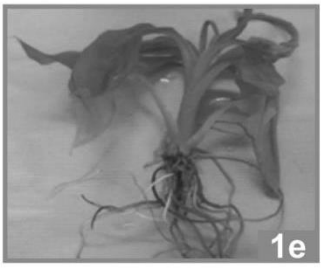
White, compact, matured somatic embryo cluster on embryogenic callus.



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

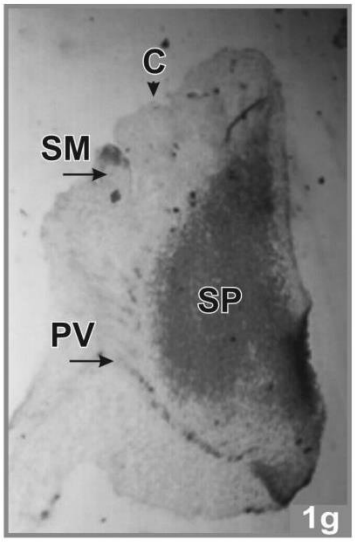


Complete plantlet formation with the vigorous growth of root and shoot on MA4 medium.

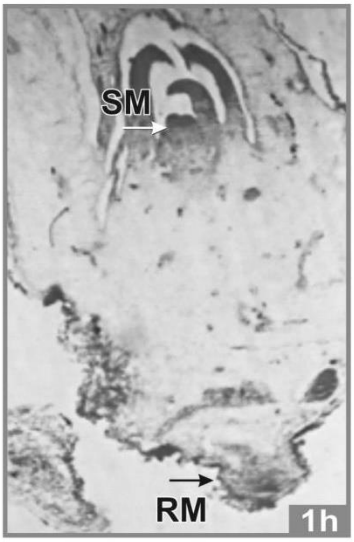


Regeneration of whole plants with elongated roots and shoots in banana cv. Rasthali.

Acclimatization of regenerated plants.

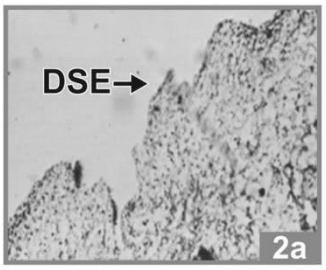


Somatic embryo (stained with mercuric bromophenol blue) SM : Shoot Meristem, C : Cotyledon, P : Primary Vasculature, SP : Storage Protein. X22

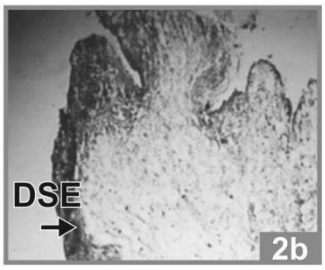


L.S. of germinated somatic embryo (stained with Toluidine blue) SM : Shoot Meristem, RM : Root Meristem. X22

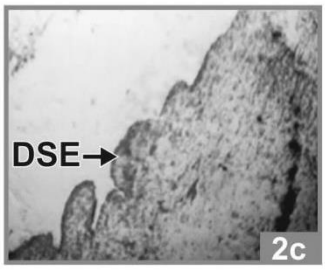
Somatic Embryogenesis
Plate - 2



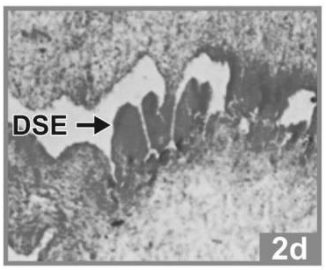
embryogenic callus showing meristemetic cells at epidermal and subepidermal cells.(stained with Toluidine blue) DSE : Developing Somatic embryos. X22



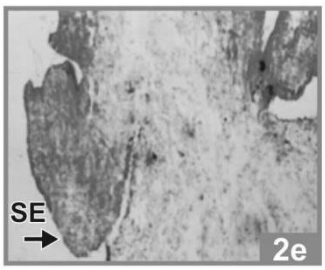
Epidermal and subepidermal layers showing meristematic activity. (stained with Toluidine blue) X46



