Cytological studies on unicellular origin pathway, early ordered cell division patterns and morphogenesis in indirect somatic embryogenesis of *catharanthus roseus* cell lines.

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ABSTRACT

Unicellular Origin of the Madagascar periwinkle indirect somatic embryogenesis developmental pathway (ISEDP) were studied and the conformation of embryogenic stability potential of suspension culture cell lines as well. We investigated the ordered pattern of cell division and early histo-differentiation over time, using light microscope. The ontogenesis of indirect somatic embryogenesis of callus was first induced from hypocotyls of in vitro germinated seeds on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxy acetic acid or in the (MS) basic medium amended with 1mg/L NAA and 1.5mg/L BA and characterized through cytological analysis of different phases of embryogenesis from the first cellular division to the last step of their development. Our investigation and observations based on basic smeared modified technique that enables us to conclude that: the onset and the ontogenesis of indirect somatic embryos developments (ISED) of *C.roseus* arise from one single embryogenic characterized cell that is very similar to zygotic cell, capable of complete proliferation through different ordered cell division patterns (e.g., transverse asymmetric division within the embryogenic cell; longitudinal division within apical cell; symmetric transverse division in suspensor cell and periclinal division to form protoderm in the outermost layer of the globular-stage), and complete their differentiation to form different morphogenetic developmental stages described: oblong-shape; heart-shape; torpedo-shape; elongated -shape "enlarged stage" and cotyledonary-shaped embryos. The morphogenesis, structure and other analogies details with the development of dicotyledon zygote are also emphasized. This finding is of considerable interest, significance, and the basic prerequisite before plant genetic improvements based genetic transformation; somatic hybridization and mutation breeding to enhance TIA yield can be employed. This study is to our knowledge the first works on *Catharanthus roseus* somatic embryogenesis clearly presenting and describing unicellular origin pathway; sequential events in early and advanced developmental stages including oblong and elongated transition stages which have rarely been reported in the majority of plant species.

Keywords: *Catharanthus roseus, somatic embryogenesis, unicellular pathway, cytology, ontogenesis, cyto-morphogenesis*.

Abbreviations: BAP, benzylaminopurine; BA, benzyl adenine; 2,4-D, 2,4-dichloro-phenoxy-acetic acid; GA3, gibberellic acid; IAA, indole acetic acid; MS, Murashige and Skoog; NAA, naphthalene acetic acid; TIAs, terpenoid indole alkaloids

INTRODUCTION

The Madagascar periwinkle [*Catharanthus roseus* (L.) G. Don] is one of the important medical plants of the genus Catharanthus, known for its biosynthesis of more than 130 alkaloids, many of which are pharmaceutically important such as ajmalicine and serpentine are prescribed for the treatment of hypertension, whereas the bis indoles vinblastine, vincristine and 3_,4_-anhydrovinblastine are used for their antineoplastic (Vonder *et al*., 2004). Due to its pharmaceutical importance and low yield, concerted efforts are being made for improvement through cell and tissue cultures (Moreno *et al*., 1995), relatively recently through somatic embryogenesis (Junaid *et al*., 2006), mutation breeding and genetic transformation (Misra and Saema 2016). *In vitro* plant regeneration can be accomplished through somatic embryo-genesis or organogenesis (Junaid *et al*., 2007a; Dhandahpni *et al*., 2008). Somatic (or asexual) embryogenesis is the production of embryo like structures from somatic cells and it is an independent bipolar structure and is not physically attached to the tissue of origin and can develop and germinate to form plants in a manner analogous to germination of zygotic embryos (Sharp *et al*.,1980). Induction of somatic embryo-genesis (SE) occurs either

directly when embryos develop from cell, tissue or organ culture without an intervening callus phase, or indirectly when an explants first produces some form of callus which later differentiate into somatic embryos (Sharp *et al*.,1980 ;Gutiérrez-Mora *et al*.,2013), as observed in the present study.

It is only recently that an efficient plant regeneration via somatic embryogenesis has been reported in *Catharanthus roseus* (Junaid *et al*., 2007b; Yuan *et al* 2011). In vitro, embryogenesis has been used extensively as an efficient plant regeneration system in raped clonal propagation system to collect raw material for the recovery of IAs (Mujib and samaj, 2006) and development of genetic transformation systems to improve the alkaloid yield. Since it can produce a large number of plants and can be coupled with cryopreservation, bioreactors, synthetic seed technologies and gene manipulation (Merkel *et el*., 2000, Loyola-vargas *et al* 2016).

The development of somatic embryo resembles the zygotic embryo in various aspects and nearly replicates the process of zygotic embryo formation (Zemmerman, 1993; Feher, 2006; Harada *et al* 2010; Gaj, 2011), and the fact is that plants can be regenerated from one single cell like their zygotic counterparts (Faure and Nougarede, 1993, Faure *et al*.,1996; Leljak-Levanić, *et al*., 2015). This character is essential and an important prerequisite step for successful stable transformation, gene manipulation, mutation breeding and somatic hybridization take place for plant improvements (de Feria *et al*., 2003; Yang and zhang, 2010; Saporta *et al*., 2016). Plants of multicellular origin cannot always be expected to be genetically uniform, and may indeed be chimeras (Secristan and Melchers, 1969; Ogura,1976; D' Amato 1978; bennici and D' Amato, 1978 ; Vasil.,1981; D' Amato 1985; Misra and Saema 2016); and unsuitable for mutation breeding; genetic analysis and genetic transformation etc. These reasons and others can explain why regeneration plants via somatic embryogenesis originated from single cell are of considerable interest and attracted geneticists, molecular biologist and biotechnologist attention.

In addition, somatic embryogenesis is the preferred method for cell to plant regeneration in several plant species; it's somatic hybrids and synthetic seed production, compared with organogenesis because somatic embryo (SE) is very similar to their zygotic counterparts and arise from single cells (Faure *et al*., 1996; Feher *et al*., 2003; Altamura *et al*., 2016), and the plantlets converted from them already possessed an integrated shoot-root system (Micheli and Standard 2016), and considerable evidence lead to the belief that, they are to rule out the creation of chimeras (Gioregetti 1995, Sharma and Millam 2004; Bertsch *et al*., 2005). The ontogeny, morphogenesis, development and conversion of plantlets have not always been studied in details as the whole process of in vitro embryogeny is quite complex (Vookova and Kormutak, 2006). Histological studies have been done in different species to describe the initiation and development of somatic embryos, several fundamental aspects of the process remain unclear (Benelli *et al*., 2001).

