Ontogenesis of the Somatic Embryogenesis of Habanero Pepper (*Capsicum chinense* Jacq.)

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Abstract. The ontogenesis of direct high-frequency somatic embryogenesis of *C. chinense* induced from hypocotyl was characterized through a histological analysis of the different phases in the histodifferentiation process during the development of the somatic embryo. The anatomical analysis was carried out since the hypocotyl segments were placed in the culture medium until 45 days of culture. The somatic embryos were induced and maintained in Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (9.5 μM). Samples of tissues and organs were taken every 24 h, fixed in formalin acetic alcohol, and embedded in plastic resin. They were cut into serial sections (5 μm) and stained with toluidine blue. The analysis revealed that the proembryogenic cells originated just from provascular hypocotyl cells. Provascular cells acquired the embryogenic competence 48 h after induction and an intense mitotic division was observed and embryogenic structures were generated first along the vascular strands, which subsequently evolved into somatic embryos. After 2 weeks, there were observed embryos at different stages of development (preglobular, globular, heart-shaped, torpedo-shaped, and cotyledonary). This is the first report dealing with the ontogenesis of the direct somatic embryogenesis of *C. chinense*, and it is the most complete histological characterization carried out on somatic embryogenesis in the *Capsicum* genus to date.

Somatic embryogenesis can be described as the process through which haploid or diploid somatic cells develop into different kind of plants through the characteristic embryological stages without fusion of gametes (Williams and Maheswaran, 1986). This phenomenon has been observed in tissue culture of several angiosperm and gymnosperm plant species. The anatomy of somatic embryos has been presented by many authors (Auboirn et al., 1990; Decout et al., 1994; Gill and Saxena, 1993; Suhasini et al., 1994). However, few studies have characterized the different ontogenetic stages of these embryos (Fransz and Schel, 1991; Toonen et al., 1994; Yeung et al., 1996).

The somatic embryogenesis, besides being an important pathway for plant regeneration from cell culture systems and a commonly used method in large-scale production of plants and synthetic seeds (Stuart et al., 1987), is also an excellent morphoge-

netic system, which allows the study of differentiation processes. Somatic embryos can easily be obtained from an embryogenic cellular line maintained under controlled external conditions such as medium composition, growth regulators, and light. Halperin (1966) was the first one who demonstrated that somatic embryos arose from particular cluster of cells, named proembryogenic masses, which, in turn, originated from single cells. However, the most important, but also the least understood, aspect of somatic embryogenesis is the transition of somatic cells into cells capable of forming an embryo (De Jong et al., 1993).

Regarding the origin of somatic embryos, the main aspects for researchers are still: the primary tissues (Aleman et al., 1996; Lee et al., 1997; Puigderrajols et al., 1996), the unicellular or multicellular origin (Aleman et al., 1996; Lee et al., 1997; Loiseau et al., 1998; Marin-Hernández et al., 1998; Nonohay et al., 1999; Puigderrajols et al., 1996), cytological aspects of the competent cells (Aleman et al., 1996; Lu and Vasil, 1985; Menéndez-Yufaa and García de García, 1997), and histochemistry of the somatic embryos through its formation and development, particularly concerning starch, proteins, and polyphenols (Aleman et al., 1996; Garin

et al., 1997; Goh et al., 1999; Loiseau et al., 1998; Marin-Hernández et al., 1998; Navarro et al., 1997; Svobodová et al., 1999). The extensive literature in this field has also considered the histological aspects of the initiation and development of somatic embryos, although several fundamental aspects of the process remain unclear or appear to differ among the systematic groups (Benelli et al., 2001). However, as knowledge increases on the genesis and evolution of somatic embryos, the level of empiricism will diminish and the ability to reproduce this complex process will be enhanced.

