

## **PLANT REGENERATION VIA ORGANOGENESIS OR SOMATIC EMBRYOGENESIS IN SUGARCANE: HISTOLOGICAL STUDIES**

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### **Abstract**

Embryogenic callus was obtained by culturing young leaves of sugarcane on modified MS medium containing 4mg/l 2,4-D. Many embryos were formed when the callus was transferred on the medium containing low concentration of 2,4-D (0.5 mg/l). The callus transferred on the medium without 2,4-D turned black after 7 to 10 days of sub-culturing. Embryos were cytoplasmically rich and showed the typical organization of grass embryos. Somatic embryos were regenerated on the regeneration media (MS salts + 2 mg/l each Kin, IAA and IBA + 2% sugar) and profuse rooting was observed on medium containing MS + 1 mg/l IBA + 6% sugar. Rooted plantlets were transferred into the field after hardening for further evaluation.

### **Introduction**

Sugarcane (*Saccharum* spp. hybrid) is a vegetatively propagated crop belongs to the family *Gramineae*. Sugarcane accounts for approximately 70% of the world's sugar and is an economically important cash crop in the tropical and sub-tropical regions of many countries (Chengalrayan & Gallo-Meagher, 2001). Due to its global importance concerted efforts are being made for sugarcane improvement through plant breeding and more recently through biotechnology. *In vitro* plant regeneration can be accomplished through somatic embryogenesis or organogenesis (Falco *et al.*, 1996). Somatic embryogenesis is the most common regeneration pathway which have been reported (Nadar *et al.*, 1978; Larkin, 1982; Ho & Vasil 1983a; 1983b; Srinivasan & Vasil 1986; Guiderdoni & Demarly 1988; Chen *et al.*, 1988; Brisibe *et al.*, 1993; Khatri *et al.*, 2002; Khan *et al.*, 2004).

In this communication we have described the events leading to the regeneration of plantlets from callus obtained from shoot meristem and immature leaves of sugarcane through histological analysis.

### **Material and Methods**

Shoot meristematic and immature leaf explants were obtained from the shoot of the sugarcane clone BL4 collected from the Experimental Farm of NIA, Tando Jam Sindh, Pakistan. Vegetative cutting were grown in the growth room at  $25\pm2$  °C with 16 hours photoperiod and after eight weeks of growth all the mature leaves were removed. The remaining apical part of the shoot which consisted of several pale-yellow furled leaves was trimmed to 3 cm in length. The shoot was surface sterilized in 70% ethanol for one minute, disinfected in 50% Clorox solution for 20 minutes and thoroughly rinsed with sterile distilled water. After removal of several layers of leaves, the innermost 5 to 6

tightly furled leaves with meristematic region were sequentially cut into 2-3 mm thick segments and cultured on the modified MS medium (Murashige & Skoog, 1962) + 4mg/l 2,4-D + 2% sucrose + 40mg/l cysteine for callus induction at 28 °C in dark. After one month of incubation, actively growing callus was separated and transferred on modified MS medium with or without 0.5mg/l 2, 4-D. One month old calli were fixed in 2 to 4% glutaraldehyde buffered (0.2 M phosphate buffer) at the pH ranging from 6.8 to 7.4 at 4 °C for 24 hours. The glutaraldehyde was washed out from the fixing material by placing the specimen in the phosphate buffer. After washing, the fixed tissues were dehydrated in an acetone series each for half an hour. After dehydration, the material was embedded in the epon for polymerization. The callus processed for semi-thin section having the sample size 2-3 mm. The ideal pH for fixation was 7.2. Embedding in epon needed three days at each step i.e., a) propylene oxide + epon (3:1), b) propylene oxide + epon (1:1) and c) epon. Polymerized specimens were sectioned with glass knife on ultra-microtome to obtain 2-3 micrometer sections. Sections were stained with periodic acid Schiff's method (McManus, 1948) and post-stained with toluidine blue. After staining the sections were washed with running tap water until water became clear. The sections were dehydrated with the series of ethanol for few seconds and then embedded in euparal, covered with glass and kept at room temperature for drying. The photomicrographs were taken with microscope fitted with a microphotographic attachment using an objective 40x.

Somatic embryos were germinated by placing them on the modified MS medium i.e., MS salts + 2mg/l Kin +2mg/l IAA + 2mg/l IBA + 2% sucrose (Siddiqui *et al.*, 1994). Rooting were induced by transferring the elongated shoots on modified MS medium i.e., MS salts + 1mg/l IBA + 4% sucrose.

## Results and Discussion

Callus induction, first started in the meristematic tissue but due to heavy phenol production in the meristematic culture, callus quickly turned brown and died. In case of immature leaves no phenol production was observed and explants close to meristematic region (1 cm) showed the highest calli induction potential. The actively growing white compact calli were transferred to the modified MS medium containing low concentration of 2,4-D (0.5 mg/l) or without 2,4-D. The calli transferred on the medium without 2,4-D started turning brown after 7 days of sub-culturing whereas the calli transferred on the medium containing 0.5mg/l 2,4-D, showed pro-embryoids structure after 14-19 days of sub-culturing. These pro-embryoids looked like a compact, whitish and globular in structure (Fig. 1). Initially small projection was developed (cambium like zone) and embryogenic callus was derived from continued proliferation of this projection (Fig. 2). Embryogenic cells with dense cytoplasm and thickened cell walls situated at the periphery of the embryogenic callus underwent internal segmenting divisions leading to the formation of globular pro-embryoids (Fig. 3). Pro-embryoids and embryos were attached to the callus surface by a small suspensor (Fig. 4). But in most of cases suspensor was either not clearly distinguishable or present as a broad multicellular structure (Fig. 5a & 5b). After that lateral notch was developed at the terminal end i.e., initiation of the scutellum and then well developed embryo was formed.