One of the main aspects for researchers of somatic embryo is still a question of debate whether somatic embryos originates from a single cell or from a group of cells. Some histological studies in different species have described unicellular origin (Williams and Maheswaran 1986; Choi *et al*., 1997; Quiroz-Figueroa *et al*., 2002a; Supena *et al*., 2008) and multicellular pathways (Maheswaran and Wlliams, 1985; Choi *et al*., 1998; Fernandez *et al*., 1999).

However, there are a few reports that describe the ontogeny and structures of somatic embryo development in *C. roseus* (Yuan *et al*., 2011; Junaid *et al*., 2014). In spite of some useful knowledge gained from their reports, both of them completely lacking the cytological and histological work to provide or to prove any evidence of the unicellular origin and early sequential events take place within the totipotent embryogenic cell and early stages of embryogenesis. Moreover, histogenesis and morphogenesis of some developmental stages in both of their reports are indeed not presented well. Therefore, these important events have not yet been presented, as a consequence, the aim of this work was to document and present these events for the first time of *Catharanthus roseus* somatic embryo formation from their first cellular division to the final stage. Understanding the origin of such developmental pathway is essential and critical in the achievement of many developing techniques based on single cell culture for plant improvements.

MATERIAL AND METHODS

Plant material

Catharanthus roseus (L.) G. Doncan seeds obtained from El-Orman garden, were washed by distilled water, surface sterilized with

commercial bleach [1.2% (v/v) sodium hypochlorite] for 20 min, and finally rinsed three times with sterile distilled water. Ten to 20 seeds were placed in GA-7 magenta vessels (Sigma, St. Louis, MI, USA) that contained 40 ml MS (Murashige and Skoog 1962) without growth regulators. Germinated seeds were grown until reached 2-4 cm in length. The different parts (leaf and hypocotyls) were excised and cultured in test tubes as explants. For embryogenic callus initiation, MS medium was supplemented with 2,4-D alone to acquire embryogenic character. Embryogenic callus induced on MS medium amended with NAA with different concentrations of 6BA. The PH was adjusted to 5.7 before autoclaving (20min., 121 ºC). The cultures were incubated under a 16-h photoperiod by cool white (Fluorescent F40 T12/CW/EG) lamp at a photon flux density of 100 *l*mol m) 2 s)1 at 26±1ºC light dark temperature

Initiation of embryogenic calluses formation.

leaf and hypocotyls segments were placed on MS medium supplemented with different concentrations of 2,4-D alone as described by Junaid *et al*., 2006. Each treatment consisted of 10 explants per dish with three replicates. The mean percentage of each explants producing friable yellowish white calluses and inducing somatic embryos were determined after 4 weeks of culture.

Somatic embryo (SE) proliferation and differentiation.

For somatic embryo (SE) proliferation and differentiation, 40–50 mg callus was cultured on MS proliferation medium supplemented with 1mg/L NAA with combination of 1 and 1.5 mg/L 6BA as described by Junaid *et al*., 2006 with some modifications.

Embryogenic cell suspension

One to 2 grams of yellowish white friable embryogenic calluses were transferred to 250 ml of Erlenmeyer flasks containing 25 ml of liquid MS medium supplemented with 1mg/L 2,4-D or 1mg/LNAA and 1.5mg/L 6BA. The flasks were placed on a shaker at 100 rpm. After 2 weeks of culture, 25ml of liquid culture was added to each of the flasks and maintained at 26 ±1Cº in the dark on a shaker at 100 rpm. After 2–3 weeks culture, 5 ml of suspension cultures was inoculated into each 250 ml of Erlenmeyer flask containing 50ml of the same liquid MS medium (i.e., MS medium supplemented with 1mg/L 2,4-D and 1.5mg/L 6BA). Embryogenic cell suspension cultures were sub cultured at two weeks intervals.

Harvesting and fixating the cells.

Harvesting of cells and fixation, cells and calli were sampled at days 7, 14, 21 and 28. Four samples were randomly collected per sampling time. Each sample harvested from 0.5-1mL culture removed in separate tubes. Cells were harvested by centrifugation at 1000 rpm for 1 minute or settled under gravity, excess of growth medium was removed by plastic pipette, then washed once in 45% glacial acetic acid and fixed in approximately 10 volumes of washing solution be used for 1 volume of tissue. Acetic acid was used in this study due to its chemical properties as a fixative i.e., its definite mordanting effects on cells and tissues: that attach itself to the nucleoprotein and also to dye,in addition to its main function of cytological and histological techniques.

Acetocarmine stain preparation

Acetocarmine stain was prepared by adding one gram of carmine powder into 100ml of 45% glacial acetic acid placed in 500-ml Erlenmeyer flask capped by aluminum foils, the solution was stirred continuously over a low flame spirit lamp for complete dissolving and gentle saturating boiling for about 5 to 10 minutes until acetocarmine solution changed color. The flask was removed to allow stain to cool to room temperature before filtration to remove a few un-dissolved powders.

Embryogenic potential assay

For confirmation stability of embryogenic potential of unicellular origin pathway in an earlysequential morphogenesis developmental stages of indirect soma-tic embryogenesis (ISE) in our cell lines. Finely dispersed suspension culture were periodically cultured on proliferation medium supplemented with NAA (1.0 mg/L) and BA (1.5 mg/L), and incubated in the same light dark cycle. After three to four weeks of culture, somatic embryos at different stages: globular, heart, torpedo and cotyledonary were observed and bright field images of embryos were taken with a dissecting light microscope. In addition, stained and un-stained temporary slide preparation and light microscopic examination were used. *For staining*, the slides are first cleaned (cleaned by soaking overnight in 70% ethyl alcohol solution containing 0.5% of 1 N Hcl, followed by thorough washing with distilled water), drayed and one to two small drops of acetocarmine added.

