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## Somatic Embryogenesis and Embryoids Formation in Chicory (*Cichorium intybus* L.)

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Malik, B., Pirzadah, T. B., Abdin, M. Z. and Rehman, R. U. (2017). Somatic embryogenesis and embryoids formation in chicory (*Cichorium intybus* L.). International Journal of Agricultural Technology 13(1):91-104.

**Abstract** The *Cichorium intybus* L. (Chicory) regenerated from the leaf explant of the *in vitro* grown-seedlings. Callusing was induced on Murashige and Skoog (MS) medium containing IAA (2.0 µM) + KN (5.0 µM) + CH (1000 mg/l). The maximum callusing observed on this medium was 85%, while only 71% showed regeneration. One week old friable calli were taken and fixed for scanning electron microscopy (SEM) and sectioned/stained for light microscopy. Definite pro-embryonic structures could be seen in or emerging from the edges near the wounded region. The morphological sequence of events tracks the *de nova* production of shoot bud by the process of organogenesis. The sequence of morphogenic events include presence of meristemoids; growth extending; and presence of several leaf primordia. Embryo development occurs through exceptionally organized sequence of cell division, enlargement and differentiation. During early development the embryo assumes a clavate, globular shape and remains essentially an undifferentiated, but organized mass of dividing cells with a well-defined epidermis. It has been observed that the somatic embryos were formed from hypo-cotyledonary epidermic cells and also from superficial embryogenic cells of pre-embryonic complex situated on peripheral and on internal cell layers of the callus. Diverse morphological differences were observed among somatic embryos at different developmental stages.

**Keywords:** *Cichorium intybus* L. *In vitro* culture; callus; embryoids; SEM; somatic embryogenesis

### Introduction

During somatic embryogenesis, somatic cells develop into whole plants through characteristic morphological stages that resemble sexual or zygotic embryos, such as globular, heart and torpedo stage embryos, in dicotyledons. This process does occur *in vivo* (i.e. "apomixis"), but mostly it is induced *in vitro* from explants through manipulation of the culture medium, as was initially described for carrot (Stewart *et al.*, 1958). Since somatic embryogenesis in principle is a versatile method of rapidly and massively

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reproducing whole plants, ways have been developed to initiate somatic embryogenesis in many different plant species. For reasons unknown so far, the yields of somatic embryos in some plant species is low. This is also true for the economically interesting cultivars of chicory. In contrast to these recalcitrant cultivars, explants from the *Cichorium intybus* L., var. *sativum* 'C. *endivia* L., var. *latifolia* hybrid clone '474' appeared to become highly embryogenic upon applying somatic embryogenesis-inducing culture conditions (Dubois *et al.*, 1988; 1990; 1991; Guedira *et al.*, 1989). This, together with the speed of embryo formation (less than 12 d) and the abundance of embryos produced (12 embryos per mm<sup>2</sup> of leaf tissue) made clone '474' attractive as a material to further investigate somatic embryogenesis in *chicory*, even more so, since by including glycerol in the embryo-culture medium (induction medium) the first cell division of the embryogenic cells can be delayed. Transferring the explants to medium without glycerol (expression medium) results in synchronized initial cell divisions of the induced cells (Robatche-Claive *et al.*, 1992). Unlike in many other plant species, somatic embryogenesis (SE) in *Cichorium* is direct and arises from single cells (Vasseur *et al.*, 1995). Also, the exogenous addition of spermine (spm) and putrescine (put) had a positive effect on improving the yield of somatic embryos in chicory but also in other SE models (Wu *et al.*, 2009). In various models of SE, the exogenous supply of abscisic acid (ABA), a well-known stress hormone of plants, also led to increase the incidence on somatic embryogenesis (ISE) (Sholi *et al.*, 2009). Also, the report of an extracellular matrix connecting cell wall-plasmalemma-cytoskeleton led to the hypothesis that the cell wall could be sensitive to osmotic stresses (Seifert *et al.*, 2010). Supporting this possibility, several groups reported that treatments with plasmolysing agents led to increase the ISE of gymnosperms (Attree *et al.*, 1991). Numerous studies have reported that soluble molecules are released in the medium during the acquisition of totipotency. These enriched medium, called conditioned medium (CM), contain factors able to promote the SE of primary explants (Tsuwamoto *et al.*, 2007). In SE models involving cell suspensions, the co-culture of embryogenic and non-embryogenic lines led to increase SE competencies (Komamine *et al.*, 1990). However, the effect of co-culturing tissue explants with different ISE has not yet been investigated. Somatic embryogenesis (SE) in *Cichorium* involves dedifferentiation and re-differentiation of single cells and can be induced by specific *in vitro* culture conditions. Jean-Paul *et al.* (2012) studied the effect of various treatments on the incidence of SE (ISE) of an interspecific embryogenic hybrid (*C. endivia* x *C. intybus*) and of different commercial chicories (*C. endivia* and *C. intybus*) that are typically recalcitrant to SE in standard culture conditions and found that the ISE of the hybrid is significantly increased by pretreatment of tissues by

