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Histochemical studies to detect the developmental stages of somatic embryos of banana cv. Rasthali

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Abstract
The present study was undertaken with the main aim to evaluate the histochemical changes during the development of somatic embryos from embryogenic callus of male flower buds of banana cv. Rasthali. 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 cvs. At successive stages of development were used for the present study. Histological sections were used for comparison of the histochemical changes occurring during the development of somatic embryos from embryogenic callus of male flower buds of banana cv. Rasthali. In conclusion, results on histochemical changes during somatic embryogenesis in Rasthali showed the presence of higher amounts of biomolecular substances such as insoluble polysaccharides, proteins and nucleic acids during different stages of embryo formation. This is necessary for the germination of somatic embryo into a plantlet. Localization of these macromolecules could be seen in matured embryos indicating, the importance of macromolecules for better embryo development.

Keywords: Banana cv. Rasthali, histochemical changes, polysaccharides, protein, nucleic acids, somatic embryos, Embryogenic callus

Introduction
Banana is one of the most important food crops in the world. It is cultivated in more than 130 countries and is an important staple crop for millions of people in several developing regions of the world [1]. Banana and plantain occupy 10.3 million ha and the total production was estimated at 139 million tons in 2012 [2]. Rasthali is one of the most popular commercial banana cultivars of Southern India adored for its special flavour, sub acid taste blended with sweetness and commands a higher price in Indian markets than the Cavendish cultivars. The cultivar belongs to the 'Silk' subgroup and is a triploid with AAB genome. It is highly susceptible to the wilt caused by *Fusariumoxysporum* f. sp. *cubense* and Banana Bunchy Top Virus. Propagation through conventional planting materials in banana is slow paced due to a low number of suckers which could be also a potential source of dissemination of fungal pathogens, nematodes, weevils, and viruses [3]. Alternatively, rapid production of healthy planting material of desired clones, within a short time period, can be facilitated by large-scale micropropagation through tissue culture using shoot tips. Even though several such reports in a banana are available, commercial micropropagation of AAB clones *in-vitro* is limited due to poor multiplication rate as compared to AAA clones.
Embryogenic cell suspension (ECS) cultures have been found to exhibit good regeneration response in the different genome groups in banana. Further cell suspension can give rise to large-scale induction of SE which can be regenerated into plants. Micropropagation using banana male floral meristems has also been reported in earlier studies [4]. In bananas and plantains different types of explants such as shoot tip [5], zygotic embryos [6], proliferating meristems and scalps [7] and immature male flowers [4] have been tried to develop and regenerate plants from ECS. Of these explants, immature male flowers appear to be the most responsive starting material for initiating ECS and plant regeneration. In addition, the ECS is the most suitable material for genetic manipulation through transformation [7]. In this connection, an understanding of somatic embryogenesis and the success in the application of biotechnological research cannot be achieved if the morphogenesis process is not well comprehended. It needs to identify the cell associated with induction process and the formation of structure capable of organized growth and eventual development into the plant.
SE induction and progression can be validating using histological and histochemical analysis reported by [8].

This study can help to understand the differentiation of somatic embryogenesis. With this use of the technique, it is possible to evaluate the growth and proliferation of calli and suspension via somatic embryogenesis [9]. Furthemrore, somatic embryogenesis of banana cultivars of different groups has been successfully achieved [10-12], 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. Hence, the present study was designed to carry out to evaluate the histochemical changes during the development of somatic embryos from embryogenic callus of male flower buds of banana cv. Rasthali.

Materials and Methods

Materials

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

Histochemical analysis: Fixation and killing of the calluss were 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 58-60 ºC 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. The deparaffinised sections were subjected to histochemical staining for the localization of different cellular compounds *viz*., total insoluble polysaccharides, total insoluble proteins and nucleic acids, cytoplasm and nucleus by using standardized protocols and techniques.

Results

Histochemical localization of macromolecules like insoluble polysaccharides, proteins, nucleic acids at different stages of embryo development were investigated. The intensity of staining was taken for assessing the extent of accumulation of the different macromolecules in different cells and tissues. Depending on the intensity, staining was categorized as intense, rich and poor (Table 1, 2).

