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RESEARCH ARTICLE

ORIGIN AND EARLY DEVELOPMENTAL PATTERNS OF SOMATIC EMBRYOS FROM FREELY SUSPENDED CELL CULTURE OF *ENICOSTEMMA HYSOPIFOLIUM* (WILLD.) VERD. - A VULNERABLE MEDICINAL PLANT

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ABSTRACT

Early somatic embryogenesis from single cells or a multi cells differentiated into embryos from *Enicostemma hyssopifolium* (Willd.) Verd. cell suspension culture were obtained by fractionation through sieving, using iron mesh and centrifugation. These cells differentiated to embryos when they were cultured on a Murashige and skoog's medium containing 0.5 mgl-[1] α-Napthaleneacetic acid (NAA) + 1.0 mgl-[1] Zeatin (Ze) for 14 days, followed by transfer to a medium containing 1.0 mgl-[1] Ze lacking auxin. This indicates that there are at least two phases in the differentiation of embryos. The progression of the first phase required exogenous auxin, whereas that of the second phase was inhibited by the same growth regulator. The embyrogenic cells were richly cytoplasmic, contained numerous starch grains and were about 30 µm. Proembryo like structures with few cells and having mainly transverse or longitudinal division was observed among many elongated and vacuolated non embryogenic single cells (50-200 µm). From serial observations of the pre-embryo like structures after transfer to a micro chamber, it was confirmed that they are developed in to successive stages of somatic embryos. Therefore, a study of the origin and pattern of development will provide a better understanding of the order-generating processes.

INTRODUCTION

Enicostemma hyssopifolium (Willd.)Verd. Syn. E.littorale Blume (Gentianaceae) is a vulnerable medicinal plant (Seetharam *et al.,* 1998), found throughout India up to 450m altitude and extensively used in both folk and ethno medicine. Somatic embryogenesis is a process by which somatic cells undergo a developmental sequence similar to that seen in zygotic embryos. This process is an important plant propagation technique and provides an essential tool for basic research into plant embryo development. The original descriptions of somatic embryogenesis came from observations of carrot cells in culture, and carrot has remained the primary experimental system for studying somatic embryogenesis (Steward *et al*, 1958; Halperin and Witherell, 1964; Mc Willium *et al*., 1974; Nomura and Komamine, 1985). It has been widely accepted that somatic embryogenesis occurs from single cells of unorganized clumps in suspension culture (Halperin, 1966; Haccius, 1978). However the mechanism of direct embryogenesis from freely suspended culture is still obscure. The isolated cells did not embark directly upon embryo development; the single cell divide unequally and smaller derivatives gave rise to callus like mass later somatic embryos differentiated from the surface of the callus. Studies of Dudlts *et al*. (1991) have highlighted the utility alternative system, particularly for studying the induction of embryo development from cultured cells. Somatic embryos are induced from cultured callus cells by a relatively simple manipulation of the culturing conditions, this generally involves (1) the establishment of a callus cell line from small leaf explant, (2) the selection of an embryogenic subpopulation of the cultured cells through sieving or gradient fractionation, (3) the removal of auxin from the culture medium, and (4) the dilution of the cells to a relatively low density. These cell clusters can be selected out of the total population by sieving is greater than 90% and relatively synchronous in development, at least through the early stages of morphogenesis. In present study we focus on the origin and developmental patterns of somatic embryos from freely suspended cultures as the initial cell by serial observation.

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MATERIALS AND METHODS

Plant Material and Culture Methods

A top cutting of 5-7 leaves emanating from *E. hyssopifoium* collected during the month of August in a natural forest