Cytological Assay of Embryogenic potential

Unicellular origin of early cell division in somatic embryo-genesis process were determined after adjusting the age of subculture and the appropriate sample of each calli and cell samples were fixed, then placed in the stain and smeared under a cover glass by pressing the slides as described by (Darlington, and La Cour 1976) with minor modifications and preheated over a low flame spirit lamp before microscopic examination.

RESULTS AND DISCUSSION

Embryogenic callus induction

In Vitro, friable white to yellowish callus was induced on hypocotyl cutting (6-9) obtained from *C.roseus* seedling grown within two to three weeks on MS solid medium supplemented with 2,4-phynoxy acetic acid (Fig.1a). The callus maintained mitotic activity subcultured on the same medium amended with 2,4-D every four weeks acquired the embryogenic competent during the ontogenesis form somatic embryo**s** within four weeks. Yellowish embryogenic calluses presented nodular features and a smooth surface, the somatic embryos were then proliferated and differentiated on medium supplemented with 1.0 mg/L NAA and 1.5 mg/L 6BA. (Fig.1b and c)

During callus induction, potent synthetic auxins such as 2,4-D is important in controlling embryogenesis and required to induce embryogenic callus have been reported in many plant species (Pretova, A. and Dedikova, B.1992; De jong 1993; Mordhorst *et al*.,1998; Gaj 2004). Incubation of Madagascar periwinkle [*Catharanthus roseus* (L.) G. Don] hypocotyl explanation medium with 2,4-D was very efficient in callus induction and necessary for embryogenic competent to occur, meaning that 2,4-D played a key role in initiated the necessary physiological changes in the calli during subcultures promoting cell division and the embryogenic determination programming of cells and early embryogenesis process. This finding was observed and reported in several plant species during callus induction and early somatic embryogenesis (Su *et al*., 2009; Zheng *et al*., 2016).

Cytological Analysis of Cell Division Pattern in Early Stages of Somatic Embryogenesis

In order to demonstrate unicellular origin of embryogenesis events in an earlydevelopmental stages of indirect somatic embryogenesis (ISE), hence the type and the orientation of cell divisions and the fate of cells during their differentiation takes place during early embryo formation, samples were taken from cultures at regular intervals (as described in material and method) and examined with aceto-carmine staining to visualize the nuclei and cell plate take place within the embryonic cell and early cell division pattern take place in proembryos. The first stage of embryogenesis, has been reached within the first week in culture on embryogenesis induction medium. Embryogenic response appear to be restricted to small spherical and oval shape cells, which often exhibit large a centric nuclei "nucleus migrate near the cell wall"; a high ratio on nuclear to cytoplasmic volume and well defined cell wall (Fig. 2-a). While, enlarged cells not producing embryos, suggesting a correlation between cell type and its embryogenic capacity in agreement with (Nomura1985, William and Maheswaran 1986).

The first division of embryogenic cell is transverse asymmetric division (figs. 2-b and 2-c). The fates of progeny cells formed as a result of this division in embryogenic culture are different: from one a somatic embryo is formed, whereas the other partly reforms the proembryo genic mass in callus or cell suspension cultures by further cell division (Ten Hove *et al*., 2015).

Unequal division documented in (Figs. 2-b and 2-c) are not "simply" representing two cells or group of coupled cells with unequal size, but represents the cyto-kinesis i.e., a clear cell plate located near one end of the newly dividing cell, so that there is no any doubt the cell division is be indeed asymmetric (Yeung *et al*.,1981; Rasmussen *et al*., 2011). These events were rarely presented and proved to be difficult to capture and recognize by some other protocols or methods e.g., serial paraffin and plastic sections (Yeung 1995).

The two cells formed as a result of this division are analogues of the basal and apical cells formed during zygotic embryogenesis (Souter and Lindsey 2000). The polarity of the first division of embryonic cells is fundamental phenomenon of zygotic proembryo genesis (De Jong, Schmidt and De Vries, 1993), but have rarely been reported for somatic embryo development. In *Catharanthus roseus,* we have clearly presented that the polarity of the somatic embryo is established at the first division as shown in (Fig.2-b). Bicellular proembryos are asymmetric, possessed two cells with different structure, the upper "apical cell" pole is the main seat for growth of the

embryo; the lower "basal cell" pole produces a stalk-like "column-like" suspensor.

Three-celled proembryo, resulting from the first longitudinal division within the "apical cell" leading to a 2-cell embryo proper (Fig. 2-c), these two cells immediately underwent a second longitudinal division to form a 4-cell embryo proper, and the lower "basal cell" divided transversely to produce the suspensor (Fig. 2-d). The proembryo is now at the six-celled, the suspensor is now distinct from the four terminal cells "apical cells", which develop into the embryo proper. Disregarding the two suspensor cell, the proembryo is now at the four-celled stage (Fig. 2-d). These change in division plane are considered a landmark of differentiation pattern of the embryo and the suspensor during zygotic embryogenesis (Dodeman *et al*.,1997; Souter and Lindsey 2000). Both of three and sex-celled stages of proembryo (Fig. 2-c and Fig. 2-d) have not been reported yet in the majority of plant species, and proved to be difficult to catch.

Eight stage embryo is proper and divided transversely, producing an upper and a lower tier of 4 cells (another four nuclei are present behind the four nuclei in focus) (Fig. 2-e) and elongated suspensor. At this stage, uni-seriated stalk or column like suspensor structure consisted of four cells produced by transverse division and eight-celled proembryo stage, contribute to formation of embryo proper. All eight pro-embryo cells, possessed small cells with large central nucleus divide through a series of relatively fast and apparently without any differentiation forming small globular proembryos as illustrated in (Fig.3-b and 3-c). The embryo at this stage consists of mass of about 15-20 dedifferentiated cells, very similar to the early "division" i.e., cleavage (mitotic division without growth) in animal embryos "morula" stage (Fig.3-a).