Table 1: Histochemical changes during different stages of embryo formation from male flower buds of banana cv. Rasthali

Biomolecules	Peripheral embryogenic cells	Globular Embryo	Cordate Embryo	Matured Embryo	Germinated Embryo
DNA	+++	+++	+++	++	+
RNA	+++	+++	+++	++	+
Total Proteins	+++	+++	+++	+++	+
Total insoluble polysaccharides	++	+++	+++	++	++

Table 2: Histochemical changes during maturation of somatic embryo from male flower buds of banana cv. Rasthali

Biomolecules	Epidermis	Vascular Strand	Shoot Primordia	Root Primordia
DNA	+	+	+++	++
RNA	+	+	+++	++
Total proteins	+	++	++	+
Total insoluble polysaccharides	++	+++	++	++

Total insoluble polysaccharides

The peripheral zone of embryogenic calli showed meristematic zone having intense insoluble polysaccharides content (Plate 1, Figs. c & e). The region of the embryogenic calli other than the peripheral region showed poor staining for the presence of insoluble polysaccharide content. As the proembryo expanded to form globular, heart shaped, torpedo type to different forms, intense accumulation of the insoluble polysaccharide content could be seen. Furthermore, globular embryos showed differentiation in the accumulation of starch. The cortical region showed more intense accumulation of starch than the medullary region. The formation of provasculature zone was in centre region and the secondary wall layer (lignin) deposition could be seen (Plate 2, Fig. e). As the embryo developed into heart shaped, pear shaped structures, the accumulation of total polysaccharide became intense to rich. The epidermis, provasculature zone, shoot apices and root meristems were rich in polysaccharides. In the provasculature secondary wall formation in the form of spiral thickenings could be seen (Plate 2, Figs. g, h, i) in the matured somatic embryo. Formation of secondary somatic embryogenesis could be traced to the presence of intense starch in the globular embryos (Plate 1, 2). Primary globular somatic embryos which showed intense staining for the polysaccharides could only develop into secondary somatic embryos and the rest of the embryos with less storage material were not forming the secondary embryos (Plate 2, Figs. a & c).

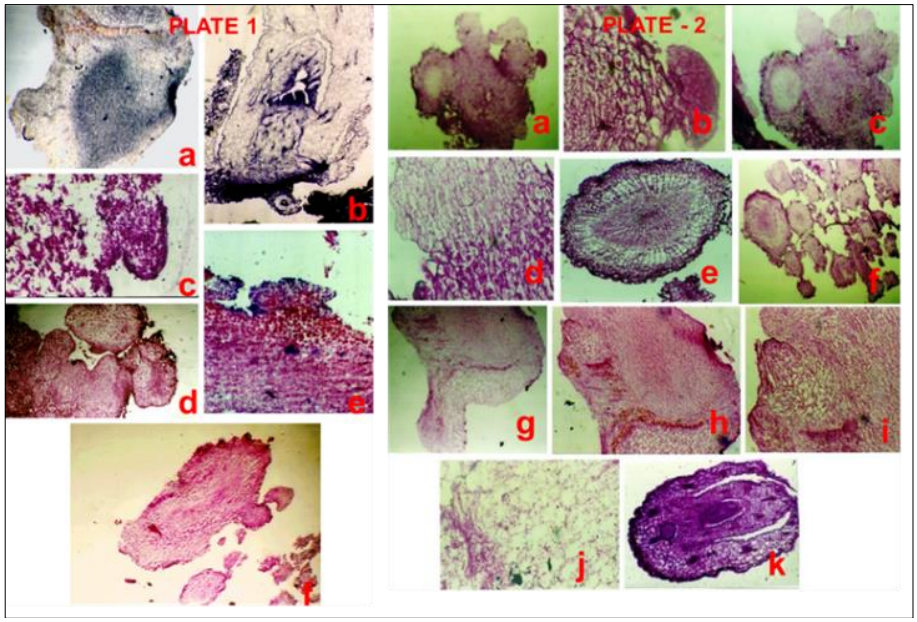

Plate 1

Figs. a-f Sections of somatic embryos during various stages of development stained with Haematoxylin and PAS reagent in banana cv. Rasthali.

a-b Sections stained with Haematoxylin.

c-f Section stained with per-iodic acid Schiff's reagent for localizing total insoluble polysaccharides.

a. Matured cylindrical shaped somatic embryo with cotyledon, provasculature, storage product, beginning of shoot apical formation.

b. Germinated somatic embryo with root initials, root and shoot initials and well developed vasculature.

c. Embryogenic callus showing intense localization of insoluble polysaccharides in the peripheral lobed region.

d. Somatic embryo cluster showing intense accumulation of starch.

e. Intense localization of insoluble polysaccharides at the periphery of embryogenic callus.

f. Primary somatic embryo showing secondary and tertiary somatic embryo with intense accumulation of insoluble polysaccharides.