submersion in solutions of glycerol, abscisic acid, spermine, putrescine or of combinations of these compounds. Interestingly, the most efficient of these pretreatments also had an unexpectedly high effect on the ISE of the *C. intybus* cultivars.

The aim of this paper was to study the somatic embryogenesis in wounded leaves of *Cichorium intybus* with emphasis being laid on various morphological events leading to the formation of pro-embryos and leaf primordia.

## **Materials and methods**

### ***Plant material***

The plant material was *Cichorium intybus* L. maintained *in vitro* as described by Rehman *et al.* (2003). Leaves from the four week old seedling was incised crosswise 2-3 times through the main vein were placed in petri-dishes each containing half-strength MS medium (3% sucrose) supplemented with vitamin-free casein hydrolysate (CH, 1000mg<sup>-1</sup>), and gelled with 6g/l agar. MS medium is also supplemented with different growth regulators: Indole-3-acetic acid (IAA), kinetin (KN). The pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 120 °C. The cultures were incubated at 27 °C under 16/8 hours (light/dark) photoperiod with white florescent light flux of 150-200 μE m<sup>-2</sup> S<sup>-1</sup> and relative humidity of 50-60 %. The Cultures were observed after 10 days under light microscopy for the presence of different differentiation phases.

### ***Light Microscopy and SEM analysis***

One week old calli were fixed in FAA, embedded in paraffin, sectioned and stained with Safranin. The slides were viewed and photographed under Olympus Vanox S AHB 2, (Japan) light microscope. The samples collected from the *in vitro* grown cultures were fixed in 2.5 % glutaraldehyde overnight. Glutaraldehyde is normally supplied as 2.5 % or 50 % solution and to prepare 500 ml of 25 % glutaraldehyde upto 250 ml of 0.2 M phosphate buffer (pH-7.2) was added and the mixture was stored at 4 °C. For SEM fixed samples were transferred into phosphate buffer (pH -7.2) and washed thoroughly 2-3 times and then dehydrated in gradual series of acetone. The critical point of CO<sub>2</sub> is 31.5 °C at 1100 psi Freon 13-28 °C at 560 psi (pound per square inch).

### ***Coating of samples***

Metal film coating of SEM samples were carried out under vacuum in an inert atmosphere using Argon gas; coating of uniform thickness was obtained by evaporating the metal (target) on to the specimen kept at specified distance (cathode specimen distance). Gold was used as a target and coating of about 35 nm (350<sup>0</sup>Å) thickness was obtained under the following conditions: current 21.5 mA; pressure 0.05 m bar; gas Argon; distance cathode specimen 30 nm; time 1min. The samples were observed and photographed under SEM (LEO 435 VP); 35mm fast film (125, 200 or 400 ASA) were used. Exposed films were developed in D-76 fine grain develop, fixed in Agfa 301 fixer, washed in running water and dried.