segment of botanic garden Gulbarga University, Gulbarga, Karnataka, India was used as an explant. The cuttings were washed in 1% (v/v) Labolene (Labolene India Pvt Ltd., Mumbai) detergent for 5-7 minutes and then in running tap water for 15 minutes. Surface sterilization was done by immersion in 0.1% (w/v) mercury chloride for 3-5 minutes and followed by 4-5 washes in sterile distilled water. Leaf explants of 0.5-1.0 cm were dissected out washed once in sterile water and blotted over sterile filter paper discs before transfer to nutrient medium. The explants were implanted abaxial surface on the medium. The nutrient medium contains salts and vitamins of Murashige and skoog's, 1962 medium supplemented with 3% (w/v) sucrose, 0.7% agar and the medium was adjusted to pH 5.7 before autoclaving at 121°C for 20 min. Varied concentration (0.5 – 2.5 mgl^{-1}) of 2, 4-Dichlorophenoxy acetic acid, NAA, and Indole-3 acetic acid (IAA) alone or in combination with BA, Kinetin (Kn) and Zeatin (0.5 – 2.5 mgl^{-1}). Once the optimum phytohormone concentration had been established for callus induction, calli were maintained on MS medium supplemented with and 0.5 mgl^{-1} NAA + 1.0 mgl^{-1} Ze. For each subculture 250 mg of fresh callus was transferred.

Induction of Embryos Formation

Suspension culture was initiated by transferring 250 mg calli into a 250 ml Erlen-meyer flask containing 30 ml of the same liquid MS medium and maintaining by subculture at 1:4 (suspension: fresh medium). All cultures were incubated in a culture room maintained at 25 ± 2^0c, RH 50-60% and 14 hr photoperiod, a photon flux density of 50-60 $\mu Em^{-2}s^{-1}$was provided by cool white fluorescent tubes (Philips India Ltd., Mumbai). The method of induction of embryo formation was induced by transfer of the cell clusters to a medium containing zeatin and lacking auxin (embryo-inducing medium). Cell clusters and embryos were transferred to a counting chamber. The number of cell clusters and embryos was counted under an inverted microscope.

Fractionation Of single Cells

A stock cell suspension culture showed a heterogeneous population composed of single cells and small and large cell clusters with different sizes and densities. Single cells from this population were collected as follows. The suspension was cultured for 14 days in medium containing NAA+ Ze until it was a very high density, and then passed through iron sieves of 74 µm size. The filtered cells were collected by density gradient centrifugation and resuspended in growth regulator-free medium. To directly observe the early morphogenetic events from the suspended single cell or a few cells, the cells were extracted using a handmade pipette under an inverted microscope, transferred to micro chamber (10x10x3 mm) with paraffin walls containing the same medium on the glass slide and then observed serially by the stereo microscope (Yong Eui Choi and Woong Young Soh, 1997).

Effect of Auxin and Cytokinin

The single cell or small cell cluster collected by the above procedure were transferred to various media. The cells were cultured in each medium for 14 d (preculture period). After this culture period, all the cells or cell clusters were collected and washed with the basal medium. The number of embryos counted after 14 d culture in the embryo-inducing medium. The cells were cultured in a medium without auxin, no embryos were formed after transfer to the embryo-inducing medium. When auxin was added to the preculture medium, embryos were induced. This suggested that auxin was essential for single cells to form embryogenic cell clusters. MS medium supplemented with 0.5 mgl^{-1} (NAA) + 1.0 mgl^{-1} Ze was most effective and caused a higher frequency of embryogenesis.