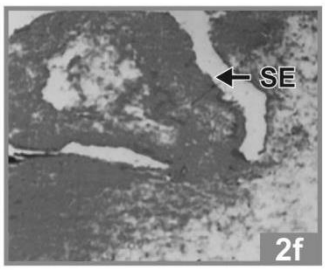
Lobe formation resulting in proembryos. (stained with mercuric bromophenol blue) X22



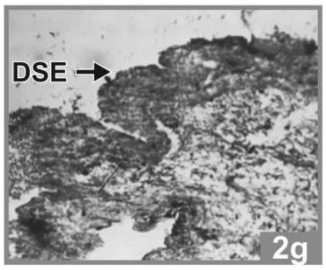
proembryos at the periphery of embryo genic callus. (stained with mercuric bromophenol blue) X22



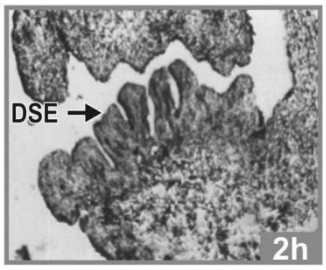
Inner nondividing embryogenic callus and peripheral actively dividing embryonal cells. (stained with mercuric bromophenol blue) X22



Proembryo with broad base indicating multicellular origin of somatic embryos. (stained with mercuric bromophenol blue) X22

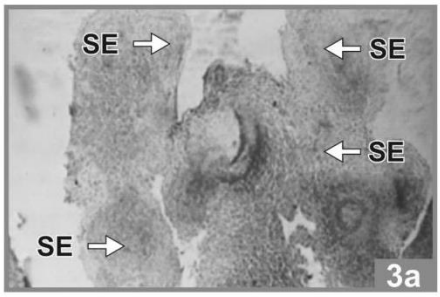


Proliferating proembryos on all the sides of embryogenic callus. (stained with PAS reagent) X22

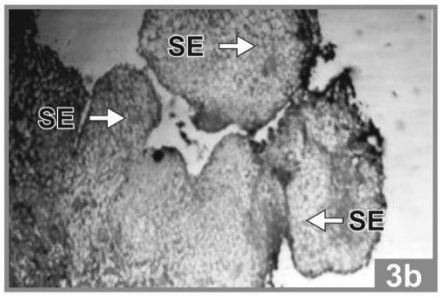


Gradual differentiation of globular proembryos at the periphery of embryogenic callus. (stained with Haematoxylin) X46

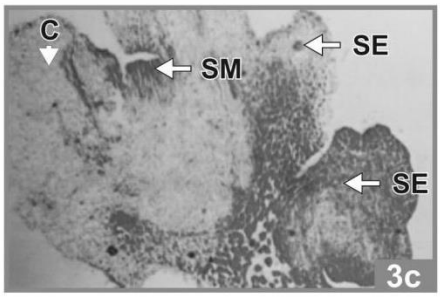
Somatic Embryogenesis
Plate - 3



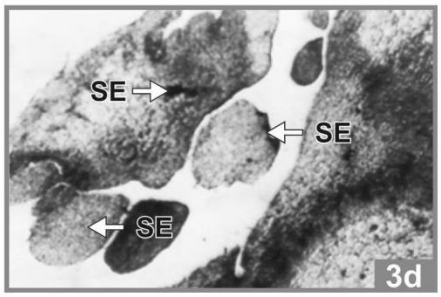
Fused Somatic embryos at different stages of development. (stained with Toluidine blue). X46



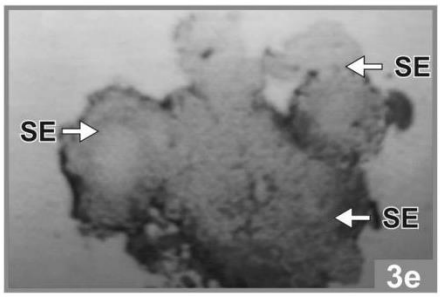
Fused Somatic embryos at different stages of development. (stained with PAS-REAGENT). X46