The slow growth of somatic embryos during the first week on proliferation medium largely reflect the fact (the increase of cell numbers usually precedes the increase in fresh weight due to the time delay for cell expansion). This results in a sharp decrease in the cell size of embryo prober during this time and explain a very rapid growth observed during the second weeks of cultures. Although the dedifferentiation of embryo prober cells is fundamental phenomenon of zygotic embryogenesis (De Jong, Schmidt and De Vries, 1993), it has been rarely reported for somatic embryos. In the present study however, this phenomenon demonstrated as can be seen in (Fig.3-b and c). Fifteen to twenty embryo prober cells go through another important ordered cell division pattern occurred in which the cells in the embryo proper underwent a periclinal division, to produce 15-20 protodermal cells and 15-20 inner cells, but the protodermal cells were relatively undistinguishable.

Somatic embryo proliferation and differentiation

After two weeks, somatic embryo proliferation on medium (MS+1mg/LNAA and 1.5mg/L 6PAB), the embryonic calli and cell suspension culture grow very rapidly, indicating high mitotic activities take place in the cells forming globular embryos. The spherical structure, "globular-shaped embryo", which eventually initiates cell differentiation and establishes the basic structure of the future plant with the formation of more distinguishable and well defined protoderm " future epidermis" in the outermost cell layer of the globular stage embryo (Fig.3-d).

At the end of the rapid division stage, the small round cells of the globular somatic embryos (SE) originating from one single embryogenic cell pass through different sequential of morphogenetic characteristic changes nearly identical to four main zygotic embryos described in many di-cotyledon plant species: which are globular shaped, heart-shaped, torpedo shaped and cotyledonal shaped embryo (Ikeda-Iwai *et al*., 2003; Etienne *et al*., 2013). Somatic embryos (SE) at different developmental stages are shown in (Figs.3-a through f).

After three to four weeks of culture, the globular-shaped embryos gradually assumes a two-lobed form "the cotyledon", which observed as small protrusions at the apical end of embryos and their formation indicates the beginning of the heart-shaped stage. With continued division of initiated cotyledons, particular cells rearrangements of the developing embryo forming morphogenetic characteristic shape, is often called the heart-shape (Fig. 3-e). As embryo development continue, the cotyledon and axis elongated forming torpedo-shaped embryo (fig.3-f).

Some line of evidences indicated that several gene expressions were up-regulated including SE-specific tubulin, actin-binding proteins and cell wall which changed dynamically prior to alterations in cell morphology (Malinowski and Filipecki 2002; Brownlee 2002; Wondrakova *et al.*, 2014). Suggesting that these proteins and cell wall in

the cells forming "globular-shaped embryos" somehow embark upon a series of cellular rearrangements that alter their spatial relation-ships with one another converting globular-shaped embryo to oblong, heart-stage and participates in the control of growth and differentiation of cells during embryogenesis in plants. After a while, cotyledonal primordium formed and both of the cotyledons and axis elongate forming "torpedo-stage" with continuing embryo development (fig.3-f).

Secondary somatic embryogenesis

Repetitive secondary somatic embryogenesis (SSE) in which new embryos are initiated from developing somatic embryos were observed and their unicellular origin were demonstrated (in press). Although this phenomenon lead to non-uniformity of culture for handling of cloned somatic embryos for agriculture species (Lindsey and Jones 1989), it's important to generate an unlimited number of secondary somatic embryos that can be generated in a cyclic manner from a culture of primary embryos (Raemakers *et al*. 1995), and can be utilized for scale up of new varieties for large scale propagation programs based on genetic transformation and/or somaclonal variation that aim to improve the alkaloid yield (Walter 2004; Azad *et al*., 2009; Arrouda 2012; Corredoira *et al*.,2016;). In addition the regenerated plants originated from unicellular pathway are usually not mosaics (Misra and Saema 2016).

Somatic embryo maturation

Removing cotyledonary somatic embryos (SE) from proliferation medium and putting them on maturation medium (MS basal medium supplemented with 1mg/L Gibberellic acid (GA3), and 3-6% maltose or 3% fructose), the embryos develop relatively slowly compared with other stages (*i.e*., globular through to cotyledon-nary). Precocious germination of developing embryos was observed (Fig. 1-D), adding 0.5mg/L 6BA to maturation medium improved normal appearance of germinated embryos. Despite the disadvantages of this phenomenon in some applications, it important to shorten somatic embryogenesis duration and can be manipulated in different ways for another applications.

As observed in our study, the process of induction of somatic embryos in dicots occurs through an orderly series of characteristic embryological stages divided in four phases; globular, heart, torpedo and cotyledonary stage (Etienne, 2005; Padua *et al*., 2014). These stages have been described in several species eg., *Daucus carota* (Schiavone and Cooke 1985), *Caffea arabica* (Quiroz-Figueroa 2002a), *Arabidopsis thaliana* (Gaj 2001, Nowak *et al*., 2015), *Theobroma cacao* L. (Ajijah *et al*., 2016) *Vitis vinifera* L.(Bouamama-Gzara *et al*., 2017), *Gladiolus hybridus* (Wu J. *et al*., 2015), and (Naz *et al*., 2015), *Cucumis melo* L (Raji *et al*., 2017).

Although single cell of somatic embryos (SE) has been demonstrated (Nomura and Komamine 1985; William and Maheswaran 1986), very a few reports were followed their development from single cell, with the occurrence of pro-embryogenic masses being rather confusing. Like *Dactylis glomerata* (Trigiano *et al*., 1989) and (Dubois *et al*., 1991, Malik *et al*., 2017) on their studies in *Cichorium spp*.

In *Cathuranthus roseus*, the ontogeny, structure and transition developmental stages have rarely been reported (Yuan *et al*., 2011, junaid *et al*., 2014). In spite of some useful information gained from their studied, they lake to provide any cytological or histological evidence about unicellular origin and early developmental stages operative in this system. Furthermore, they claimed that the somatic embryos of *C.roreus* arise from pro-embryonic mass of cells is very confusing and is in line with observations previously made by the above cited authors. Additionally, they did not present the embryos at some later developmental stages very well. It has been known that somatic embryos (SE) "embryoids" develop from single cell since it was hypothetically suggested (Waris 1957b; Steward *et al*., 1958), and several reported studies in different species provided valuable information and relatively improved our understanding about the topic, but they are indeed failed to demonstrate unicellular origin and ordered orientation of early division pattern and subsequent developmental stages of somatic embryo (SE) except very few reports (*C.roseus* not included).