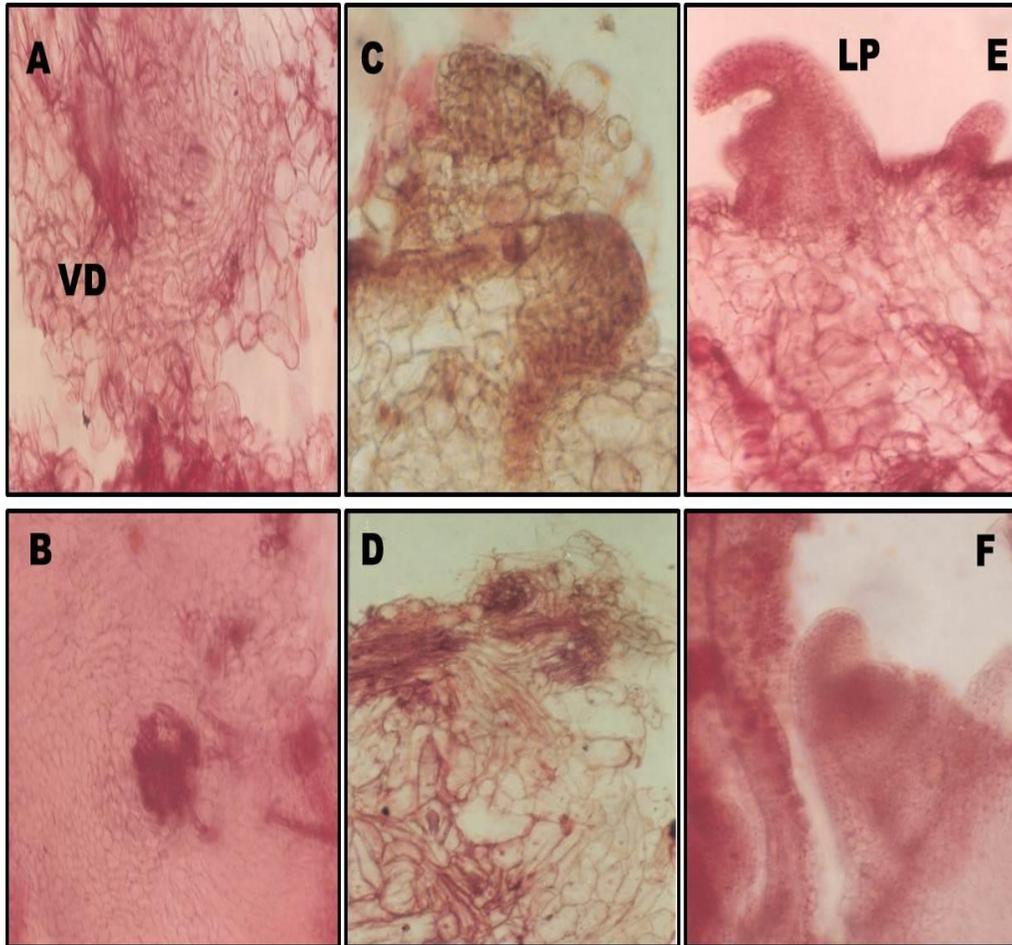
### **Results**

The initiation of the embryonic cells requires *in vitro* culture of the appropriate explants on medium that contains specific plant growth regulators (PGRs). Somatic embryos were always present on both basal and distal sides of the sections. The pro-embryonic cells underwent successive division leading to the formation of isodiametric cells, the dedifferentiation of pro-embryonic cells leads to the embryo formation of various types.

