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Somatic embryogenesis in cactus pear "*Opuntia ficus indica* (L.) Mill.": Morphological and anatomical determination of ovular tissue involved in the process.

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Abstract - *Opuntia ficus-indica*, known as prickly pear, is the most important plant species in the *Cactaceae* family. It is known as a multipurpose use plant with great worldwide interest because of his ecological and socioeconomic virtues such as the combat against erosion and desertification. In prickly pear multiplication by somatic embryogenesis was very important as a first step to induce mutation from suspected fertilized ovules. In this study, our interest has been oriented to identify the origin of somatic embryogenesis initiation. For this purpose, morphological study linked to anatomical investigation, made in the early stages of induction revealed that the tissue involved in this callogenesis was none other than the cambial section of vascular bundle of the funicular tissue.

Keywords: *Opuntia ficus indica*; Somatic embryogenesis; Ovular Tissue; Anatomical studies.

1. Introduction

The cactus pear (*Opuntia* spp.) is native of Mexico but is currently distributed in North and South America, some regions of Europe, Africa, and Asia (Mohamed-Yasseen et al. 1996). These species have been used since ancient times in traditional medicine. Cactus, and more precisely prickly pear, has gained an increased interest due to his ecological and socio-economic virtues such as their use in the combat against erosion and their medical properties which have been recently evaluated (Astello-Garcı́a et al. 2015). Somatic embryogenesis (SE) is defined as a process analogous to zygotic embryogenesis, which gives rise to somatic embryo (Tautorus et al. 1991). *In vitro* somatic embryogenesis has been induced in the Cactaceae family from various explants (Santacruz-Ruvalcaba et al. 1998; Moebius-Goldammen et al. 2003; Gomes et al. 2006; Jedidi et al. 2015). Especially in *Opuntia ficus-indica*, embryogenesis was obtained from isolated explants achieved by axillary budding from young cladodes (Gomes et al. 2006) or from cotyledonary tissues of *in vitro* germinated zygotic embryos (da Costa et al. 2001), following the process of dedifferentiation-redifferentiation process (Ochatt et al 2010).

The phenomenon of cell dedifferentiation is at the origin of what is called "indefinite embryogenesis" which is specific to the plant kingdom, lato sensu and means that any plant can be reproduced without limits regardless of its origin and age as long as it still has living totipotent cells. Dedifferentiation, which corresponds on the cytological level to the return of any plant cell still having a viable nucleus, to the state of meristematic and primary cells by borrowing a new program of mitotic divisions (Ochatt et al 2010). This reprogramming, which can take place even in planta, is the basis of vegetative *in vitro* propagation which is based on inductive conditions empirically defined by making use of an example of correlative factors where the culture medium plays the preponderant role (Thompson, 2008; Gerdakaneh et al. 2011; Omar et al. 2013; Kumar-Verma et al. 2016).

Dedifferentiation is a necessary but not sufficient step for the accomplishment of the entire ontogenetic program leading to the regeneration of an entire plant. Indeed, obtaining a primary meristematic focus only makes sense if the correlative context in which it is formed is also favorable to a redifferentiation phase where its cells acquire a structural and functional specialization resulting in fine, to the construction of the architecture of the plant which is also marked out by a whole complex of intrinsic physiological and molecular mechanisms.

This two-way process of "dedifferentiation-redifferentiation" is in the simplest case developed directly on the tissue itself of the cultured explant; This is referred to as "direct adventitious" regeneration where

dedifferentiation first passes through a phase of callogenesis in which the cells are of the parenchymal type regardless of the nature of the starting cells (Von Arnold 2008; Gueye et al. 2009; Omar et al. 2013).This work indicates the persistence of some uncertainty about the typicality of the observed embryogenic structures. It was only afterwards research developed by Chakroun et al (2013) that a true protocol for indirect somatic embryogenesis has been developed, this time from immature seeds decorticated on an induction medium containing gibberellic acid. Thus, it would be wise to identify the tissues actually involved in this embryogenic activity and thereby to perfect our control of the developed regeneration protocol.To achieve this objective, we will make use of spatio-temporal, morphological and anatomical explorations during the induction phase, while insisting on the earliest events that would make it possible to better understand the structural modifications that led to the embryogenesis in question.

2. Material and methods

2.1. Plant material and explants targeted

The plant material used for carrying out this experiment is the clone N° 1321 belonging to the species *Opuntia ficus-indica*. It was imported by the INRAT (Animal and Feed Production Laboratory) for its relative nitrogen content. It is a relatively elevated tree with large oblong racquets, yellow petal flowers and mature yellow-orange fruit. Explants used to induce somatic embryogenesis were immature seeds taken from flowers ten days after anthesis.