Fig. 1. Compact white globular structure.

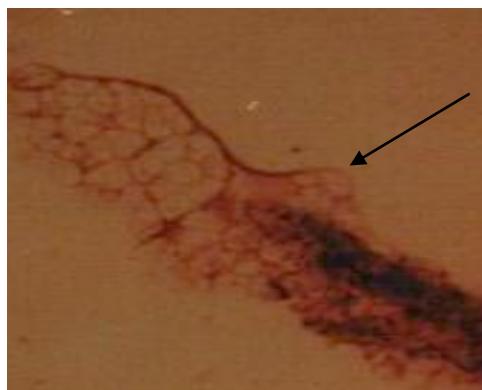


Fig. 2. Development of small projections on the surface of callus.

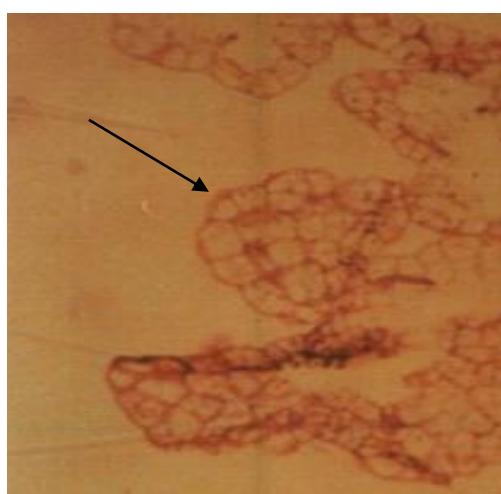


Fig. 3. Globular Pro-embryoids.



Fig. 4. Pro-embryoids attached to the callus surface by a small suspensor.

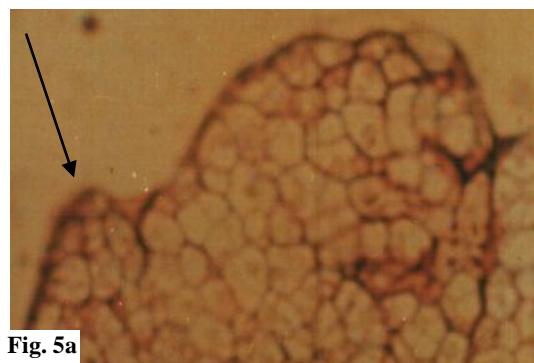


Fig. 5a

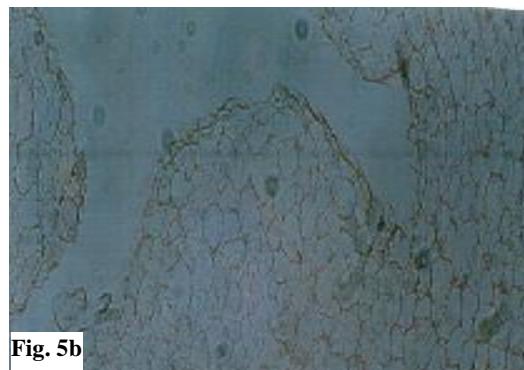


Fig. 5b

Fig. 5a & 5b. Initiation and development of scutellum which turns into embryos

The main morphological characteristic of somatic embryos is the bipolarity and the absence of connection with the explant vascular tissue (Reinert, 1977). These characteristics were described for sugarcane by Guiderdoni (1986) through the histological analysis of somatic embryos which showed a typical bipolar orientation, scutellum, coleoptile and absence of vascular connection with adjacent tissue. More or less same results were observed in our study. Ahloowalia & Maretzki (1983) demonstrated that the absence of coleoptile was due to a premature germination of the embryos in culture medium with plant growth regulators. According to Ho & Vasil (1983a) atypical development of sugarcane embryos may have led to erroneous interpretations of the regeneration pathways. Although shoot apex of zygotic embryos was similar to the shoot apex formed during organogenesis (Falco *et al.*, 1996), In the present study it was observed that some structures were connected to the callus by broad bases and lacked root apex or any oriented vascularization which would lead to a root pole in other cutting planes. It became clear that somatic embryogenesis was not the only regeneration pathway occurring in this sugarcane material. Regeneration of plants from callus can occur by both organogenesis and somatic embryogenesis, simultaneously.

Embryos were transferred to the medium containing MS salts+3% sucrose+2mg/l IAA+2mg/l IBA+2mg/l Kin for germination and plant development. Well developed roots and shoots were obtained while in some cases where root system was not properly developed the regenerants were transferred on the medium containing MS salts +1mg/l IBA+ 4% sucrose. Plantlets were transplanted to potted soil, acclimatized at 28°C in growth chamber with 16-hour photoperiod for 10 days and then grown to maturity.

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