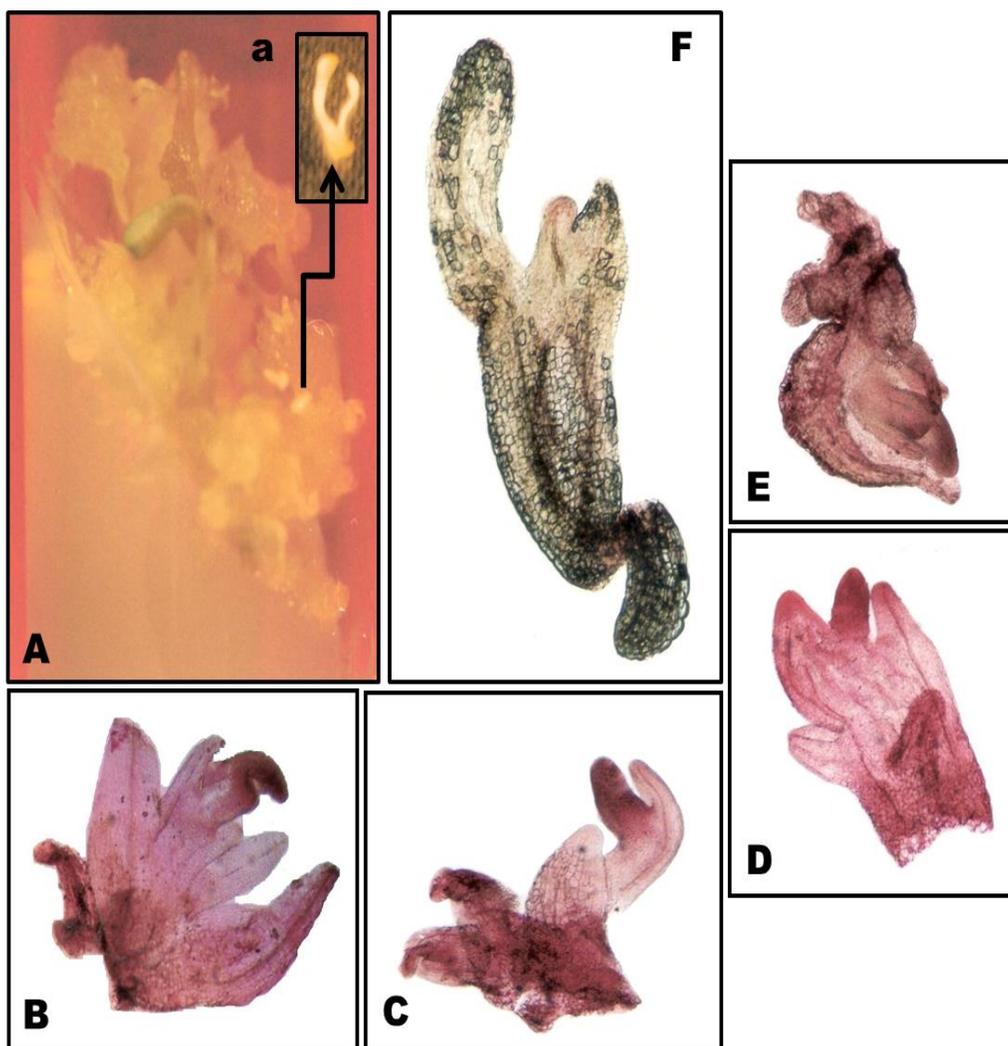
### ***Histological Examination***

The light microscopy was performed on one-week-old callus from the leaf explant grown on MS medium. The following developmental stages could be identified. Vascular differentiation occurs near the wound of the developed callus forming meristematic aggregate and epidermal region has number of cells which are isodiametric (Fig1, A); Section through callus showed a single layered epidermis with rectangular cells; the cotyledonary tissue is parenchyma with cells larger than epidermal cells. The parenchyma is seen traversed by the vascular bundles. Presence of meristemoids as denoted by the darkly stained nuclei in the clustered group of small cells composed of non-vacuolated, compactly arranged as being pre-embryonic complexes (Fig1, B); It was clearly observed that the meristematic region is rapidly increasing in size (pre-embryonic complexes). These regions formed by the thick walled cells displays a well-defined demarcation line between embryonic cells of the meristematic region and the larger vacuolated cells of the callus and thus served as a source of somatic embryos (Fig1, C); rapidly developing bud meristem beginning to protrude above the surrounding which clearly showed that the somatic embryos