Histological studies to describe the development of somatic embryos have been done on *Quercus robur* (Zegzouti et al., 2001), *Olea europea* (Benelli et al., 2001), Safflower (Mandal and Gupta, 2003), *Solanum tuberosum* (Kumar and Millam, 2004), *Feijoa sellowiana* (Cangahuala-Inocente et al., 2004), *Schlumbergera truncata* (Al-Dein et al., 2006), and *Phalaenopsis amabilis* (Chen and Chang, 2006). Within the Solanaceae family, it has been reported for *N. tabacum* (Gill and Saxena, 1993; Stolarz et al., 1991); *L. esculentum* (Gill et al., 1995; Newman et al., 1996), *S. tuberosum* (De García and Martínez, 1995; Gill and Saxena, 1993; Kumar and Millam, 2004; Pretova and Dedicova, 1992); and *S. melongena* (Tarré et al., 2004). However, there are a few reports on the genus *Capsicum* (Binzel Marla et al., 1996; Harini and Sita, 1993) and all of them on *Capsicum annuum* L. The objective of this study was to describe the events associated with direct somatic embryogenesis induced from the hypocotyl in *C. chinense* and to determine the structures and tissues of the explant involved in the development of the morphogenetic process through the monitoring of morphological and histological changes during the ontogenesis of the somatic embryo.

Materials and Methods

Induction of somatic embryogenesis. Somatic embryos of the cultivar BVII-03 of red Habanero pepper (*C. chinense*) were obtained from hypocotyls segments of 15-d-old plantlets from seeds germinated in vitro (Santana-Buzzy et al., 2005). Somatic embryogenesis was induced directly from the explant following the protocol of López-Puc et al. (2006) in a medium containing Murashige and Skoog (1962) mineral salts supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 9.05 μM). The cultures were maintained at $25 \pm 2^\circ\text{C}$ with a 16-h photoperiod (40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) during 45 d.

Histological analysis. The embryos took form inside the explant and became visible when a longitudinal cut was made in the hypocotyl epidermis. The somatic embryos were isolated and classified by stages of development (globular, heart-shaped, torpedo-shaped, and cotyledonary). During the process of somatic embryogenesis, biological samples were taken every 24 h throughout the 45 d of culture. The samples were placed in

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the fixing solution of formalin acetic alcohol containing 5 mL of 96% ethanol, 10 mL of 37% formaldehyde, 5 mL acetic acid, and adjusted to a 100 mL of volume with distilled water for 24 to 48 h following the Berlin and Miksche (1976) protocol at 10 °C. After fixing, the samples were dehydrated by successive changes in ethanol solutions at different concentrations (30%, 50%, 70%, 85%, 99%, and 100% v/v). Subsequently, after infiltration, the samples were embedded in plastic resin (JB-4 Glycol Methacrilato; Polysciences, Warrington, PA). Serial sections of 5 µm were cut off in a rotary microtome HM340E (Microm International GmbH), mounted on cytological glass slides, and stained with 0.05% toluidine blue (3 to 5 min). Observations and images were obtained with an inverted microscope (Axiovert MC80; Carl Zeiss D-73446).

Results

Figures 1A and 1B show a longitudinal section of the hypocotyl before being exposed to the culture medium containing 2,4-D. It was possible to distinguish: the cortical parenchyma, characterized by the presence of long, irregular cells of different sizes; the provascular zone had very small cells; and the central zone of the hypocotyl was performed by differentiated xylematic and phloematic elements. The first mitotic activity was observed at 48 h of culture from the center to outward with the xylem and phloem surrounded by a fine layer of cells that formed the provascular tissue. It can be seen around provascular tissue at the four- to five-cell layer of cortical parenchyma. After 2 d of culture with 2,4-D, an intense cell division of the hypocotyl provascular cells was observed in both periclinal and anticlinal directions, resulting in the presentation of slight bulges along the procambium with a radial orientation to the vascular strands (Fig. 1C–D). These bulges evolved into preglobular embryos after 6 to 7 d of culture that rapidly turned to the globular stage and only became visible when the epidermis of the hypocotyls was broken by the pressure applied from the inside the explant by the embryos (Fig. 2A–B). A line of tiny embryogenic structures along and around the vascular strands was observed (Fig. 2B). These groups of cells, presumably proembryoids, were individualized and separated from each other by a fine wall identified as the protoderm (Fig. 2C–D); the proembryoids evolved into bipolar and independent structures (Fig. 2E–F). The somatic embryos originated directly from the explant cells, and only the provascular cells were involved in the process, apparently without the intervention of the explant's xylem or epidermis cells (Fig. 2C–D). The proembryoids showed an apical region of very small cells with a very dense, stained cytoplasm as well as protodermis and suspensor (Fig. 2C). The suspensor, unlike the protodermis, showed long vacuolated cells with a slightly stained