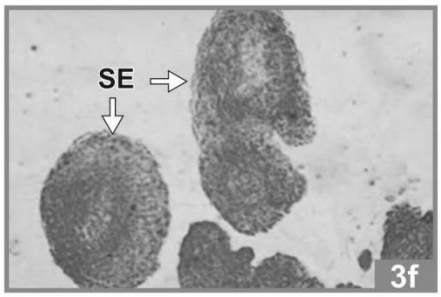
Globular and heart shaped epidermised somatic embryos with provasculature showing intense localization of proteins (stained with mercuric bromophenol blue). X22



Different shapes of Somatic embryos. without proper shoot meristems (stained with Haematoxylin) X46

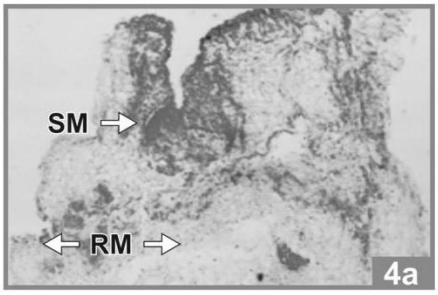


Asynchronus growth of somatic embryos (stained with PAS-REAGENT). X46

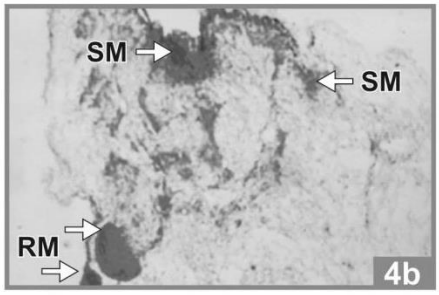


Globular & heart shaped somatic embryo on embryogenic callus. (stained with MBB). X22

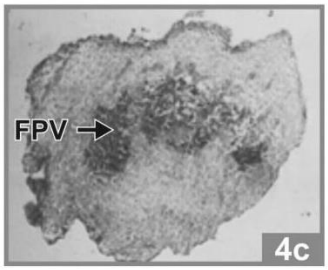
Somatic Embryogenesis
Plate - 4



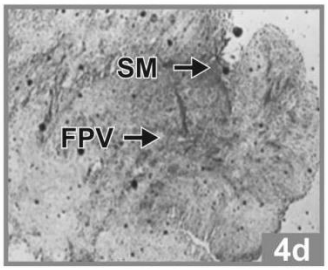
Fused somatic embryogenic cluster showing root initials at different places. (stained with MBB). X22



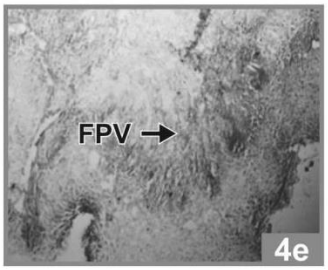
Abnormal somatic embryo with two root initials and expanded shoot apical meristems. (stained with MBB). X22



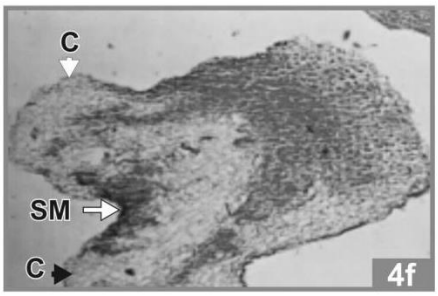
Fused embryo showing spreading of provasculature to all the parts of somatic embryo. (stained with Toluidine blue). X46



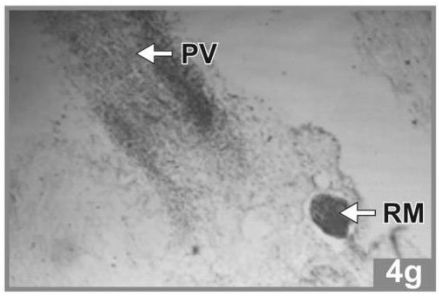
Somatic embryos with shoot apical meristem & fused vasculature (stained with Toluidine blue). X22



Somatic embryos without shoot apical meristem & fused vasculature (stained with Toluidine blue). X22



Unusual torpedo shaped embryo in monocot like banana showing 2 cotyledons, apical meristem and vasculature with protein accumulation at the base. (stained with mercuric bromophenol blue). X22



Abnormal somatic embryo with root meristem, provasculature & without shoot meristem (stained with Toluidine blue). X22

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