were originated from the superficial cells of the meristematic regions (Fig1, D); meristematic dome with leaf primordia protruding high above the surrounding epidermis and the transition from the compact mass to meristematic primordia occurred at the periphery of the lobes accompanied by cell dedifferentiation and also numerous anticlinal and periclinal divisions followed by successive random oriented divisions in the dome to the left and right of the centre of the dome can be seen. Incompletely developed adventitious buds enclosing outer primordial leaves and procambial strands that are visible in the area of leaf primordia with vascular tissue differentiation was also observed (Fig1, E, and F). Embryoids are initiated from single high cytoplasmic cells of the leaf epidermis of plantlets of *Cichorium intybus* L. derived from callus cultures. Plantlets derived from such callus embryoids have the ability to initiate embryoids along their hypocotyl surface. Callusing was induced on MS + IAA (2  $\mu$ M) + KN (5  $\mu$ M) + CH (1000 mg/l) resulted in embryogenic callus formation with friable nature. After two weeks of culture subjected to darkness, somatic embryos were developed on the callus which are white or pale yellowish concentric circle like tightly compact mass of cells and are easily crumbled (Fig. 2A). Light micrographs of embryoid formation with different structures are as follows: Somatic embryos (one, two or more) are linked by their basal portion to the pre-embryonic complex with multicotyledonary cylindrical somatic embryos. These embryos show morphologic variations; trumpet and cotyledonary somatic embryos with or without shoot apex (Fig. 2B); Bulboid/rhizoid type of structures having meristematic dome and leaf primordia with definite vascular strands that linking the surface cells of proembryoids (Fig. 2C,D); Somatic embryos with multiple foliar structures (Fig. 2E). Usually cylindrical embryos are weakly developed shoot apex while as only meristematic cells which are densely stained were observed. Somatic embryos showing a suspensor like structures with the meristematic regions at the caulinar end (Fig. 2F). On one hand it was observed that the somatic embryos were linked by a narrow organ similar to suspensor and appeared to be originated superficially. But on the other hand the one that showed wide connection with the callus may be originated from the meristematic region situated at the inner area of the callus.