Fig. 1. (A) Longitudinal section of the hypocotyl before in vitro culture (vb = vascular bundles; pc = parenchymatic cells); (B) longitudinal section showing periclinal cell divisions (pd); (C) longitudinal section of the hypocotyl showing thickening in the procambium (prc) as a result of intense mitotic activity of the provascular cells; (D) longitudinal section showing a cluster of mitotically active cells (pg = proembryonic globules).

Fig. 2. Ontogenesis of the somatic embryogenesis. Longitudinal section of: (A) segment of hypocotyl with irregular epidermis because of the pressure of the embryos born inside (ge = globular embryo); (B) longitudinal section of the explant during ontogenesis of the somatic embryos in the provascular zone of the hypocotyl (ep = epidermis); (C–D) embryos in early stages of their development (proembryoids). The suspensor formed through successive divisions of the base cells (s = suspensor); (E–F) somatic embryos in different stages of development (hse = heart-shaped embryos; te = torpedo embryos).

cytoplasm (Fig. 2C). After 12 d of culture induction, a procambium in the alveolated and polarized-shaped embryo was observed. The longitudinal section of the explant (Figs. 2E–F) showed embryos in heart-shaped and torpedo stages, respectively. These perfectly bipolar structures, once formed, did not show a vascular connection with the hypocotyl cells. The bilateral symmetry of somatic embryos was observed in later stages of development.

The transition of proembryoids to pre-globular and globular stages (early stages) was characterized by the formation of concentric rings of cells with a small vacuole and a prominent cytoplasm (Fig. 3A). In these meristematic centers, intense mitotic activity was observed (Fig. 3B). The subsequent cellular division in anticlinal and periclinal directions gave rise to meristematic centers consisting of small embryogenic cells with dense cytoplasm, small nucleus, and with small starch grains (Fig. 3A–B). A complex of cells around the embryogenic mass was perfectly distinguishable, producing small masses or isolated zones identified as proembryogenic structures (Fig. 3C–D). The appearance of globular structures was coupled with the development of the protoderm, the outermost layer of a developing embryo (Fig. 3E–F). The protoderm was distinguished in the early globular stages, but it was better defined in the late globular stage (Fig. 3G). A marked reduction in the mitotic activity of the cells and a well-defined cell organization at later stages of the embryos' development were observed, but differentiation did not occur; this event was observed mainly in cells located inside the cellular mass. It was evident by the presence of small vacuolated cells.

Somatic embryos preceded toward the different development stages: globular (Fig. 4A), heart-shaped (Fig. 4B), torpedo (Fig. 4C), and cotyledonar (Fig. 4D–E). The globular stage was followed by the appearance of cotyledonary zones, indicating the heart-shaped stage (Fig. 4C). Most of the mature somatic embryos showed bilateral symmetry and typical histology (Fig. 4E). The apical and root meristems and provascular strands of the embryos were clearly visible and displayed characteristic, intense staining (Fig. 4F–I). The root pole was developed in the cotyledonary stage of the embryo in group of cells with dense cytoplasm (Fig. 4G). Vascular connections between somatic embryos and explants were never observed. The apical meristem of mature somatic embryos in most of the cases was concave in the longitudinal section and composed of two to three cell layers (Fig. 4G), and the root meristem was covered by large cells with starch grains, a typical feature of the root cap (Fig. 4H). In a longitudinal section, tissues observed consisted of protodermis, parenchyma, and provascular strands (Fig. 4I). However, because of unknown reasons as yet, a high frequency of somatic embryos did not reach the plantlet stage despite the rapid emission of the radicle. This behavior is most likely associated with some physiological or genetic factors and undoubtedly confirms the recalcitrance of *C. chinense* to in vitro regeneration in the same way as other species of the genus *Capsicum*. On the other hand, the high frequency of deformed somatic embryos, including deformed cotyledons (Fig. 5A–E), abnormalities in the histodifferentiation of the apical meristem (Fig. 5F), absence of cotyledons (Fig. 5G–H) and the fusion of embryos (Fig. 5I–J) in *C. chinense*, seems to be a common phenomenon as in other species

of the *Capsicum* genus. The persistence of this behavior in Habanero pepper also contributes to a marked reduction in the rates of germination and conversion of somatic embryos into normal plants.