RESULTS

The leaf segments were cultured on MS medium containing NAA (0.5 mgl^{-1}) in combination with different concentrations of BA, Kn and Ze (0.5-2.5 mgl^{-1}) induced somatic embryos and the per cent responses are summarized in Table - 1. Suspension culture was initiated by transferring 35 days old callus onto 250 ml Erlen-meyer flask containing 30 ml of liquid MS medium supplemented with 0.5 mgl^{-1} NAA + 1.0 mgl^{-1} Ze and maintained by subculture at a 1:4 (suspension: fresh medium) dilution for a 14 days interval. The filtered suspension just after transfer to medium lacking NAA but fortified with 1.0 mgl^{-1} Ze consisted of a cell mixture of two to five cells dividing at random and heterogeneous single free cells. Most of embryogenic cells (Fig.2- A&B) were about 25 µm in size, richly cytoplasmic and contained abundant starch grains except for some single cells which are vacuolated and elongated from 30 µm to 60 µm (Fig. 2-B). After two-week culture proembryo like two-several celled structures appeared in suspension (Fig. 1, B-J) but the other single cells were larger in number and elongated further by 100-200 µm. The proembryo like cells showed the characteristics of transverse and longitudinal cell division without conspicuous cell enlargement. Cell contained dense cytoplasm with numerous starch grains similar to zygotic embryos. From the serial observation, the pro embryo – like structures were classified into four groups single-celled, two-celled, and four-celled or pre globular stage (Fig. 1, B-F). Somatic embryogenesis was not observed from single free cell. The cells of two-celled structures developed directly into globular somatic embryos at a rate of 48%, in four-celled proembryo like cells, about 80% of them were developed into globular somatic embryos (Fig. 1-F) (Table- 1). About the developmental pattern of proembryos, the embryo proper was most frequently intercalary positioned cells of filamentous proembryos, thus suspensor was observed from the lower end of embryo proper (Fig. 1, G-J) and proembryos derived from such embryo proper gave rise to viable embryos. Most suspensors were highly vacuolated compared to embryos proper and were constituted uniseriated cell structures of various sizes (Fig. 1, G-J). The suspensor divided randomly and persisted until the late globular stage (Fig. 1-H).

DISCUSSION

After two week incubation in NAA omitted medium, but fortified with Ze, pro embryo like structures in a freely suspended state were observed, interspersed among elongated single cells. The promotive effect of Zeatin on embryogenesis at every phase, being most effective during phase 2, when active cell division occurs has been reported (Atsushi Komamine, 2001).