2.2. Methodology and culture conditions

2.2.1. Tissue disinfection method

The collected flowers were first brushed to rid them of their irritating glochids. They were then washed with running water and Tween 20 and then disinfected for 20 minutes with sodium hypochlorite 12°(bleach) diluted to 20%. After 3 rinses with sterile distilled water under laminar flow hood, they are cut longitudinally with sterile lancet to extract the immature seeds (Fig.1).

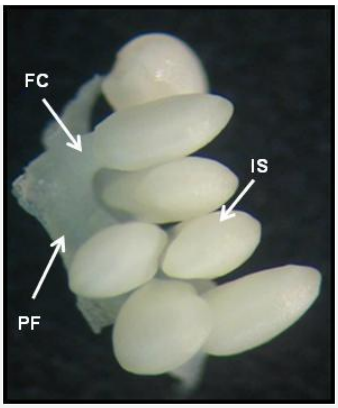

Figure 1. Immature seeds excised from the flowers of *Opuntia ficus-indica* clone 1321 ten days after anthesis. (FC). Funicular cord; (PF). Placenta fragment; (IS). Immature seed.

2.2.2. Decortication

In order to avoid the inhibitory effect of the external seed coat on its response to callogenesis and embryogenesis, these integuments are removed by longitudinal incision using a sterile scalpel blade.
Explants resulting from decortication consist of embryos surrounded by their internal integument and retaining part of the funicular cord (Fig.2).

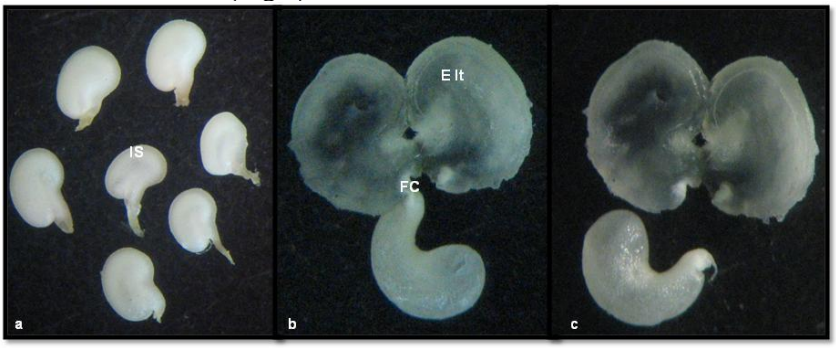

Figure 2. Decortications of immature seeds (IS). **(a).** Whole seeds with funicule (F); **(b)**. External integument (EIt) open and attached to the embryo with the rest of the funicular cord (FC); **(c).** Embryo completely detached from the outer integument.

2.2.3. Culture establishment

Single embryos were cultured on petri dishes (Ø 90 mm) containing the culture medium containing Murashige and Skoog (1962) supplemented with 1 mg. $l^{-1}AG_3$, 3% sucrose and 0.6% agar. The pH is adjusted to 5.7.

The dishes were incubated in the dark in an enclosure at a constant temperature of 25 °C ± 2°C.

2.3. Parameters observed

The changes occurred during incubation were observed at both morphological and anatomical levels. The morphological aspect was monitored daily under binocular magnifier (Leica MZ6) and was illustrated by digital photographs taken with a Canon S50 camera. These aspects were related to any apparent change in the explant in its general form or any other neoformation from this cultured organ.

Anatomical observations of cultured explants aimed the identification of tissue (s) involved in callogenesis process that may induce embryogenesis and the identification of the principal anatomical changes associated to this induction process. Observations were made at 10 days intervals during one-month period on samples taken from the cultured explants. For each sample, the five most representative explants of the corresponding batch are selected and stored in a fixative.

They are first visualized with a binocular microscope before being soaked for 24 hours in an FAA fixative (formalin, glacial acetic acid, absolute alcohol, 2: 1: 1 V / V). After rinsing with running water overnight, they undergo a series of gradual dehydrations in 50% to 100% increasing concentration ethanol baths for 30 minutes each. After two additional absolute alcohol baths, the samples are transferred to a mixture (50:50) of absolute alcohol and xylene followed by two gradual passages in xylene alone. The use of paraffin begins with a xylene-paraffin transition impregnation, in an oven at 57 °C, followed by two other pure paraffin baths. The samples are finally included in melting paraffin, cast in molds designed in the laboratory for this purpose. The blocks formed are sectioned with the RM 2125RT microtome at a thickness of 8µm. The ribbons obtained are spread on slides to undergo the rest of the protocol after drying at 37°C for 24 hours, mainly dewaxing and double staining with hematoxylin and safranine (Appendix 2). The sections covered with lamellae are observed under a Leica DMLB2 microscope. Interesting views are photographed from the same camera mentioned above.