The results indicated that we have achieved a direct somatic embryogenesis, in which the embryos evolve normally through the different stages of development. Most of the embryos have bilateral symmetry, provascular tissue along the axis of the embryo, and both apices (caulinar and apical) easily distinguishable and well defined. This is one of the most complete studies carried out on the origin and ontogenesis of somatic embryos in the genus *Capsicum*, and it is the first report on the species *Capsicum chinense*. However, although it provides information of great importance for understanding the origin and evolution of the morphogenesis in this genus, further studies are required to shed light on the origin and nature of this phenomenon affecting the conversion of the embryos of this species of *Capsicum* into plants. Nevertheless, the results do allow us to infer that the inability of the embryos of Habanero pepper to convert into plants is not related to the ontogenesis of the embryo, but may be caused by genetic factors associated with the genus *Capsicum*.

Discussion

The culture of *C. chinense* hypocotyl in a solid medium supplemented with 2,4-D promoted the formation of somatic embryos in high frequency directly from the explant cells (López-Puc et al., 2006). However, to date, there are no reports on studies carried out on this species related to the ontogeny of the somatic embryo development or the cells that acquire the embryogenic competence during the ontogenesis of the somatic embryo. With the results of this anatomical study during induction and development of somatic embryogenesis of *C. chinense*, it was possible to identify the hypocotyl cells with the capacity and predisposition for direct formation of somatic embryos. It was also evident how these competent embryogenic cells evolved up to the formation of the somatic embryo. Although these two events are the most important in somatic embryogenesis, they are the least understood. Kwaaitaal and De Vries (2007), working with *Arabidopsis*, determined that the gene SERK1 (somatic embryogenesis receptor kinase 1), which was originally identified as a marker for embryogenic competent cells in plant tissue culture, was expressed in procambium cells and in immature vascular cells. The anatomical analysis revealed that in *C. chinense*, the embryos originated from the procambium cells, whereas the cortical parenchyma and epidermal cells did not participate in the process, at least not directly; this agrees with the results of Kwaaitaal and De Vries (2007). In plants, somatic embryos can develop from a competent cell (Nomura and Komamine, 1985); however, the origin of these cells is still uncertain. Many reports state that the

Fig. 3. (A) Presence of a ring of cells with small vacuole and prominent cytoplasm during the formation of the somatic embryo (preglobular); (B) Intense mitotic activity of the cells; (C–D) preglobular embryos; (E–F) globular embryos without vascular connection to the explant; (G) embryo in late globular polarized stage.

Fig. 4. Different somatic embryo development stages of *C. chinense*: (A) globular; (B) heart-shaped; (C) torpedo; (D) early cotyledonary; (E) late cotyledonary. Longitudinal section of the cotyledonary embryo *C. chinense*: (F) complete embryo; (G) caulinar apex; (H) radical apex; (I) vascular tissue developed.

Fig. 5. Abnormalities observed in high frequency during the somatic embryo development of *C. chinense*: Deformed cotyledons (A–E); abnormalities in the histodifferentiation of the apical meristem (F); absence of cotyledons (G–H); fusion of embryos (I–J).

totipotency is not an intrinsic property of all plant cells but can be acquired when the explant is exposed to potent synthetic auxins such as the 2,4-D (De Vries et al., 1988;

Mordhorst et al., 1998). Similar studies have been conducted on other species (Bespalko-Filho and Hattori, 1997; Guerra and Handro, 1998; Ho and Vasil, 1983; Hutchinson et al.,

1997; Laparra et al., 1997; Ozias-Akins and Vasil, 1982; Sagare et al., 1995; Van Hengel et al., 1998), some of them over periods of up to 40 to 60 d (Schwendiman et al., 1988;

Thomas et al., 1972), whereas Masuda et al. (1995), by anatomical analysis of samples, saw somatic embryo formation only from the epidermal cells of explants of carrot hypocotyl.