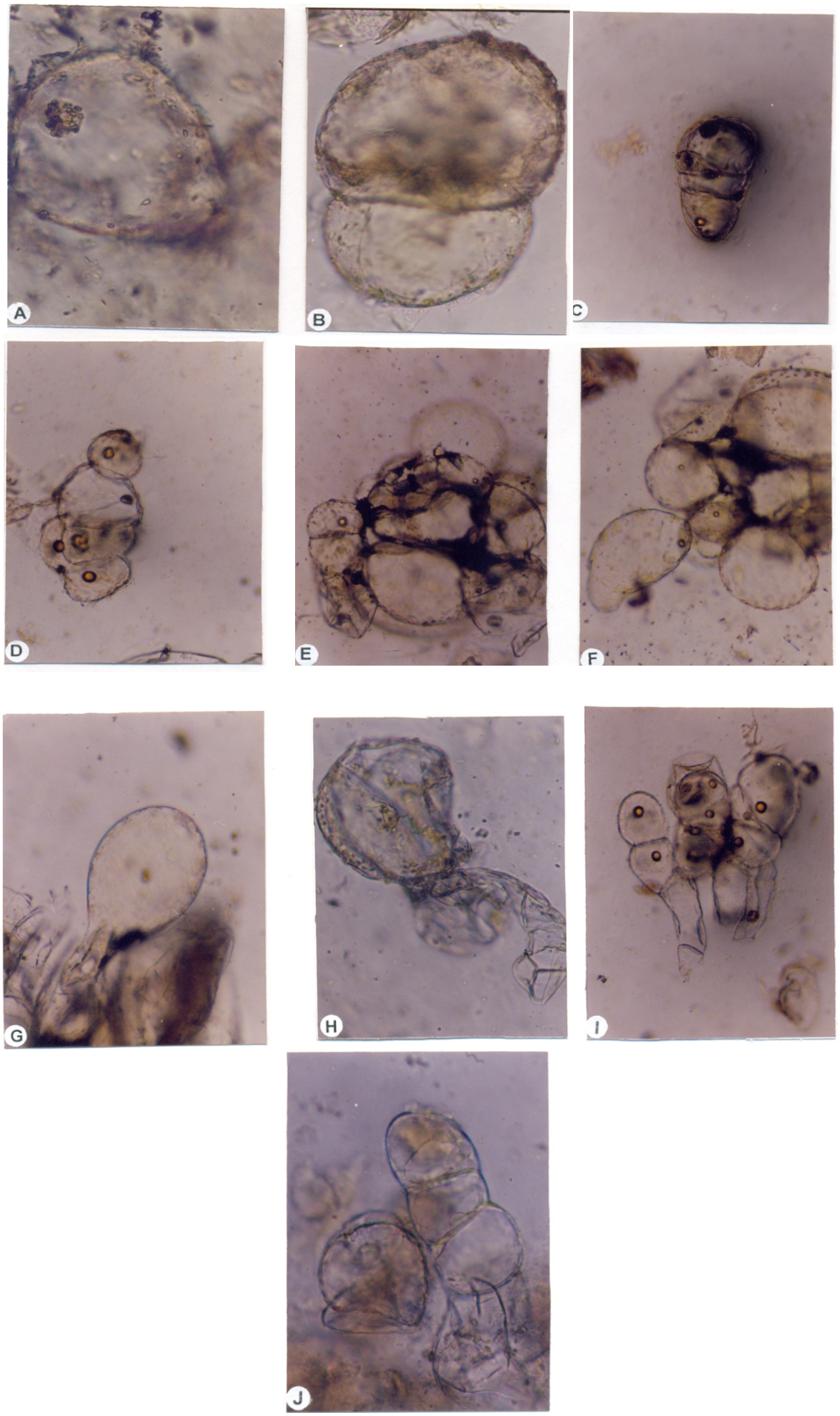

Fig. 1. Serial observation of early somatic embryogenesis from isodiametric two-celled prembryos
A. A single celled proembryo; B: Two celled proembryos, C-D:Four celled proembryos. E: Proembryos with embryo proper. F: Embryo proper derived from intercalary positioned cells of filamentous proembryos resulted into globular embryo with suspensor, G :Globular embryos with vacuolated suspensor like structure, H-I: Embryos with obvious suspensor, J: Heart shaped embryo

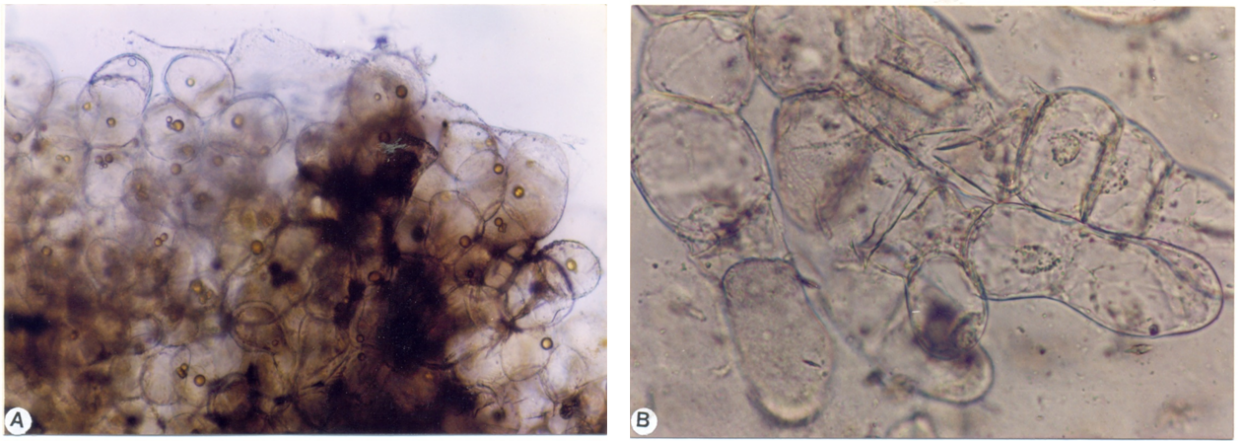

Fig. 2. Structural comparison between embryogenic cells and proembryo like structures

A: Embryogenic cell with dense cytoplasm and starch grains among elongated nonembryogenic cells in the filtered suspension just before transfer to NAA free medium and fortified with Ze. B : Proembryo like structures presented among the highly elongated non embryogenic cells for two week of culture

Table 1. Frequency of formation of somatic embryos from suspension culture of four different stages of pro–embryo like cells