Our investigation and observations based on the basic smeared modified technique enable us to conclude that: the onset and the ontogenesis of indirect somatic embryos developments (ISED) of *Catharanthus roseus* arise from one single embryogenic characterized cell nearly identical or very similar to zygotic cell, underwent asymmetric "unequal" division and differentiation leading to the formation of somatic embryos possess four main sequential morphogenetic developmental stages: globular-shape; heart-

shape; torpedo-shape and cotyledonary-shaped embryos as can be seen in (Fig. 4 and Figs. 5-a; 5-c; 5-e and 5-f) moreover to oblong and enlarged-stage "elongated stage" showed in (Fig. 5-b and Fig. 5-d).

Four stages were reported and described in several plant species, but the oblong and enlarged-stage have rarely been described. Globular stage embryos (Fig. 4-a and 4-b and Fig. 5-a) were observed during the second week and distinctly observed within the third week in culture. Several smooth surface globular-shaped embryos with different globular morphological with up to 30 embryos per cluster with different sizes were presented in the front callus (Fig.4-a and b), middle and back calluses of the same plate showing globular stage, heart stage and torpedo stage embryos in heterogeneous mixtures, indicating that the embryos were occurred and developed in a non-synchronous fashion.

The next recognized developmental stage after three weeks on proliferation medium, the oblong and heart-shaped embryo (Fig. 4-c; Fig. 5-b and 5-c) were about one to one and half times larger than the globular-stage one. In heart stage, the initiated cotyledons were observed as small protrusions at the apical end of embryos and their formation indicates the onset of heart-shape,whenever both of torpedo (Fig. 4-a and Fig.5-e) and Cotyledonary stages (Fig.5-f) were observed after four weeks onwards. The oblong and the elongated "enlarged" transition developmental stages which have rarely been reported in several plant species have reported for the first time in this study during an indirect somatic embryogenesis (ISE) of *Catharanthus roseus* as illustrated in (Fig. 5-b and Fig. 5-d respectively). The Oblong-shaped embryo have previously only been reported for *Daucus carota* (Schiavone and Cooke 1985), and in *Coffea arabica* somatic embryogenesis histogenetic study (Quiroz-Figueroa *et al*., 2002). The elongated-shape is a transition stage between heart and torpedo-shape that has only been reported in *C.arabica* and was also observed and demonstrated in *C.roseus* (Fig.5-d). This stage is formed because the initiated cotyledon and axis elongate (the so-called elongated "enlarged" stage of embryo development) as embryo development continue.

From our point of view there are more than one transition stages between globular and heart stage. The same is through between heart and torpedo-shaped somatic embryo. As far as we know, these stages were not observed or reported until now for many reasons. During the course of development, some deferent transition stages of somatic embryo and certain events can be difficult to capture histologically. The recognition and catching of these stages and events depend on the method used. Consequently, accurate observation can be done in a large number of embryos to avoid error in interpretation and erroneous conclusions based on a few samples and "two dimensional sections" (Sanderson 1994).

In this study, we demonstrated the unicellular origin of the sequential organized events of early embryogenesis and the main four developmental stages, moreover to oblong and elongated transition developmental stages in indirect primary somatic embryos (IPSE) of *C. roseus*. The unicellular origin described in this study is the first report of such developmental pathway and considered a key step in all such developing techniques based on single cell culture (Bajaj 1995, Ochoa-Alejo 2016). The observation and information extracted from this work provides a base for further experimentation for improving (SE) procedure and conditions to be more uniform and reproducible formation of good quality somatic embryos for quantitative assessments.