Somatic embryogenesis from the hypocotyl has been reported in *Genciana cruciata* (Mikula et al., 2005); *Gossypium hirsutum* (Ul, 2005); *Cicer arietinum*; *Lilium martagon* (Kedra and Bach, 2005); *Daucus carota* (De Vries et al., 1988; Guzzo et al., 1994; Kikuchi et al., 2006; Masuda et al., 1995; Nishiwaki et al., 2000), whereas in the genus *Capsicum*, only *C. chinense* has presented this regeneration model (López-Puc et al., 2006; Zapata-Castillo et al., 2007). In the case of *C. annuum*, somatic embryos have been obtained only from zygotic embryos in direct and indirect ways (Binzel Marla et al., 1996; Buyukalaca and Mavituna, 1996; Harini and Sita, 1993; Steinitz et al., 2003). We described the specific features of the origin and development of somatic embryos in *C. chinense* belonging to a genus (*Capsicum*) known for its recalcitrance to the morphogenesis in vitro. To date, there is no clear evidence of how this process took place. However, all the species of this genus in which somatic embryogenesis has been reported have one problem in common (Binzel Marla et al., 1996; Buyukalaca and Mavituna, 1996; Harini and Sita, 1993; López-Puc et al., 2006; Steinitz et al., 2003; Zapata-Castillo et al., 2007); once the somatic embryos are formed, directly and/or indirectly, most of them become deformed and do not reach the later stages of development (torpedo and cotyledonar). Steinitz et al. (2003) found that the most frequent deformations of the somatic embryos can be classified into three categories: 1) fusion of embryos (Benelli et al., 2001; Carraway and Merkle, 1997; Rodriguez and Wetzstein, 1994; Stipp et al., 2001; Tomaz et al., 2001); 2) absence of cotyledons, only one cotyledon, or deformed cotyledons (Carraway and Merkle, 1997; Jayasankar et al., 2002); 3) anomalies in the histodifferentiation of the apical meristem (Chengalrayan et al., 2001; Jayasankar et al., 2002; Nickle and Yeung, 1993; Padmanabhan et al., 1998; Stipp et al., 2001; Suhasini et al., 1994). This phenomenon could be provoked by genetic, epigenetic, or physiological factors. The permanent exposition of the somatic embryos of Habanero pepper to 2,4-D during induction and throughout their development could be one of the reasons of the mentioned abnormalities. Similar results were reported by Christou and Yang (1989), when their embryogenic cultures were maintained in synthetic auxin for long periods of time. Santos et al. (1997), in an ontogeny study of the somatic embryos from immature cotyledons of soybean [*Glycine max* (L.) Merr.], described numerous abnormal histodifferentiated embryos like torpedo-like embryos resembling a trumpet. This kind of embryos had well-defined procambium, but neither an apical meristem nor a root meristem was detected. Fernando et al. (2002) found soybean trumpet embryos with normal root meristem, but the shoot meristem

was absent. The analysis of histological sections in mature trumpet embryos in peanuts showed a poorly developed meristematic region and low conversion rate (Wetzstein and Baker, 1993) like in soybean (Buchheim et al., 1989; Fernando et al., 2002; Kiss et al., 1991). High frequency of abnormal embryos of *Capsicum annuum* in later stages has been reported restricting germination and conversion into plants (Steinitz et al., 2003). Thus, the removal of the somatic embryos from the auxin-containing medium must be crucial to normal cotyledon development. However, the Habanero pepper explants only responded to the somatic embryogenesis when they were maintained in a medium containing 2,4-D throughout the whole process of histodifferentiation. The histological analysis showed that, after maturation, the fused embryos developed into fasciated cotyledonary embryos. These fused embryos also showed an epidermal layer common to all of them.

The results showed not only the nature of the hypocotyl cells from which the somatic embryos of Habanero pepper originated, but also the phases through which the embryo passes during its development. These results also represent an important contribution toward a better understanding of morphogenesis of recalcitrant species to in vitro plants regeneration not only for the *Capsicum* genus, but also for many other similar species affected by this phenomenon.

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