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RESEARCH ARTICLE

Histological studies of somatic embryogenesis of immature inflorescence derived calli of *Sorghum bicolor* using SEM

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Abstract

The sequence of histological events occurring during somatic embryogenesis from *Sorghum bicolor* genotype IS 3377 (pigmented variety) immature inflorescence were studied. Sections were made and studies were carried out from the initiation of callus induction to somatic embryogenesis. The Processes leading to embryogenesis were studied elaborately. Our observations indicated that somatic embryos arose from floral primordium region of embryogenic callus. Internal divisions gave rise to detached groups of cells on the callus surface. The transverse section of immature inflorescence segment showed central inflorescence axis was enclosed with peripheral floral primordia that appeared as protuberances of meristematic cells, richly dividing cytoplasmic cells at the tip of each floral primordium.

Introduction:

Sorghum, a special cereal crop worldwide because of its drought tolerance, is an important staple food in developing countries of the semiarid tropics and is also used as an animal feed. Various strategies were described based on investigations in tissue culture and new approaches were discussed focusing on the considerable recent progress made using transformation techniques for the enhancement of tissue culture transformation efficiency in *Sorghum*. In *Sorghum* Sudhakar Pola reported high frequency plant regeneration with various explants including immature inflorescences (2003 and 2005), immature embryo (2007, 2008 and 2009) mature embryo (2009), leaf (2006 and 2011) however, in *Sorghum* still it requires standardized protocols using various explants. So, the origins of various developmental stages are essential to study.

Somatic embryogenesis implies rapid clonal multiplication of explants leading to more number of embryoids reaching to maturity and it is advantageous for its rich germination potential (Indra K. Vasil 1983). Somatic embryos arise from single cells either directly or after the formation of a pro-embryonal complex like their zygotic counterparts. Plants of multicellular origin (eg. Shoot tip) cannot always be expected to be genetically uniform due to the chance of formation of Chimeras. Therefore, it would be advantageous to obtain plants from isolated single cells such as protoplasts or through the formation of somatic embryos. So, a basic understanding of the process of somatic embryogenesis is essential. The first detailed documentation for Somatic embryogenesis in tissue cultures of grasses was worked out by Vasil & Vasil 1981a in *Pennisetum americanum*. The Objectives of present study is to microscopically analyze the mechanism of Somatic embryogenesis of six different *Sorghum* cultivars and provide histological evidences for callus culture differentiation which have capability to regenerate

variant plants. In *Sorghum*, plant formation took place largely by the depression and proliferation of presumptive shoot primordia in the process of microtillering (Indra K. Vasil 1983).

The study of Vasil & Vasil 1981a, Lu & Vasil 1981a reported that for immature embryo scutellar tissue gave rise to embryogenic callus and for leaf tissue, cells of the lower epidermis as well as mesophyll cells near the vascular bundles proliferated to form embryogenic callus. The initiation and conversion step has been comparatively less investigated although they directly contribute to the final plant yield and influences the ability of the resulting embryos to germinate and develop into growing plantlets. Two main problems have been observed concerning the initiation and conversion step. The first one is the difficulty in obtaining stable and suitable embryogenic callus. The second is the lack of knowledge of regenerating embryo development and the risk of morphological callus. In angiosperm species, multiplication of embryogenic cells can be achieved either by regular sub culturing of explants taken from compact or friable embryogenic calli, or by formation of new embryos from the previously developed somatic embryos themselves. This second case is referred to as secondary embryogenesis. In *sorghum*, too little ontogenic studies have been made on somatic embryogenesis either with light or SEM. Dunstan *et al.,* (1978) and Pola (2005) studied immature embryo under light and SEM. Brettell *et al.,* (1980) and Pola (2005) observed the cultures of immature inflorescence. Wernicke *et al.,* (1982) described the morphogenic pathways and morphogenesis from cultured leaf and leaf & mature embryo explants by Pola (2005). The nature of embryogenic callus in sorghum is white, compact and globular with smooth surface as also described by Cai and Butler (1990). Zhong *et al.,* (1998) demonstrated embryogenesis from shoot apices with the aid of SEM.