Plate 2

Figs. a-k Sections of somatic embryos stained with per-iodic acid Schiff's reagent for localizing insoluble polysaccharides during different developmental stages in banana cv. Rasthali.

a. Cluster of primary somatic embryos showing intense accumulation of starch along with epidermal origin secondary somatic embryo.

b. Mitotic cell division in the proliferating secondary somatic embryos formed on the epidermal cell of primary somatic embryo. Intense localization of starch in primary somatic embryo indicting energy for formation of secondary embryo.

a. Primary somatic embryo cluster along with secondary embryo cluster.

b Starch granules more in primary somatic embryo than newly originated secondary somatic embryo.

c. Primary globular somatic embryo showing intense accumulation of insoluble polysaccharides towards periphery and procambial region in the centre.

f. Mesh work of primary, secondary, tertiary somatic embryos.

g. Matured primary somatic embryo showing root initials.

h. Matured primary somatic embryo showing well defined plumule, provasculature region.

i. Intense accumulation of insoluble polysaccharide at plumule region in matured primary somatic embryo.

j. Germinated embryo with less localization of insoluble polysaccharides.

k. Regenerated plantlets with plumule, leaves, root initials and vasculature.

Germinated somatic embryo showed intense staining at plumule region, root meristem, vascular region and in leaf primordial region. Distributed vascular bundles also showed rich in total polysaccharides content (Plate 2, Fig. k). Parenchyma cells of germinated embryo showed starch granules scattered in the peripheral regions of the cells (Plate 2, Fig. j).

Total proteins

The embryogenic calli at the peripheral meristematic zone showed intense accumulation of proteins (Plate 3, Fig. a-e). The rest of the calli other than the peripheral zone was poor in accumulation of total insoluble proteins. Histochemical investigations revealed that the intense accumulation of total insoluble proteins in all the developing stages of somatic embryo i.e., globular, heart shaped to cylindrical shaped embryos was the striking aspect seen in this study (Plate 3, Figs. f-i). When the embryos started to mature from heart shaped to more mature forms with having root and shoot meristem, the intense protein level decreased and started to accumulate in one particular position i.e., cotyledonary expanded region (Plate 4, Figs. b & c). Provasculature region also showed intense accumulation of the total insoluble proteins. The distribution of total insoluble proteins in the

clumps (joined) of somatic embryos was not uniform and depended on the maturity of the concerned developing embryos (Plate 4, Figs. c & e) i.e., in the embryogenic clump the accumulation of proteins gets reduced once shoot meristem and root meristem were determined, whereas in the other embryos the total insoluble proteins accumulation was intense as bipolar nature was not yet achieved. Different shapes of somatic embryos other than the globular, heart shaped could be seen (Plate 3, Figs. h & i; and Plate 4) compared to the starch globules, protein deposits in the cells of embryos appeared bigger and prominent. Localization of total insoluble proteins as intense regions on one side of the matured embryo shows that the bipolarity had been reached and proteins were now kept as reserve molecules (Plate 4, Figs. g, d, e). Plumule, root primordium, provasculature of matured somatic embryo showed rich accumulation of proteins. Germinated embryos showed rich in total insoluble proteins in the shoot meristem, root meristem and in vasculature zone only. Here we could observe that all the reserve proteins had been used up (Plate 4, Fig. h).