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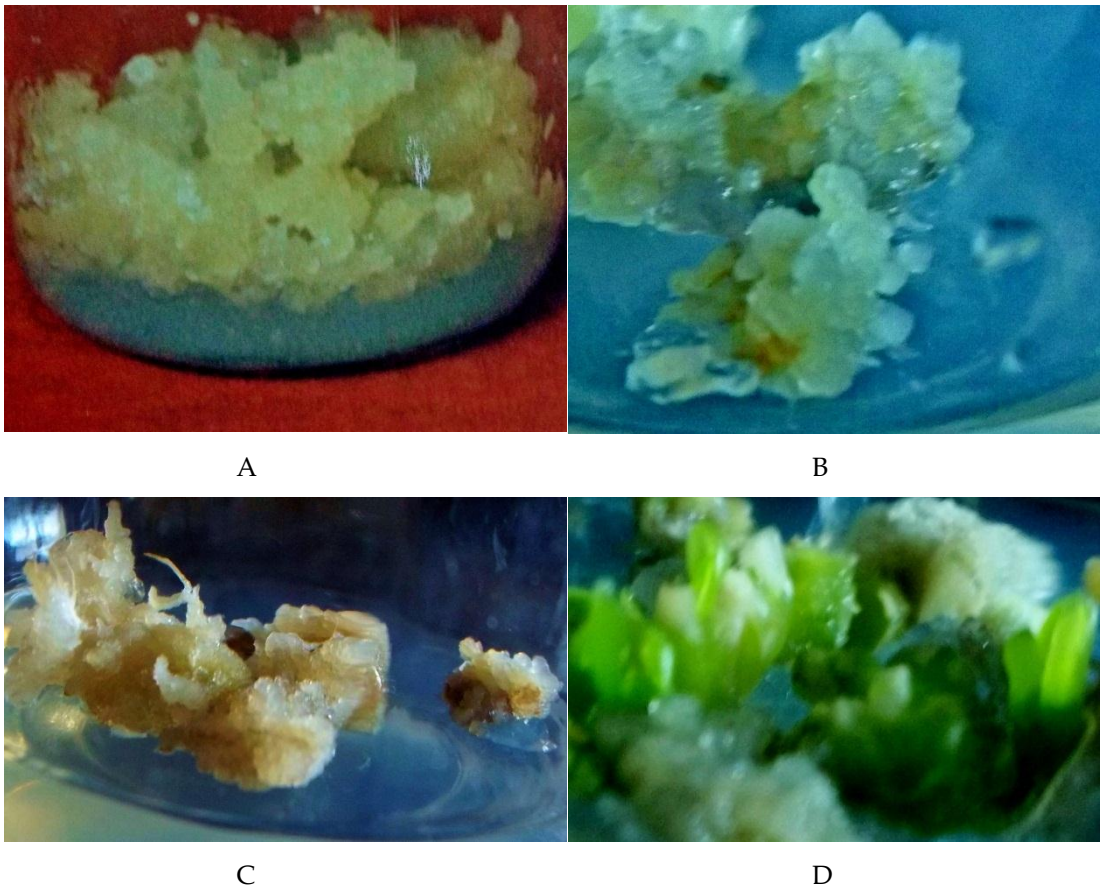
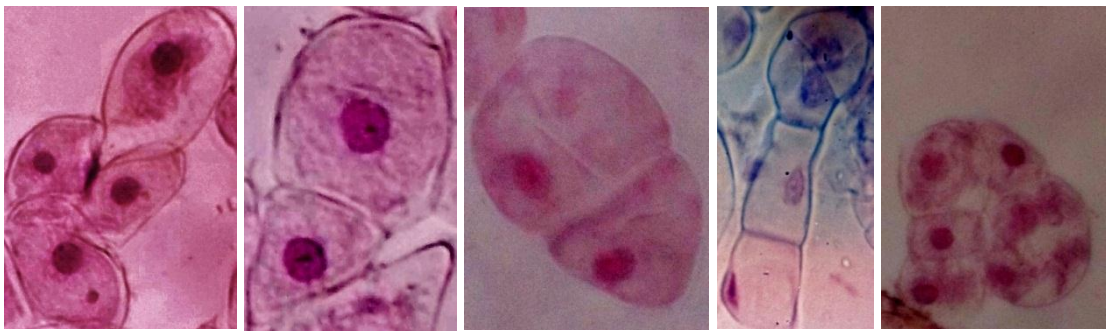
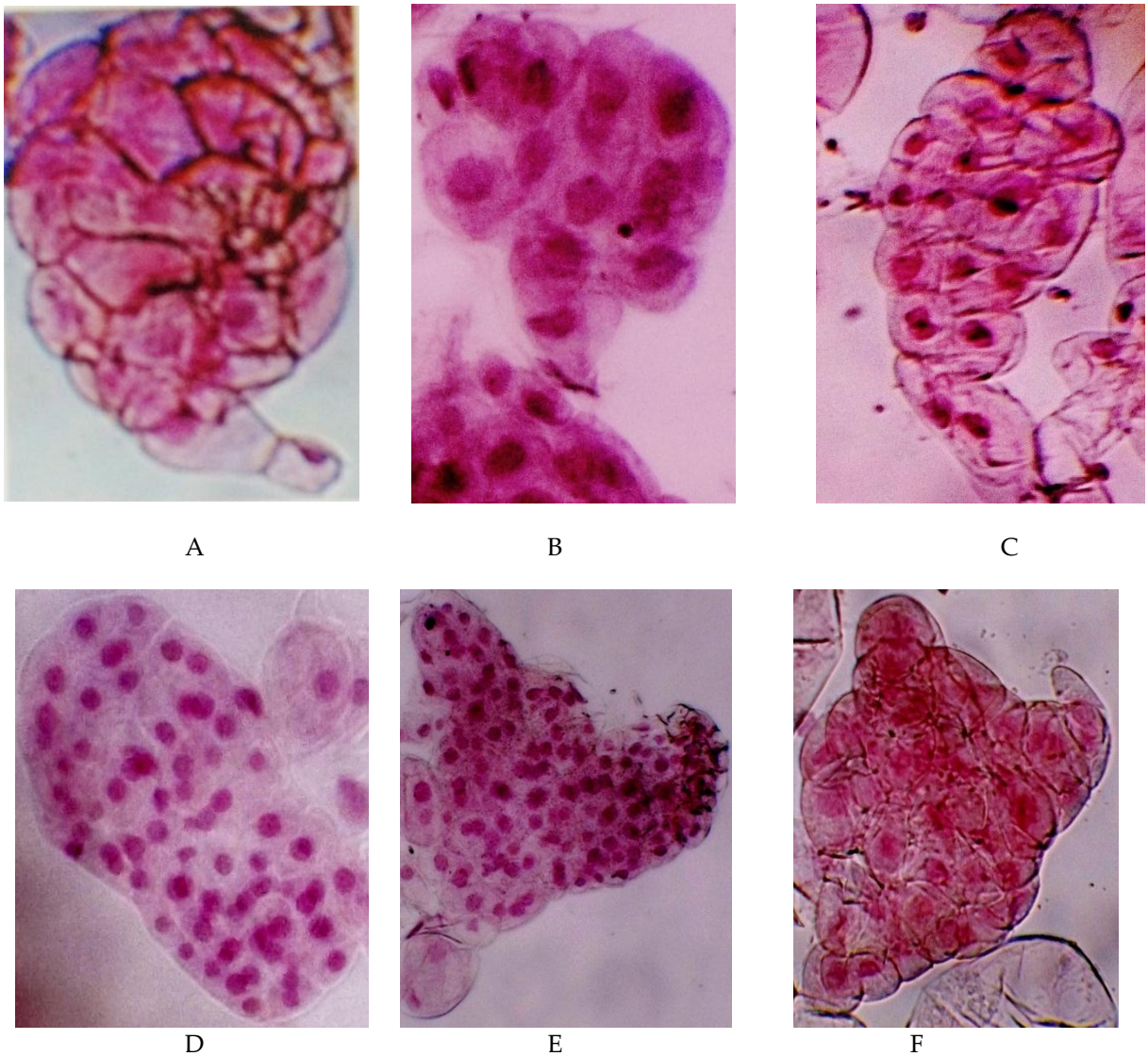


Figure 1: Somatic embryogenesis and plant regeneration from hypocotyl-derived callus in *C.roseus*. (a) Friable embryogenic callus from hypocotyls cultured in MS medium supplemented with 1mg/L 2,4-D. (b) Induced varies developmental stages (globular; heart and torpedo-shaped somatic embryos on proliferation medium containing 1.0 mg/L NAA. (c) Embryos in MS maturation medium amended with 1.0 mg/L GA3. (d) Precocious germination of developing embryos grown on maturation medium.