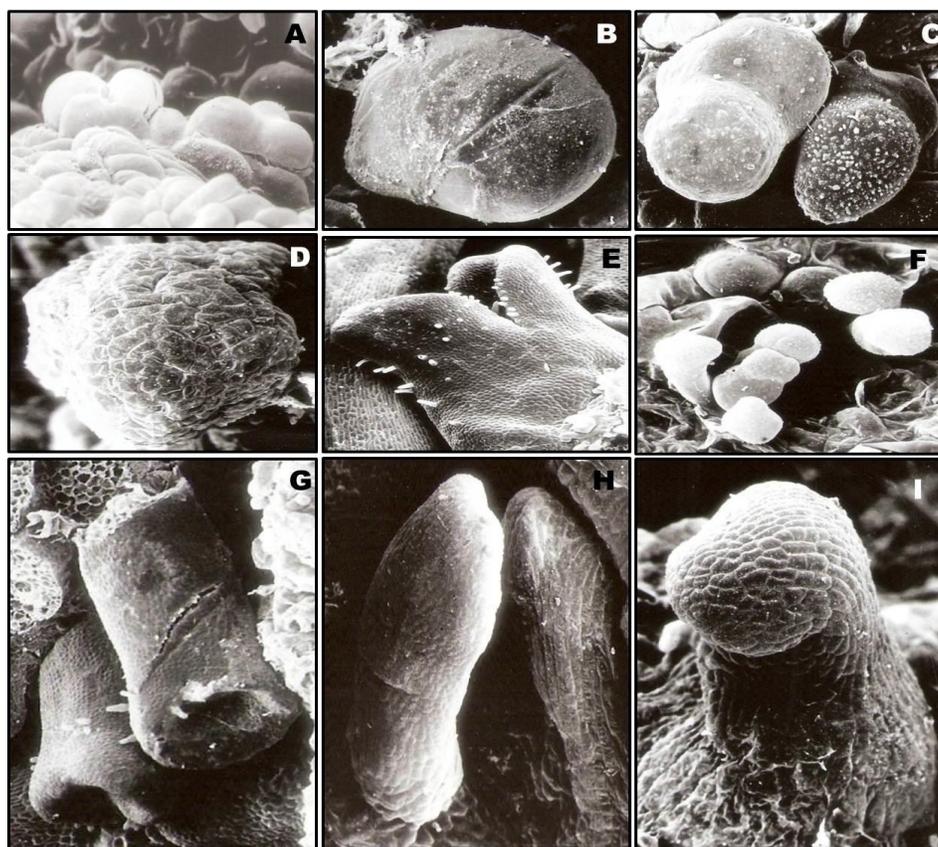
**Figure 1.** Light micrographs of cultures of leaf segments after 10 days. Medium: MS + IAA (2 $\mu$ M) + KN (5 $\mu$ M) + CH (1000mg<sup>-1</sup>). Different stages are: (A) Vascular differentiation (VD)(B). Presence of meristemoids. C). Meristematic regions are increasing (D). Protruding bud meristem. (E and F) Meristematic dome with leaf primordia (LP).



**Figure 2.** Light micrographs showing diverse morphological differences among somatic embryos: (A) Callus forming white or pale yellowish somatic embryos (B).Embryos having multicotyledonary cylindrical somatic embryos (C, D) Bulboid/rhizoids structures linking surface cells of pro-embryoids (E) Somatic embryos with multiple foliar structures (F). Somatic embryos showing a suspensor at the caulinar end.

### *Scanning electron microscopy*

SEM was performed on one week old callus from the explant grown on MS + IAA (2 $\mu$ M) + KN (5 $\mu$ M) + CH (1000mg/l). One week old callus developed numerous white globular somatic embryos highly friable in nature that could be easily isolated. This callus showed a variety of structures viz: irregular shaped cells, few-celled aggregates mainly at the periphery and cultures of meristematic cells and embryo structures at different developmental stages. The following developmental stages could be identified from the scanning electron micrographs. The globular proembryos emerging from the surface of callus appeared as protuberances (Fig. 3A), but the majority of the embryos were inserted in the leaf and some bipolar embryos were floating in the medium having small size and dense cytoplasmic contents whereas, the buds are always linked to the callus. Some of the globular structures get converted into cotyledonary embryos while some of the cells remain same. A globular proembryo with the slit in the centre is also seen clearly (Fig. 3B) and an embryonic bud emerging from the callus at the state of transition from globular to heart shaped embryo (Fig. 3C). The cultured leaves also shows a clavate globular shaped proembryo which is embedded in a fibrillar network that links the superficial cells of the proembryos (Fig. 3D). Meristematic budding took place at periphery of the compact mass and the newly formed compact mass further develops and the proliferation extended to nearby areas until most of the embryo body was covered. Some buds are also emerging on the callus near the wound these are: embryonic bud with two cotyledons and radicle ends (Fig. 3E), the first visible sign of proliferation that was observed is the slight thickening of an area at the apical portion of the hypocotyl, when the mother embryos were at a very early cotyledonary stage. At this stage, the outer cell layer of hypocotyl consisted of a single layered epidermis of radially oriented cells and underlying cortical cell layer of longitudinal oriented cells. Cylindrical and dicotyledonary buds (Fig. 3F) Asynchronous embryos (Fig. 3G) and embryos with flattened cotyledons and subtending hypocotyl. The proliferation always takes place when the epidermis of the embryo gets detached from the underlying tissue, although the cells still represented meristematic appearance (Fig. 3 H, I). Modification of the explant leads to the complete discontinuity of hypocotyl protodermis and each single protodermic cell give rise to somatic embryos. The globular structure further developed into mature embryos with two poles a root and a shoot meristem.



**Figure 3.** SEM of one week old cultures of leaf segment of *Cichorium intybus*. Medium: MS + IAA (2 $\mu$ M) + KN (5 $\mu$ M) + CH (1000mg<sup>-1</sup>). Different stages are: A) Globular proembryo emerging out from the surface of callus. B) Globular proembryo with a slit in the centre. C) Embryonic bud emerging from the callus D). Clavate proembryo embedded in fibrillar network. (E) Bud with two cotyledons F). Cylindrical and dicotyledonary buds G). Asynchronous embryos. H-I). Embryos with flattened cotyledons and subtending hypocotyl. Bar = 100 $\mu$ m (A); Bar = 20 $\mu$ m (B,C); Bar = 20 $\mu$ m (D); Bar = 100 $\mu$ m (E); Bar = 30 $\mu$ m (F); Bar = 100 $\mu$ m (G); Bar = 30 $\mu$ m (H); Bar = 20 $\mu$ m (I).

## Discussion

Embryonic cultures constitute an important tool for the study of plant development, both due to the unique convenience of *in vitro* cultures over *in vivo* growth. According to the Hicks (1980), there are two developmental sequences leading to organogenesis. They differ in the presence or absence of a

callus stage in the organogenic sequence of events. A developmental sequence involving an intervening callus stage is termed as “indirect” organogenesis. In the present work, callus developed on MS medium from the leaf explant were taken and observed under light microscopy for the presence of different differential phases. The physiological state and the regenerative ability can be affected by the origin of callus cells that vindicate the necessity for the study of morphological events in the initial stage of callogenesis (Oka *et al.*, 1995). These sequences of events that lead to organogenic shoot bud formation are in accordance with conifer which is widely applicable to most plant species producing adventitious buds (Thorpe, 1980; Yeung *et al.*, 1981). At the same time, obvious tissue differentiation begins with the development of embryonic vasculature and accumulation of intracellular storage substances (Puigderrajols *et al.*, 2000). Final stages of development towards maturation are distinguished by overall enlargement and increase in cotyledon size in dicots (Gray, 2000). In plants, pattern formation is not confined to embryogenesis, because morphogenesis continuously occurs during postembryonic development. The primary meristem of the shoot and the root, which are the two pattern elements that produce the structure of the adult plant are generated in the embryo (Canhoto *et al.* 1996). Earlier studies have also found that the embryo develops from the small aggregates of embryonic cells originated from single cytoplasm –rich cells (Yeung, 1995). Nevertheless, the whole developmental sequence of embryogenesis was fully documented in carrot suspension culture (Yasuda *et al.*, 2000). New insights into embryonic pattern formation have been achieved over the last decade by perturbing the *in vitro* development of isolated zygote or somatic embryos with Auxin and selected substances (Chasan, 1993). Isolated somatic cells can develop normally into embryos, irrevocably demonstrated that the development programme for embryogenesis is contained within and controlled by the cell itself and not by external factors. However, the exact nature of the triggering mechanism of embryogenesis whether it is physical, biochemical, and/or genetic events is unknown (Bajaj *et al.*, 1988). Mix (1985) using a wide range of modified Gamborg and MS culture medium to induce callus and plant regeneration from segments of the leaf vein of Chicory observed embryogenesis in the calli, but stated that the occurrence of organogenesis as well as embryogenesis is largely dependent on the physiological condition of the donar plant. Auxin, indole acetic acid (IAA) a natural plant hormone serves to induce the formation of embryogenic cells, possibly by initiating the differential gene activation, through repetitive cell division, while simultaneously suppressing cell differentiation and growth (Pinto *et al.*, 2002). Cytokinin, like KN is also required to induce embryogenesis in many dicotyledonous species. Earlier studies also found

similar white globular structures from callus obtained from petiole explant cultured on MS + KN ( $1.5\text{mg L}^{-1}$ ) + IAA ( $0.5\text{mg L}^{-1}$ ) + CH ( $500\text{mg L}^{-1}$ ) of Indian chicory (Abdin and Ilah 2007) and also bipolar somatic embryos were developed on the surface of the embryonic calli in *Emilia zeylanica* (Jayachandran *et al.*, 2009). However, Decout *et al.*, (1994) induced somatic embryogenesis by perturbing the physiological condition (i.e. light and temperature) and observed the effect of temperature on embryogenesis of *Cichorium* hybrid and found that somatic embryogenesis at  $20\text{ }^{\circ}\text{C}$  and  $25\text{ }^{\circ}\text{C}$  promotes the development of callus and shoots instead of direct somatic embryogenesis at  $35\text{ }^{\circ}\text{C}$ . At  $30\text{ }^{\circ}\text{C}$  all types of morphogenesis are observed. Shoot and embryoid promotion takes place with the shift of hormone balance in the medium for *Sorghum bicolor* (Wernicke *et al.*, 1982); *Solanum carolinense* (Reynolds 1986); *Glycine max* (Barwale *et al.*, 1986) and *Stylosanthes scabra* (Dornelas *et al.*, 1992). Fibrillar network was also observed around proembryos in coffee (Sondahl *et al.*, 1979; Nakamura *et al.*, 1992) and in *Citrus* (Belkoura *et al.*, 1993). The first observations of a fibrillar network linking the surface cells of the proembryos of *Cichorium* were made in critical-point-dried roots, fixed with 1 % aqueous OsO<sub>4</sub> (Dubois *et al.*, 1990). A more conventional fixation procedure including a pre-fixation with glutaraldehyde in PBS buffer, followed by OsO<sub>4</sub> postfixation gave similar results. Fixation, dehydration and critical-point drying may cause artefacts, but also remove the network-like feature (pectic strands), observed on cryo-SEM surface cells of white clover nodules (Webb and Sheehy, 1991). It has also been found earlier that the proliferation takes place only when epidermis of the embryo detaches from underlying tissue (Cruz *et al.*, 1990) in *Feijoa sellowiana*. Pre-embryonic complexes forming meristematic aggregates composed of non-vacuolated, small cells that are compactly arranged were also observed in *Phoenix dactylifera* (Tisserat and DeMason, 1980). As observed that there is difference between the embryonic cells of the meristematic region and the vacuolated cells of the callus, the somatic embryos were originated from embryonic cells settled on the surface. These same remarks were made by Oka *et al.*, (1995) in *Hordeum Valgare* and in *Japonica Lonicera* (Burciela and Vieitez, 1993). One of the earlier studies found that the somatic embryos were linked by an organ similar to suspensor and also revealed that there is diverse morphological variation among the somatic embryos in *Carica papaya* (Fernando *et al.*, 2001). The pro-embryonic cells undergo successive division leading to the formation of isodiametric cells, the dedifferentiation of pro-embryonic cells leads to the embryo formation of various types. Observations of *Cichorium* by light microscopy did not show details on the surfaces of the proembryos, because of the very high cytoplasmic density of these small cells. In the present study however, scanning

micrographs taken from one-week-old calli developed from the leaf explant showed numerous pattern formation of embryogenesis. Some other important patterns of embryogenesis were not observed, this could be because morphogenesis occurs continuously during pro-embryonic development.

## Acknowledgements

RUR is thankful to Hamdard National Foundation for financial assistance.

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(Received: 12 December 2016, accepted: 8 January 2017)