These analyses that began with the random differentiation of leafy scutella and tubular coleoptiles from the organized apical domes, while the cellular integrity of the epidermis was well presented during such folding and budding process. They demonstrated that the differentiation and development of scutella was spatially uncorrelated to that of coleoptiles. Ahmed *et al.,* (2002) studied *in vitro* morphogenesis of shoot apical meristem, somatic embryogenesis and regeneration system in *Triticum aestivum* using SEM. They reported a proliferation of the budding state that gave rise to somatic embryos and adventitious shoots and further multiplication of shoot meristems on MS+BAP and 2, 4-D medium. Aparna *et al.,* (2004) by using light and SEM observed the formation of somatic embryos and their maturation from immature inflorescence with a well organized bipolar structure showing embryogenic axis, scutellum, coleoptile and coleorhizae. Nirwan and Kothari (2004) observed the origin of shoot apices from the surface of enlarged meristemoids from the cultures of immature embryo.

However, the information available on the histology of somatic embryo induction and development of sorghum is insufficient; much more is needed for better understanding the process of somatic embryogenesis as well as developing a stable transformation system. The present study describes the developmental stages of embryo formation and maturation. Immature inflorescence was studied using scanning electron microscope. Analysis was done for the IS 3477 variety of *Sorghum* Callus cultures *in vitro*.

Materials and Methods:

Plant material:

The seeds of genotype of *Sorghum* IS 3477 was provided by Germplasm unit of International Crop Research Institute for Semi Arid Tropics, Hyderabad, Andhra Pradesh, India.

Explant:

The field collected immature inflorescence of Sorghum genotype IS 3377 was washed several times with tap water before surface sterilization. Then the explants were surface sterilized with 70% ethanol for 2min, NaOCl for 20min and $HgCl_2$ for 10min and inoculated on MS medium supplemented with 2, 4-D concentration ranging from 0.5-3.0mg/L and subjected for direct somatic embryogenesis. After the explants were inoculated into the medium, the cultures were incubated at 25 ± 2^{o}c in light conditions at intensity of 25µ mol m^{-2} s^{-1}.

Histological Studies:

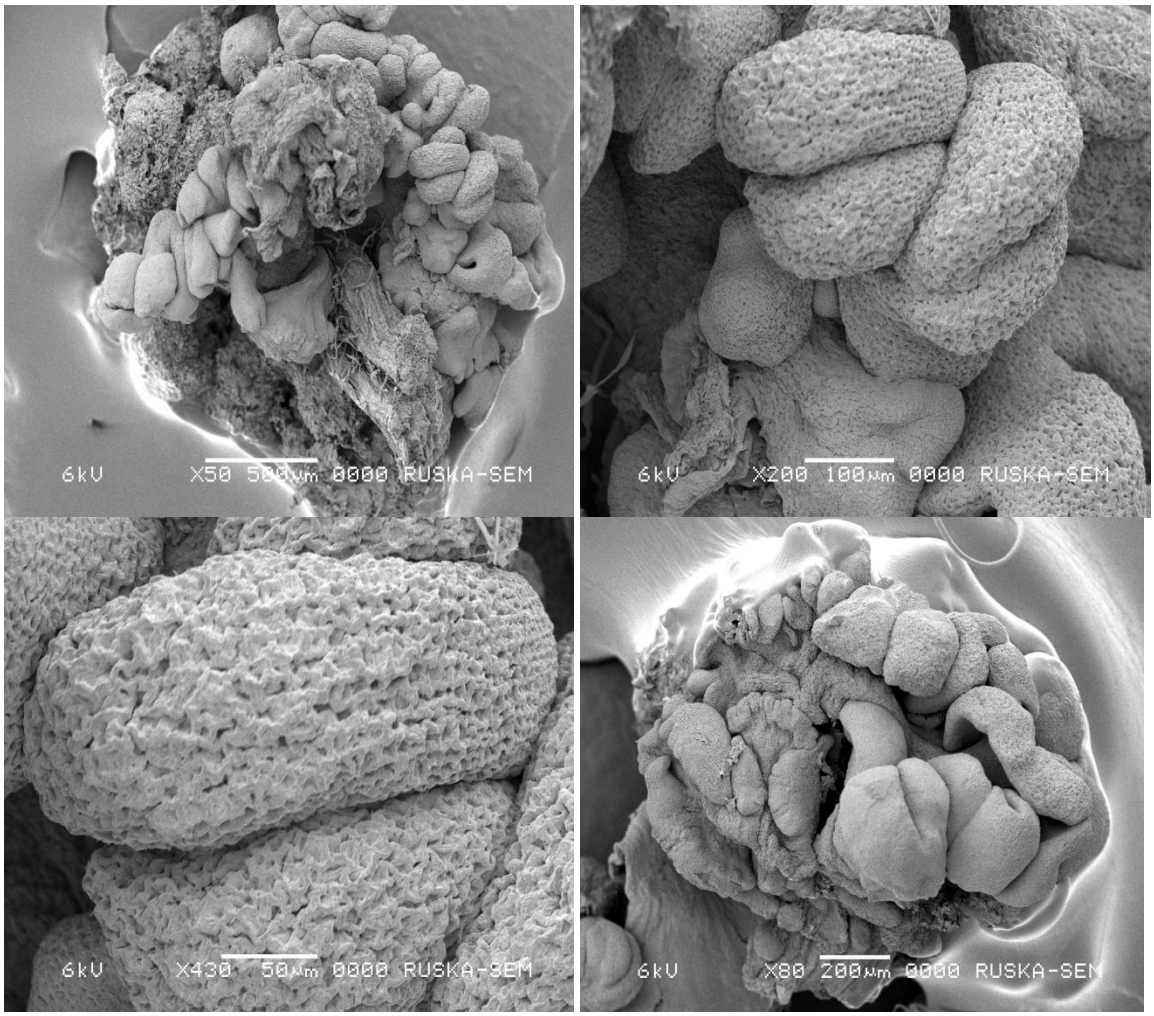
The method followed for microscopic studies was according to John J. Bozzola 1999. Samples were transferred to vials and fixed in 3% Gluteraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 hr at 4^{0}C and post fixed 2% aqueous Osmium tetroxide in the same buffer for 2 hr. After the post fixation samples were dehydrated in series of graded alcohol and processed for critical point drying with Electron Microscopy Science CPD unit. Then dried samples were mounted over the stubs. Finally, a thin layer of gold metal was applied over the sample using an automated sputter coater (JEOL JFC-1600) for about 3 min and then scanned using scanning electron microscope (Model: JOEL-JSM 5600) at various magnifications at RUSKA Lab, College of Veterinary Sciences, ANGRAU, Hyderabad, India.