S. No.	Nutrient medium	Hormones (mgl^{-1})	Stages of somatic embryos	Per cent of somatic embryos (%)
1.	MS	NAA (0.5) + Ze (1.0)	one celled	--
2.	MS	NAA (0.5) + Ze (1.0)	two celled	43
3.	MS	NAA (0.5) + Ze (1.0)	four celled	80
4.	MS	NAA (0.5) + Ze (1.0)	preglobular	91

We investigated whether the proembryo like cells were truly proembyros, or merely embryogenic clumps, using serial observation under stereomicroscope. Most of the proembryo like cells are developed directly into successive stages of somatic embryos without any intervening embryogenic cell clump formation. The results suggested that the proembryo like cells were truly proembryos. Numerous authors have described cellular structures from embryogenic cells and from suspended cells resembling early stages of zygotic embryos (Halperin, 1966; Haccius, 1978; Nomura and Komanine, 1985; McWilliam *et al.,* 1974). However very few reports on sequential developmental stages from two or few celled structure into mature embryos. Our observations traced somatic embryogenesis back from a suspended two celled structure. In the present experiment two to few of cells cultured on medium omitted auxin and fortified with Ze did not divide and eventually elongated in the same medium. This suggests that the first embryogenic division from single cells requires exogenous growth regulators such as NAA to some extent. A significant literature on auxin biosynthesis, metabolism, and transport in embryos that has grown out of extensive analysis of carrot somatic embryos (Schiavone and Cooke, 1987; Michalczuk *et al*., 1992a, 1992b) shows that auxin appears to play important roles both in the induction of embryo development in culture and in the subsequent elaboration of proper morphogenesis in embryo development. The role of exogenous auxin in somatic embryo induction appears to depend on the nature of the explant used in the experiment. For example, petiole explants (Ammlrato, 1985), hypocotyl explants (Kamada and Harada, 1979), and single cells isolated from established suspension cultures (Nomura and Komamine, 1985) require exposure to exogenous auxin for 1, 2, or 7 days, respectively, before they are competent to undergo embryogenesis upon auxin removal. Whether auxin treatment stimulates more than just cell proliferation as a prerequisite to embryogenesis is unclear. Microcallus cells of alfalfa require only a short (a few minutes to a few hours) pulse of auxin before they are competent to initiate embryogenesis in hormone-free medium (see Dudits *et al*., 1993, for review). In carrot suspension culture, Nomura and Komamine (1985) reported that embryonic clumps formation from single cells took place in medium containing 2,4-D. In our observation of early segmentation pattern of somatic embryogenesis, embryo proper were most frequently intercalary positioned cells of proembryos, thus suspensor like structures were observed on lower end of the embryo proper. McWilliam *et al.,* (1974) described that cell division sequence of early somatic embryos in suspension cultures of carrot corresponds to crucifer type. The present experimental study pattern was closer to the crucifer than to Solanad or Asterad types. In the present observation a distinct suspensor of variable size and shape was present.

The size of the suspensor was determined by whether the embryo proper was initiated at a few-celled embryo stage or after a long filamentous stage. In Haccius and Bhandari's (1975), opinion the suspensor like figure of proembryos is nothing more than a rudimentary proembryonal cell complex capable of forming super numerary embryos. The cells of unorganized embryogenic clumps separated from each other after rapid vacuolation and elongation in auxin ommited medium. But in our study suspensor like structures of embryos remained without conspicuous structural changes until heart shaped stages. This means that the suspensor cells were not

unorganized embryogenic clumps but were truly a part of somatic embryos. Similar results have been reported with carrot tissue culture (Back-Husemann and Reinert, 1970).

Conclusion

In conclusion, it can be considered from the present work that there are at least two phases in somatic embryogenesis from single cells, and determination for embryogenesis may occur in the first phase. Exogenous auxin was required for the determination, but once the determination occurs, auxin is inhibitory for development of the embryogenic cell clusters formed in the first phase to embryos. The system established here is a useful system for studies of the whole process of differentiation from a single cell to a whole plant, especially for analysis of the early process of embryogenesis, that is, for the investigation of how embryogenesis is determined.

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