A B C D E

Figure 2: Early cell divisions pattern in the somatic embryogenesis of *Catharanthus roseus*. Somatic embryogenic process (a-e). (2-a) embryogenic cells ; (2-b) two-celled stag, resulting from transverse asymmetric division within the embryogenic cell; (2-c) Three-celled proembryo, resulting from longitudinal division within "apical cell" forming two embryo proper cells; (2-d) six-celled proembryo. The suspensor is now distinct from the four (the two other cells behind them) terminal cells" apical cells", which develop into the embryo proper; (2-e) Twelve-celled proembryo showing eight apical cells, four cells are observed (the other four cells behind them), go through a series of slow divisions, contribute to formation of embryo proper and suspensor-like structure.

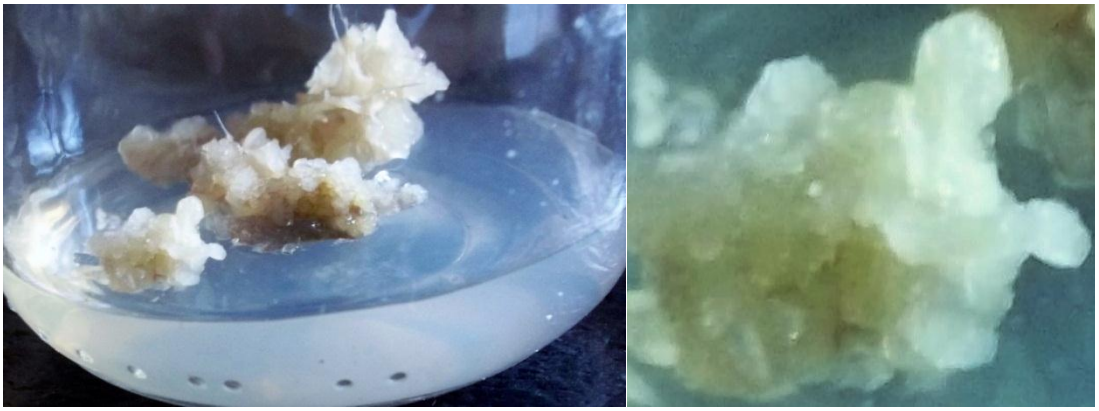


A B C

D E F

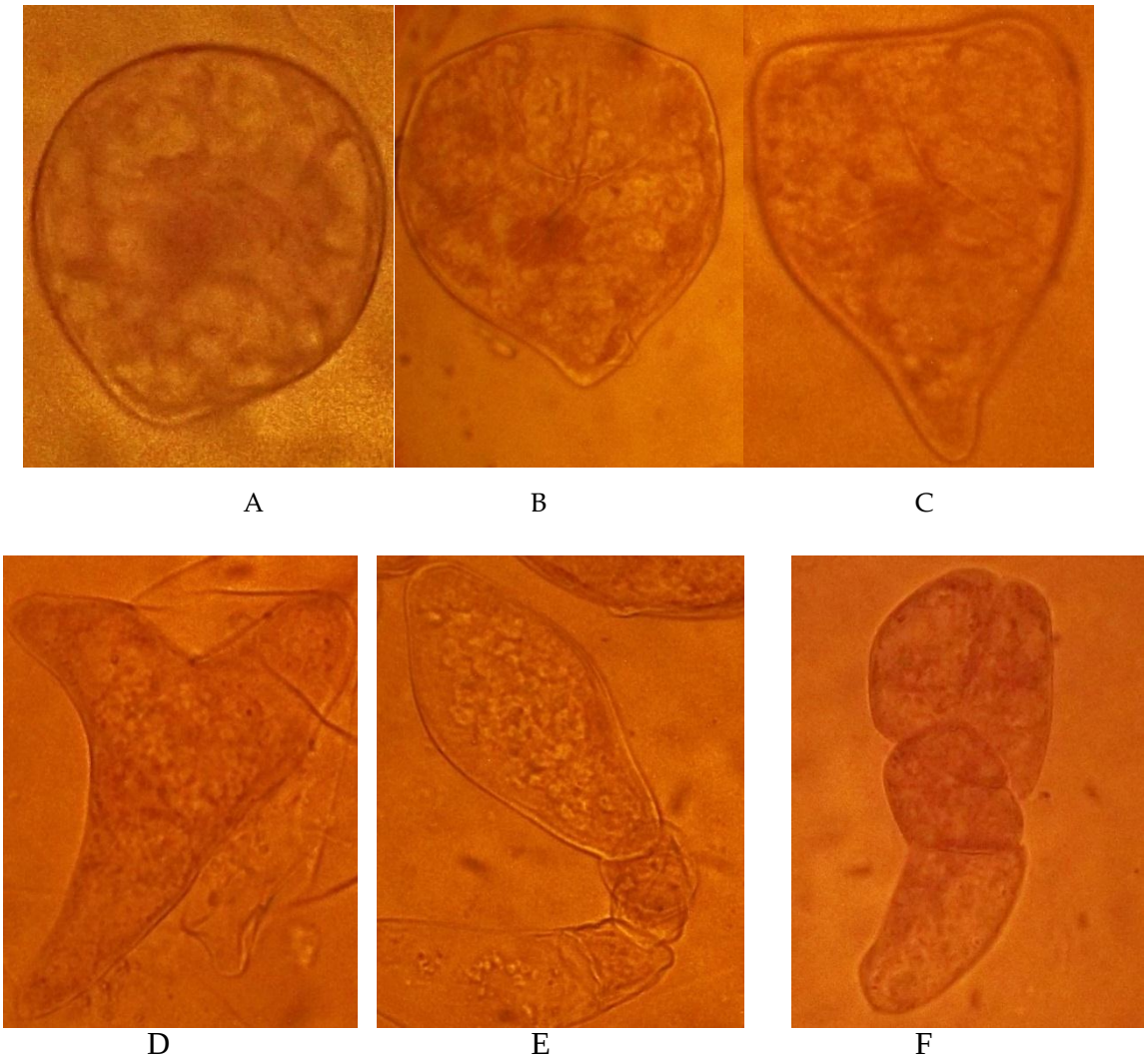
Figure 3: Developmental stages of somatic embryogenesis of *Catharanthus roseus*. (3-a) unsqueezed globular–stage (3-b) squeezed small embryo proper "small globular-stage", have nearly iso-diametric