Results:

Somatic embryogenesis:

Immature inflorescence explants cultured on medium showed callus initiation within nine days of inoculation. At the beginning of third week after culture, the developed calli became detached from the non-embryogenic portions of the explant. Each callus mass was divided into equal pieces and subcultured or transferred on to the regeneration medium. Calli of different varieties were separately transferred to subculture medium or regeneration medium to test regeneration ability. The white embryogenic callus after transferring on to the regeneration medium turned to green colour. The development of somatic embryos at various stages was observed by gently dissecting and teasing the embryogenic callus. Shoot initiation from the shoot buds occurred after 9 days of culture transfer on to the regeneration medium now containing BAP 2mg/L and Zn 0.5 mg/L. The control explant did not show any embryogenesis. The embryos on this medium turned into horse shoe shaped structures which later developed into leafy shoots.

Figures:



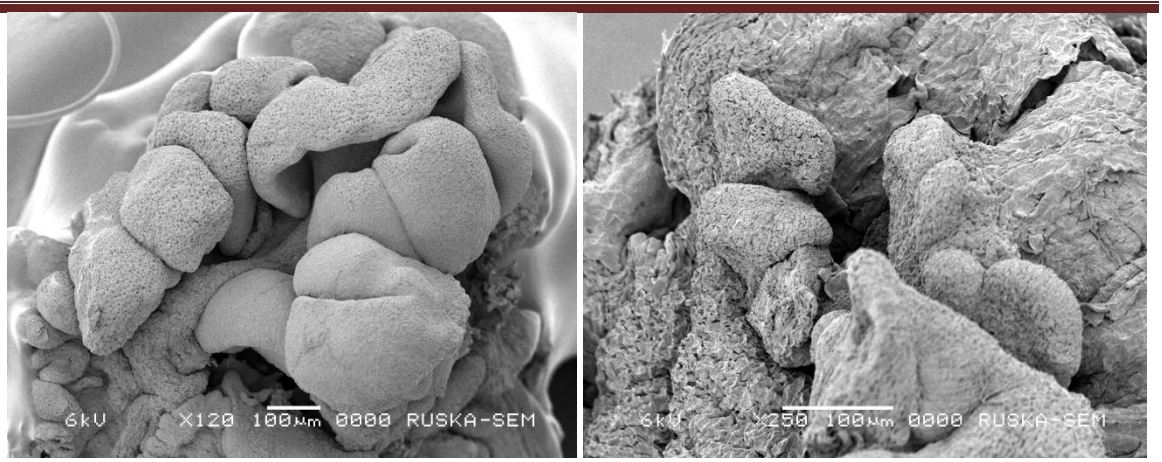


Fig 1. Scanning Electron Micrographs showing various stages of Somatic embryogenesis

SEM analysis:

The transverse section of immature inflorescence segment showed central inflorescence axis was enclosed with peripheral floral primordia. The floral primordia appeared as protuberances of meristematic cells, richly dividing cytoplasmic cells at the tip of each floral primordium. The section of the initial explant showed highly vacuolated central core with large parenchymatous cells, surrounded by outer layer of vascular bundles. This embryogenic callus was found to consist of small cells which were round in shape, richly cytoplasmic with a prominent nucleus in a compact arrangement (Fig 1.). After a week, clusters of rounded or oval structures were observed with their basal ends embedded in the callus mass.

The section of 8 day old cultured explant showed a single layered epidermis with parenchymatous cells and 10-15 day culture revealed proliferation of cells with meristematic activity. At this stage the formation of richly cytoplasmic cells, with prominent nuclei without any intercellular spaces were observed. The surface scan of the callus also showed the differentiating non-embryogenic callus. The meristematic cells observed at the periphery were small and compact. Embryogenic callus covered the entire scutellum surface in 20 day old explant.

The morphological division of the embryo was recognized by the growth of the globular embryo into oval shaped structures. After 22-35 days of culturing, the meristematic cells differentiated into medium sized, highly cytoplasmic embryogenic cells. These embryogenic cells could be easily distinguished from the protective epidermal layer due to their greater size. After four weeks of culture, these richly dividing embryogenic cells occupied several layers immediately after the epidermis, followed by various cell types representing different stages of differentiation. Internal divisions in many of these cells gave rise to detached groups of cells on the callus surface. Each group was distinct and separate from each other cell by a thick wall. The lobed callus after the development of vascular initials within the lobed structure was the embryogenic tissue which contained a layer of embryogenic cells.

Continued divisions and association in some of these separate groups formed rounded structure i.e., proembryoids with a distinct epidermis. Further differentiation of these structures led to the formation of globular embryoids, which appeared on the surface of the callus after breaking through the epidermis. These globular structures when maintained by sub culturing for over 30 days developed into somatic embryos on the surface of the callus. Scutellum initiation occurred at one end of the oval shaped somatic embryos at the globular stage. Further development of these structures led to the formation of typical embryos with a well-organized embryonic axis, scutellum and coleoptile. Later these structures developed into shoot buds which subsequently developed into complete shoots in the presence of light.

Maturation of somatic embryos

Further differentiation of these structures led to the formation of globular somatic embryos and embryoids, which appeared on the surface of the callus after breaking through the epidermis as revealed by SEM. These globular structures developed into somatic embryos on the surface of the callus were maintained by sub culturing medium for 3-4 weeks. The development of the embryoids was not synchronous, and therefore embryoids at various stages of development could be found adjacent to each other. The large, peripheral cells proliferated in many embryoids to give rise to secondary embryogenic callus tissue and secondary embryoids.

Germination of somatic embryos

The globular somatic embryos formed were cultured on MS regeneration medium for maturation and germination of the somatic embryos. The globular somatic embryos formed were round. Polarity was established in the embryoids

followed by cotyledon differentiation. Cotyledon initiation occurred at one end of the oval shaped somatic embryos at the globular stage. Further development of these bipolar structures led to the formation of typical embryoids with a well-organized embryonic axis, scutellum, coleoptile and coleorhiza. Globular suspensors were found attached to the somatic embryos as revealed in SEM. The immature inflorescence explants responded well for somatic embryogenesis as well as for regeneration when compared to immature embryo and shoot meristems. Therefore, the embryogenic callus mediated regeneration from immature inflorescence derived calli was studied by light and SEM to observe the histology of somatic embryo induction and development. Immature inflorescence of sorghum genotype IS 3477 were collected and cultured. For callusing of the explants, MS medium with 2, 4-D (2.0 mg/L) and kinetin (0.5 mg/L) was optimal; this combination gave the best response in the initial studies.