3. Results and discussion

3.1 Morphological aspects

The observations made in a very close manner over time allowed us to locate precisely the site of the explant which were involved in the first callogenesis reactions at both morphological and anatomical levels. We recall that the first interest the precise location of the cell enclave which was the target of the induction of callus in a given number (about 30%) of explants cultured under the optimal conditions of somatic embryogenesis induction;The photos in Figure3 shows that the rest of the funicular cord (FC) remaining attached to the embryo after decortication (Fig.3.a) was itself the source of profusion of the first callogenic clusters (Fig.3.b). Which tend to proliferate to give a sort of callus ball (Fig.3.c). The initial explant will eventually become necrotic and be invaded by this mass of callus.

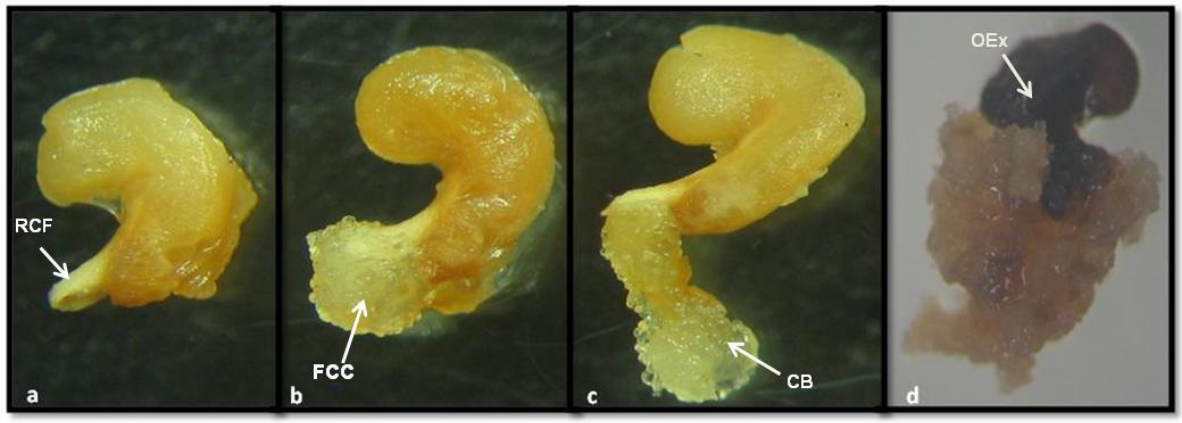

Figure 3. Primary morphological changes accompanying the early stages of in vitro induction of embryogenesis from immature shelled seeds of *Opuntia ficus-indica* flowers taken at the Anthesis stage + 10 days. **(a).** Embryo before culture, **(b).** First callogenic clusters (FCC) at the level of the rest of the funicular cord (RCF) after 10 days of culture, **(c).** Callogenesis ball (CB) after 20 days of culture, **(d).** Dark friable callus invading the oxidized explant (OEx) after 30 days in culture.

We have just seen that the cultivation on the induction medium (1mg.l^{-1} AG_3) leads to the formation of callus from the rest of the funicular cord forming an integral part of the embryo and its integument. The fact of having performed an injury by separating the embryo from its funicule and of having brought it into contact with the culture medium would have been an experimental condition which favored its reaction to callogenesis

3.2 Histological analysis

Culture in vitro on a medium rich in minerals and vitamins supplemented with growth regulators (GA_3), and likely to accentuate cell dedifferentiation in the cambial area in question. This is visible in figure (4 a, b, c) where the coloration of this tissue becomes more and more intense.

Under these conditions, the reactivated zone ends up giving a parenchymal tissue (TP) forming a callus after ten days. This callus increases in size with cultivation until the implant is invaded. Certain cellular enclaves of the formed callus are characterized by a growing hub which ends up occupying almost all of the cells which have a more or less elongated shape and fairly thin walls.

These last two characteristics are generally found in embryogenic cells. (Fig.4).

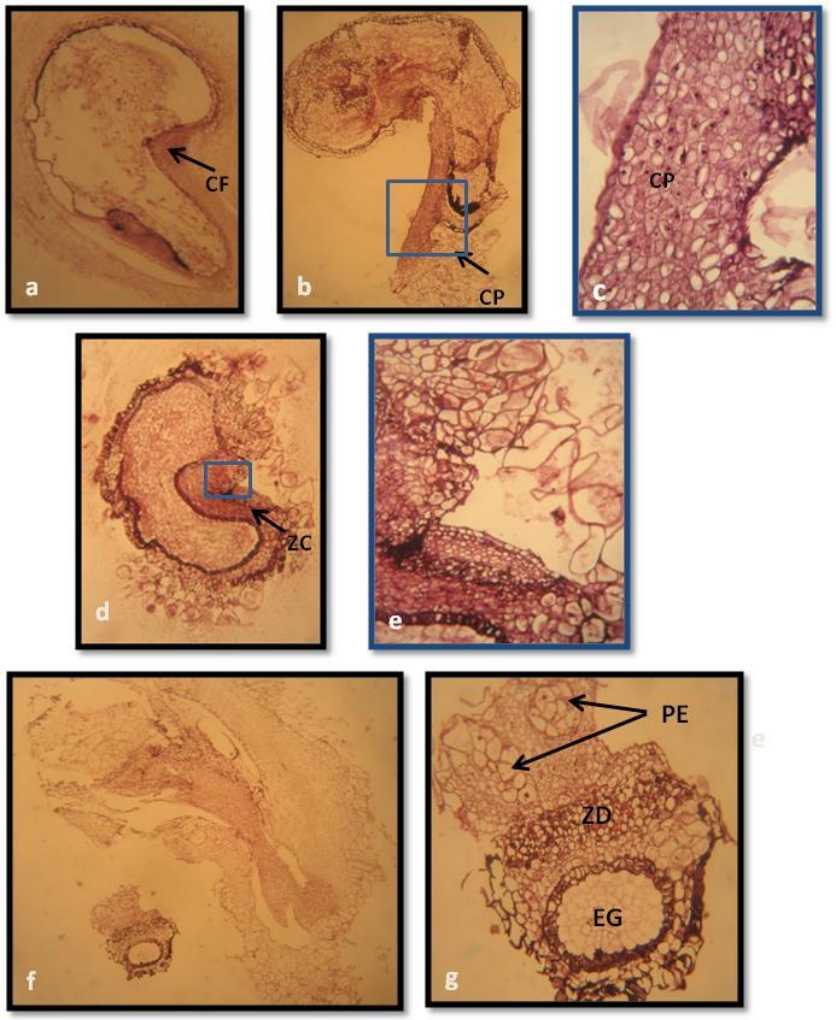

Figure 4. Anatomical changes corresponding to the morphological changes in Figure 3. **(a).** Decorticated embryo presenting a funicular cord (CF) with high stained cells Gx200; (b). Cells of the CF more stained of the CF with new formation of the first callus blanks with parenchymal cells (PC) Gx 200; (vs). **(c)** Close-up of the source cells of the Gx400 callus; **(d).** Increase of the target zone (CF) becoming even more colored with enlargement of the zone of callogenesis (ZC) Gx 200; **(e).** Callus proliferation around the target zone Gx 400; **(f)** Invasion of the explant by callus and formation **(g)**Close-up of embryos Gx400.

Histological observations made subsequently before transfer to expression medium seemed to prove that the dark-colored and friable callus that we have just described are truly embryogenic in view of the type of cells which constitute them more or less elongated cells with dense cytoplasm, large nuclei and thick walls. (Fig.5)

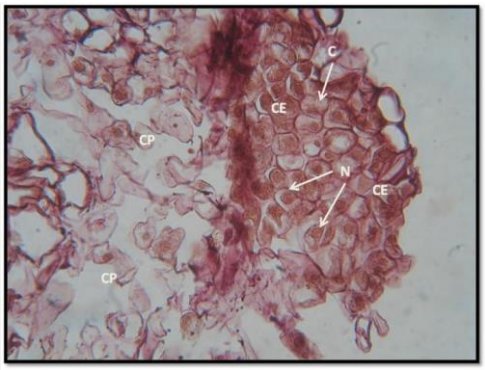

Figure 5. Histo-cytological appearance of the embryogenic callus of Opuntia ficus-*indica* maintained on MS medium supplemented with 1 mg.l^{-1} GA_3 before the expression phase. On the left, the cells are parenchymal (CP). To the right of embryogenic cells (CE) with dense cytoplasm (C) and large nuclei (N) Gx 400.

3.2 Discussion

Despite the consistent analysis made previously, and in whose testimony a nucellar enclave on the micropylar side was at the origin of the embryogenic callus induced *in vitro*, the resumption that we made revealed a small confusion in this analysis. In fact, the structure of the ovum in *Opuntia ficus-indica* is of a strong anatomical complexity which can easily lead to an amalgamation of legend.

Analysis based on closer observations in time and space on both morphological and anatomical analyses showed that the tissue involved in embryogenesis induction appeared to be the cambial tissue of the funicular cord part remaining attached to the embryo of immature seed explants. However, by referring to the role played by the funicule as an umbilical cord, it allows the organic and nutritional link of the embryo with the maternal tissue by means of vascular bundles. These vessels are in part made up of primary tissues with poorly differentiated cells (Esou, 1965).Such a specific involvement of this particular tissue could be inherent in the histogenic property of its cambial part responsible for the genesis of the phloem and xylem tissues of this funicular cord which seems, according to Esau (1965), to maintain a certain histo-cytological activity even in the seeds at fairly advanced maturity.

In cactus, as in other plants, we presume that the remnant of the funicle which remains unattached from the embryo after decortication has an anatomical structure similar to that described by Esou (1965). The sections taken before culturing confirm this analogy, in that the tissues of the funicular cord are made up of colored cells testify to the presence of a fairly dense cytoplasm corresponding to young cells in mitotic activity at the level of the funicular cord of immature shelled seeds. These young cells belong to the cambial area of the funicular cord which generates phloem outwards and xylene inwards.

The anatomical characteristics of the cambial cells described in the text constitute an argument in favor of the involvement of this zone in the initiation of the process of cell dedifferentiation leading to the formation of callus, the aptitude of which for somatic embryogenesis has been confirmed by the obtaining real somatic embryos. In the present study, the tissue of the funicular cord, ensuring the vascular connection with the embryo at the level of the hilar - micropylar pore, we demonstrated its evolvement in somatic embryogenesis through "dedifferentiation-redifferentiation" pathway".

The two types of morphological and anatomical analyses used as a tool of investigation prove unequivocally the origin of the callogenesis induced on juvenile tissues of the immature seed. Reproductive organs have been used with success in the *in vitro* regeneration of many plant species (Olivera-Freitas et al. 2016). As examples, high responses to somatic embryogenesis have been obtained using ovaries, (Kikkert et al. 2005), the stigma (Chinacone and Germana 2016), the anthers (Gribaudo et al. 2004; Chinacone and Germana 2016) and the flower (Gambino et al. 2007). Other types of explants have been also used as the primary source for the induction of somatic embryogenesis in cacti by cultivating shoot apices of leaf primordia (Gomes et al. 2006). Mature and immature zygotic embryos have shown to be the better explants for somatic embryogenesis induction in other species such as tropical palm (Olivera–Feritas et al. 2016); strawberries (Omar et al 2013).

A direct correlation has been identified between the degree of polyembryony *in vivo* and the ability to regenerate somatic embryos *in vitro* (Mitra and Chaturvedi 1972). Indeed, in some cases, the development of somatic embryos is due to the ability of the species to undergo polyembryony (Zhang et al. 2006; Nakano et al. 2013).

In olive trees, the regeneration of somatic embryos has been attempted through the use of several types of explants such as immature zygotic embryos (Maalej et al. 2002), cotyledons (Brhadda et al. 2008), and other explants such us *in vitro* radicules, leaves of mature trees (Capelo et al. 2010). Meristematic cells with dense cytoplasm have been also observed in olive trees *Olea eurepea*. These cells are

isodiametric, non-vacuolated and have a dense cytoplasm where we note the presence of one or more nucleoli showing their embryogenic activity (Féreol et al. 2005).

5. Conclusion

The morpho-anatomical analysis carried out in this study made it possible to correct the interpretations previously made by other authors regarding the ovular tissue involved in the process of somatic embryogenesis developed at the time (Chakroun et al. 2013). The removal of external integuments of this organ, containing vascular cells including cambial parts, improved the exposure of this zone to the culture medium. This contact would have activated the callogenesis process before explants oxidation has occurred facilitating embryogenesis. One might have wondered why the nucellar part containing a naturally embryogenic zone (zygotic and/or gametophytic) would not have been a potential target for embryogenesis in vitro. We believe that the preservation of the internal integument around the embryo would have been an obstacle to this possible reaction.

Further investigations on larger tissue portions of the funicular cord should be attempted to improve the regeneration rate of somatic embryogenesis.

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