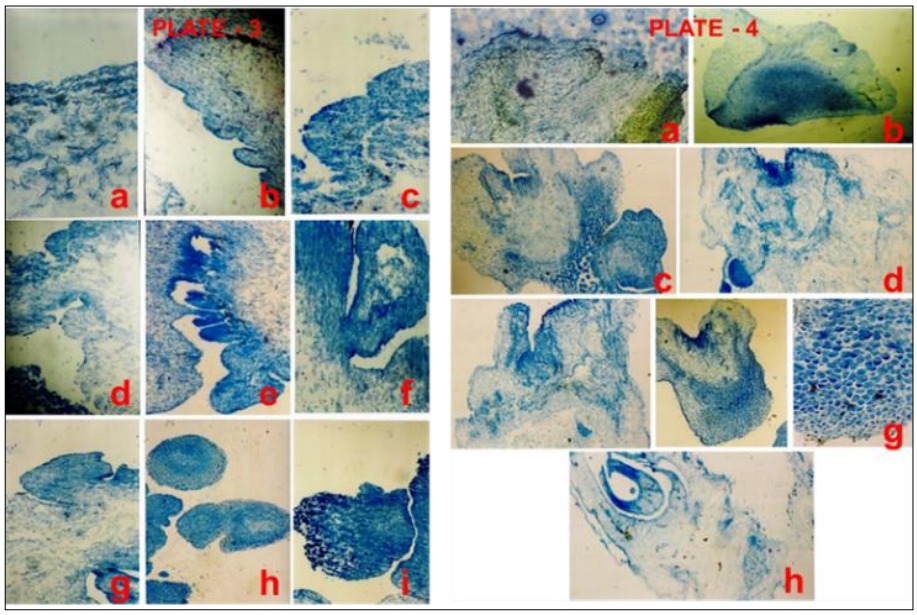

Plate 3

Figs. a-i Sections of embryogenic callus showing origin of proembryos from embryogenic callus, stained with mercuric bromophenol blue for localizing proteins in banana cv. Rasthali.

a. Peripheral epidermal and subepidermal cells showing intense accumulation of insoluble proteins.
b. Small buds on peripheral region of embryogenic callus with intense MBB stain.
c. Intense localization of insoluble proteins at the meristematic region of embryogenic callus.
d. Different stages of proembryos with intense accumulation of proteins.
e. Gradual enlargement of proembryos showing intense insoluble protein accumulation.
f. Proembryo within base still attached to the embryogenic callus with intense protein accumulation.
g. Embryogenic callus tissue showing intense accumulation of insoluble proteins at peripheral cells than inside.
h. Globular and heart shaped epidermised somatic embryos with provasculature showing intense localization of proteins.
i. Intense accumulation of total insoluble proteins in all the cells of heart shaped somatic embryo.

Plate 4

Figs. a-i Sections of somatic embryos stained with MBB for localizing total insoluble proteins at different stages of development in banana cv. Rasthali.

a. Plumule region of matured somatic embryo showing localization of proteins at the shoot tip region.
b. Matured bipolar somatic embryo with plumule, provasculature, cotyledon, leaf, root initials, localization of proteins can be seen in the middle region of the matured somatic embryo.
c. Fused embryos at different stages of development matured embryo with shoot tip shows localization of protein in cotyledonary base, whereas globular embryo still showing intense accumulation of proteins.
d. Malformed embryo with many root initials and broad shoot tip region.
e. Abnormal distribution of vasculature in the somatic embryo with two root initials and one expanded shoot apical meristem.
f. Unusual torpedo shaped embryo in monocot like banana showing 2 cotyledons, apical meristem and vasculature with protein accumulation at the base.
g. Intense localization of insoluble protein granules in all the cells of proembryo.
h. Regenerated plantlet from somatic embryo showing poor accumulation of insoluble proteins in all the cells except shoot tip.

Nucleic Acids

The embryogenic calli showed intense accumulation of RNA in the peripheral zones compared to DNA. From the embryogenic calli to geminated embryo the intensity of nucleic acids could be seen as a strong indicator for the development of somatic embryos (Plate 5, 6). The globular, heart shaped embryos showed intense RNA accumulation in the central zone than the peripheral zone of the embryos. In the embryogenic cluster differential staining of the RNA could be seen (Plate 6, Fig. e). Epidermis, provascular

bundles, shoot meristem, root meristem showed rich accumulation of DNA than RNA in the matured somatic embryos. But the intensity was lesser compared to globular and heart shaped embryos (Plate 6, Figs. h & j). In fully matured somatic embryo the nucleic acids were restricted to plumule root meristems and provasculature. The germinated embryo showed clear picture of localization of nucleic acids. The plumule, budding young leaves, root initials and connecting vascular bundles showed intense DNA presence compared to RNA and rest of the parenchymatous tissue showed absence of nucleic acids in their cells (Plate 6, Figs. j & k).

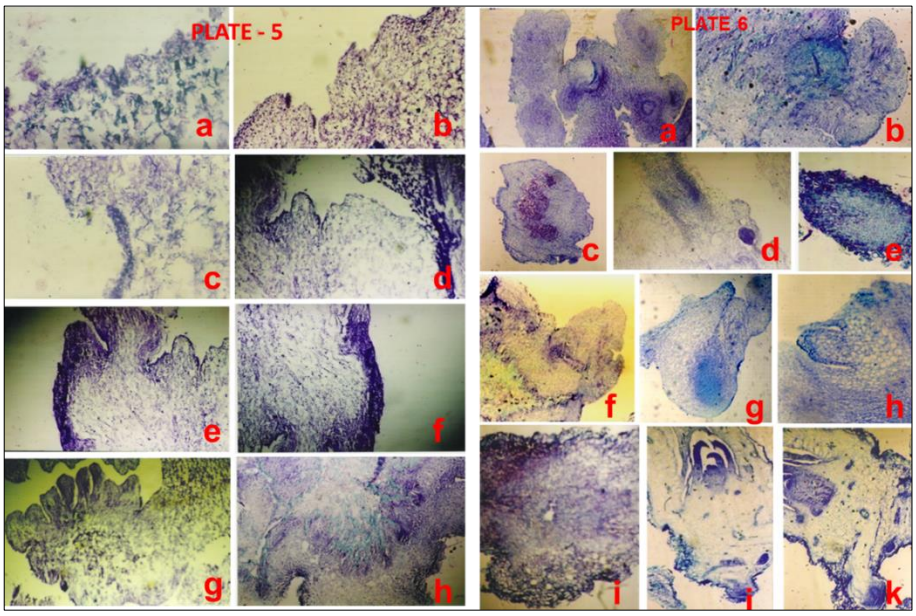

Plate 5

Figs. a-hSections showing origin of somatic embryos from embryogenic callus of banana cv. Rasthali stained with Toluidine blue (TB) for localizing nucleic acids.

a. Intense localization of RNA in the cells of epidermis and subepidermis of embryogenic callus.
b. Meristematic activity seen at epidermal and subepidermal region of embryogenic callus.
c. Gradual expansion of peripheral layers of embryogenic callus showing rich accumulation of RNA.
d. Initial stages of peripheral active cell divisions at the corners of embryogenic callus.
e. Different stages of proembryo formation at one position of embryogenic callus showing intense accumulation of RNA.
f. Embryogenic callus showing intensely stained peripheral region (RNA presence) and poorly stained inner region.
g. Segmented proembryos showing intense accumulation of RNA.
h. Fused primary somatic embryos showing fused vasculature indicating rich localization of RNA and DNA.

Plate 6

Figs. a-k Sections of somatic embryos showing different stages of development stained with Toluidine blue for localization of nucleic acids in banana cv. Rasthali.

a. Fused somatic embryos showing provasculature with intense accumulation of RNA.
b. Fused embryo showing spreading of provasculature to all the parts of somatic embryo.
c. Somatic embryos fusion showing fused provasculature along with secondary somatic embryogenesis at the base.
d. Fused somatic embryogenic cluster showing root initials at different places.
e. Globular somatic embryo showing intense accumulation of RNA at the peripheral region and DNA in the central region.
f. Development of shoot meristem in fused embryo showing intense DNA accumulation in provasculature.
g. Abnormal somatic embryo with fused cotyledons.
h. Plumule region of matured embryo showing RNA at shoot tip region.
i. Somatic embryo without shoots apical meristem formation but showing intense accumulation of RNA at the periphery.
j. Longitudinal section of germinated somatic embryo showing localization of RNA at plumule, leaf primordia, root initials, vasculature.
k. Germinated somatic embryo showing poor localization of nucleic acids and many roots initial formation.

Discussion

Histochemical localization of insoluble polysaccharides was noted in the peripheral region of embryogenic callus of male flower buds of banana cv. Rasthali. Thomas *et al.* considered starch to be an indicator of the development of tissue towards somatic embryogenesis [13]. Indeed, other works have shown that before following organogenesis or embryogenesis the cells synthesize and store considerable amounts of starch [14, 15]. It has been suggested that starch may function as an energy source during intense meristematic activity or may provide osmotica in the form of free soluble sugars [16]. Starch degradation results in the formation of glycolytic intermediates that will subsequently catabolized and yield high amounts of ATP [17].

Mikula *et al.* 2004 reported the ultrastructural study of embryogenic callus of *Gentiana punctata*. They observed distinct accumulation of starch in the peripheral region of embryogenic callus which gave rise to somatic embryos even

in our present work distinct starch rich peripheral zones in embryogenic callus were observed [18]. Dhed'A *et al.* reported in suspension cultures of banana cv. Bluggoe, starch granules and proteins were seen from single cell to globular and mature stages of somatic embryos derived from scalp [19]. However, in contrast to findings of our study Escalant *et al.* reported absence of starch in the developmental stages and localized presence in the mature embryos [20].

Furthermore, presence of starch in the unicell / multicells which forms somatic embryo has been reported in plants like cork oak [21], sugarcane [22], Cassava [16], highlighting the importance of starch in the ontogeny of somatic embryos. Many reports suggested that starch deposition in the matured somatic embryo is necessary for germination into normal plant as in Norway spruce [23], bamboo [24], in *Hevea brazilensis* [15].

Neumann reported that a continuous change in the composition of the protein moiety occurs with initiation and termination of protein synthesis of one or other group in a sequential and hierarchical pattern during the induction of somatic embryogenesis in carrot. Even in our work we have noted the presence of protein from the origin to the maturation stages of somatic embryo of banana cv. Rasthali [15].

According to Misra *et al.* seed storage proteins are the source of amino acids for new proteins needed in germination. Storage proteins are located in protein bodies that may contain amorphic proteins such as enzymes, phytase containing globoids as well as protein crystals [26]. Leal and Misra, considered that accumulation of storage protein to be a marker of zygotic embryo maturation [27]. In embryos, proteins start to accumulate during later stages of rapid growth. Their synthesis declines during desiccations and late embryogenesis abundant (LEA) proteins increases [28]. Presence of proteins in the cells which develop into somatic embryos similar to our work have been reported in cell suspension culture of banana cvs. by number of authors like Dhed'A *et al.*, Escalant *et al.*, Grapin *et al.* [12, 19, 20]. They have reported that protein rich cells will develop into somatic embryos of banana cvs. Distinct accumulation of proteins at the peripheral meristematic zone, in this study indicate the cells were preparing for cell division and for further growth of somatic embryos in accordance with the report by Michaux-Ferriere *et al.* in *Hevea brasiliensis* [15]. But they reported the presence of high soluble protein accumulation during embryogenic callus and at the globular stage but disappearance and appearance again at the germination stage whereas in our study from the embryogenic callus stage to the globular, heart shaped till the cylindrical matured stage of somatic embryos, protein localization was continuous and decreased only at maturation stage of somatic embryos of banana cv. Rasthali.

According to Hu *et al.*, before embryogenic cells were formed the synthesis of RNA was activated first followed with increase of synthesis rates of DNA and protein [29]. The histochemical and ultrastructural properties of meristematic zones during somatic embryogenesis suggest intense RNA synthesis and metabolic activity as stated by Maheshwaran and Williams *et al.* in Trifolium [30]. Similar results were seen in the histochemical sections of embryogenic callus of banana cv. Rasthali.

In the present histochemical study, RNA localization was seen prominently in the peripheral meristematic zone of embryogenic callus, embryo formation stage, embryo developmental stages, and only the germinated embryo showed least localization of RNA. Whereas DNA was seen in globular to mature stage of embryo formation similar to the report by the Hu, Z. *et al.* [29]. According to them during formation of globular embryo, DNA synthesis reached peak then the activities of RNA and protein reached the peak. Increased RNA synthesis was also observed ultra-structurally by Mikula *et al.* in *Gentiana punctata* during somatic embryogenesis [18]. Specific changes in nucleotide biosynthesis during carrot somatic embryogenesis was reported by Claudio Stasolla *et al.* [31].

Conclusions

In conclusion, our studies on histochemical changes during somatic embryogenesis in Rasthali showed the presence of higher amounts of biomolecular substances such as insoluble polysaccharides, proteins and nucleic acids during different stages of embryo formation. This is necessary for the germination of somatic embryo into a plantlet. Localization of these macromolecules could be seen in matured embryos indicating, the importance of macromolecules for better embryo development.

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