Effect of PGR on somatic embryogenesis

The consequences of this study support the common concept that 2, 4-D plays an important role in conferring embryogenic capability in tissue culture of cereals. The hormone 2, 4-D is an auxin, which has ability to induce cell proliferation at low concentrations in wide variety of gramineceous cultures (Vasil and Vasil 1982a). Form a list of 65 dicot species of reviewed by Raemakers (1995), somatic embryogenesis was induced in 17 species on hormone-free media, in 29 species in auxin-containing media and in 25 species on cytokinin-supplemented media. Among auxins, the most responsive hormone was 2, 4-D (49%) followed by naphthalene acetic acid (27%), indole 3-acetic (IAA) (6%), indole-3-butyric acid (6%), Picloram (5%) and Dicamba (5%). In the case of cytokinins N6-benzylaminopurine was most responsive (57%), followed by Kinetin (37%), Zeatin (3%) and Thidiazuron (3%). The importance of auxin to cytokinin ratios in the control of regeneration were reported by Ouf *et al.*, (1986). The 2, 4-D to KN ratios significantly influenced the responses for callus induction and differentiation in this study. Inflorescence in which the primordia were just being initiated (1-4cm) was found to be a good source of embryogenic calli. The formation of compact tissue comprising of small and richly cytoplasmic cell, as observed in the present investigation, is a common feature in most of the cereals *in vitro* cultures. These cells then differentiated by attaining embryogenic competence during early stages of culture as also reported by Lu and Vasil (1985).

Discussion

Organogenesis and somatic embryogenesis are two different mechanisms, by which explants can regenerate the complete plants. Generally, in the first case shoots and roots form in response to appropriate culture conditions mainly to the type and concentration of plant growth regulator present in the culture medium. This type of development is also characterized by the presence of vascular links between the mother tissue and the regenerating section. On the other hand, somatic embryogenesis can be described as the process by which haploid or diploid somatic cells develop into structures that resemble zygotic i.e., bipolar structures lacking any vascular link with the explant (Victor 2001). An outstanding feature of the somatic embryo is its constant growth with no development arrest (Faure *et al.*,, 1998). Both organogenesis and somatic embryogenesis may occur in the same explant (Falco *et al.*,, 1996).

In the present study, the occurrence of enlarged single cells spotted in the embryogenic callus was observed. Scattered occurrence of pro-embryoids without any polarity in the early stage prior to the appearance of globular and club shaped structures was observed. The presence of white swelling with collar like structures on callus surface from which plantlets arose subsequently indicated that the pathway of regeneration is through somatic embryogenesis rather than organogenesis. Cells of the non-embryogenesis callus initially appeared as hyaline and irregular structures of different shapes and sizes. With increase in the age of the callus, the size of its callus cells also increased and the cells appeared highly curved and irregular in shape with thick walls. In contrast to these, cells of embryogenic calli were relatively smaller, mostly round oval and possessed dense cytoplasm and prominent nuclei. The observations of Vasil and Vasil (1981) on calli were reported as three different types: (i) yellow to white ridge of compact, opaque embryogenic callus (ii) soft, translucent, friable unorganized callus (iii) soft, watery, mucilaginous callus from immature embryos as well as immature inflorescence cultures. The formation of compact tissue comprising of small and richly cytoplasmic cells, as observed in the present investigation and also by (Vasil and Vasil 1982a), Sudhakar pola (2005) is a common feature in most of the cereals *in vitro* culture. These cells then differentiated by attaining embryogenic competence during early stages of culture as also reported by earlier investigations (Lu and Vasil 1983).

Gradual differentiation of callus from peripheral meristematic cells led to the formation of layers of embryogenic cells, which further differentiated into globular embryos. Age of the seedlings in relation to number of actively dividing cells that have greater potential for *in vitro* response, was supported for the first time in sorghum with scanning electron microscopy study done by Sai Kishore *et al.*, 2005. Histological studies by Vasil and Vasil (1982) in pearl millet revealed the presence of small and richly cytoplasmic cells with starch containing cells and compact

clumps were termed as embryogenic cells and they also observed the presences of elongated, thick walled cells with large vacuoles in the young inflorescence cultures. Lu and Vasil (1985) described the histology of the formation of embryogenic callus and somatic embryogenesis from cultured immature zygotic embryos of Panicum maximum. Morphology and ultra structure of embryogenic cell suspension cultures of *Pennisetum maximum* and *P. purpureum* was studied by Karleson and Vasil (1986a). Oka *et al.*, (1995) reported detailed histological observations on initiation of morphogenesis in immature embryo and mature embryo derived of barley. Taylor and Vasil (1996a) described the ultra structure of somatic embryos development in pearl millet.

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