de-differentiated cells with a big nuclei and precocious development of the suspensor like structures. (3-c) squeezed relatively advanced globular embryo with uniseriate cells forming suspensor, showing relatively iso-diametric de-differentiated cells without protoderm (3-d). embryo in late globular polarized stage with a protoderm which has been initiated at the outermost layer of embryo proper. (3-e) embryo at heart-shape (emergence of cotyledons) stage. (3-f) torpedo-shaped embryo.



A B

Figure.4-a: Embryonic callus grown on induction medium showing globular-shaped embryos cluster on the surface of the front callus, heart-shaped and torpedo-shaped embryos on the middle and back callus. 4-b, magnification of the front callus.



A B C

D E F

Figure 5: Lightly stained somatic embryogenesis developmental stages of indirect somatic embryo-genesis (ISE) of *Catharanthus roseus* (a-f). 5-a. Representing globular-shape embryo 5-b. Oblong shape ;

5-c. Heart-shape; 5-d. Enlarged "elongated"–shape embryo ; 5-e. Torpedo-shape with suspensor like structure; 5-f. Cotyledonary-shaped embryo.

دراسات سيتولوجيه لمنشأ المسار الخلوى وحيد الخليه والأنماط المملاه للإنقسام المبكر وتكوُن المراحل التطوُرية للأجنة الجسمية للطرز الخلوية لنبات الونكا الوردية الناتجة بطريق غير مباشر

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الملخص العربى

تم دراسته المنشأ وحيد الخليه للتكون الغير مباشر لمسار تطور الأجنة الجسمية لنبات الونكا الوردية علاوه على تأكيد ثبات إمكانية تكوُنها بالمزارع السائله. ولقد تحرينا أنماط السمات المُملاة لانقسام الخلايا والتشكل النسيجى المبكر على مدار الوقت مستخدمين الميكروسكوب الضوئى. ولقد بينت الدراسه أن إستمرارية إبتداء تكوُن الأجنة الجسمية من الخلايا غير المتشكلة "نسيج الكالس" تم إستحداثها من السُويقه الجنينيه العُليا للبُذور النابته على البيئة الغذائيه للباحثين مُوراشيج وسكوج المُضاف إليها 2-4 الداى كلورو فينوكسى حمض الخليك أو1مليجرام/ ليتر من مادة نفتالين حمض الخليك و1.5مليجرام/ ليتر من مادة 6بنزايل أمينو بيورين. ولقد تم التوصيف الخلوى للمراحل التطورية المختلفة لتكون الأجنة الجسمية بداية من الانقسام الخلوى الأول للخلية الجنينية لآخر خطوه من تطورها. وتحرياتنا وملاحظاتنا بُنيت على التقنية المُعدّله لفرد الخلايا، التى مكنتنا من القول بأن إبتدء وإستمرارية تطوُرتكوين "نشؤء" الأجنة الجسمية غير المباشر لنبات الونكا الورديه ينشأ من خليه واحده ذات مواصفات تُشبه كثيرا خلية البيضه المُخصبة "خلية الزيجوت" وقادرة على إكمال الإنقسام خلال طرُز أو أنماط مُختلفة مُملاة عليها (على سبيل المثال الانقسام العرضى غير المُتناظر داخل الخلية التى ستُكون الجنين والانقسام الطولى داخل الخلية القميه ,الانقسام العرضى المُتناظر فى الخلايا المُكونه للمُعلق والإنقسام العمُودى لتكوين "البروتوديرم" فى آخر طبقة خارجية من الخلايا المُكونة للمرحلة الكُروية)، وتُكمل تشكلها لتُكون الأشكال التطوُرية المُختلفه للجنين وهى: الطور الكُروى المسحُوب من اتجاه واحد "الأوبلونج" والطور القلبى والطور القلبى المسحُوب "الممطُوط" والطور التُوربيدو والطور الفلقى للأجنة. ولقد تأكد أيضا من كون الأشكال التركيبية للمراحل التطوُرية المختلفة للأجنة الجسمية والتشابهات التفصيلية الأخرى مُتشابهه كثيرا مع مراحل تطوُر الأجنة الزيجوتية فى النباتات ثنائية الفلقه، وما وجدناه فى هذه الدراسة من الأهمية بمكان وجوهرى ومُتطلب أساسى قبل إستخدام التحسين الوراثى للنبات المبنى على النقل الجينى له أو التهجين الجسمى أوالتربيه بالطفرات لتحفيز زيادة إنتاجية قلويدات الإندول التربينيه. وهذه الدراسه من وجهة نظرنا هى الدراسة الأولى من نوعها التى عرضت ووصفت بوضوح منشأ المسار وحيد الخلية والأحداث المُتعاقبة للمراحل التطورية المُبكرة والمُتقدمة لتكوُن الأجنة الجسمية بنبات الونكا الوردية، علاوة على الطورين الانتقاليين (الطور الكروى المسحوب من ناحيه واحدة "طور الأوبلونج" والطور القلبى المسحوب أو"الممدود") والذان لم يتم ذكرهما فى الغالبية العظمى من الأنواع النباتية التى درست إلى الآن.

الكلمات الاسترشادية: