Title: Development of the ovule and seed of Habanero chili pepper (*Capsicum chinense* Jacq.):
Anatomical characterization and immunocytochemical patterns of pectin methyl-esterification

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Abstract

Ovule and seed development in plants has long fascinated the scientific community given the complex cell coordination implicated in these processes. These cell events are highly conserved but are not necessarily representative of all plants. In this study, with the aim of obtaining information regarding the cellular patterns that follow the usual development of the ovule and the zygotic embryo, we carried out an integral anatomical study of the *Capsicum chinense* Jacq., floral buds and seeds at various days during maturation. This study allowed us to identify the main histo-morphological stages accompanying the transition of somatic cells into the macrospore, female gamete, and the zygotic embryogenesis. This knowledge is fundamental for future biotechnological research focused on solving the morphological recalcitrance observed during the *in vitro* induction of somatic or microspore embryogenesis in *Capsicum*. For the first time in *C. chinense*, we have described the hypostases, a putative source of plant growth regulators, and “the corrosion cavity”, a space around the embryo. Additionally, the cell wall pectin-esterification status was investigated by immunohistology. At early stages of morphogenesis, the pectin is highly methyl-esterified; however, methyl-esterification decreases gradually throughout the process. A comparison of the results obtained here, together with the histo- and immunological changes occurring during the somatic and microspore embryogenesis, should help to elucidate the biochemical mechanisms that trigger the morphogenic events in *Capsicum* spp.

61 **Keywords**

62 *Capsicum chinense* Jacq.; ovule development; zygotic embryogenesis; methyl-esterified/de-
63 methyl-esterified pectin; immunohistology

64 **Abbreviations**

65 Bovine serum albumin (BSA), chalaza (Ch), cotyledons (Co), days post-anthesis (DPA), 4', 6-
66 diamidino-2-phenylindole (DAPI), embryo (Emb), embryonic sac (ES), endothelium (Ent),
67 endosperm (End), electrical conductivity (EC), functional megaspore cell (FM), funiculus (Fu),
68 ground meristem (Gm), integument (Int), hypostases (Hyp), megaspore mother cell (MMC),
69 micropyle (Mi), phosphate-buffered saline (PBS), potential of hydrogen (pH), pre-anthesis (PreA),
70 procambium (Pc), protodermis (Pd), ribonucleic acids (RNAs), root apical meristem (RAM), shoot
71 apical meristem (SAM), suspensor (Su), testa (Te).

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1. Introduction

The transition of the morphological and biological events, which extends from the establishment and development of the vegetative meristem to the flower formation, has been a motive of scientific fascination for years (Endress, 2011). Those "apparently simple cell changes" enclose the power to differentiate somatic cells into macrospore or microspore (megasporogenesis/microsporogenesis), which subsequently give rise to the female or male gametes (megagametogenesis/microgametogenesis), respectively (She et al., 2013). The fertilization of the female gamete allows zygote cell formation with defined cell divisions to form the embryo (zygotic embryogenesis) until it completes its maturation into the seed, thus producing a successful offspring (Yadegari and Drews, 2004).

Proper ovule and embryo sac development is critical to reproductive success. The multiple cell layers or cell types of the ovule and the embryo sac form the various components of the future seed. Research on the development of megasporogenesis, megagametogenesis, and embryogenesis in plants involves the analysis of the histo-morphological changes which begins with the differentiation of somatic cells into the archesporium or megaspore mother cell (MMC), the two meiotic divisions undergone by the MMC, the differentiation of one of the resulting meiotic cells into the functional megaspore cell (FM), followed by three continuous mitotic divisions which, in the end, differentiate into the egg cell among other cell types. In the double fertilization process, the egg cell fertilization produces the zygote, and the continuous cell divisions permit embryo development (Reiser and Fischer, 1993; Xu et al., 2011).

Anatomical details of ovule development or embryogenesis have been reported in several *Capsicum* species (Dharamadhaj and Prakash, 1978), but information on the whole process from megasporogenesis to embryo formation in *Capsicum chinense* is still lacking. Increasing evidence has indicated that many plant developmental processes, including embryogenesis, involve changes in the esterification status of cell wall pectins, which may cause cell wall remodeling during the process (Bárány et al., 2010; Dobrowolska et al., 2012; Kurczynska et al., 2012; Lora et al., 2017; Müller et al., 2013; Solís et al., 2016; Xu et al., 2011). Pectins are major components of primary plant cell walls; they are secreted into the cell wall in a highly methyl-esterified form and can be de-esterified in muro by pectin methyl-esterases (Lionetti et al., 2007). The degree and pattern of methyl esterification affect the cell wall structure, texture, porosity, and its chemical and mechanical properties (Wolf et al., 2009) dramatically. For example, pectin de-methyl-esterification is essential for the correct function of the guard cells (Amsbury et al., 2016).

Monoclonal antibodies that recognize plant cell wall epitopes, specifically those that recognize low and high degrees of methyl-esterified pectin, have been reported as particularly useful in revealing the patterns of pectin esterification during developmental processes (Rodríguez-Sanz et al., 2015). The JIM5 antibody immunorecognizes differentiated cells that usually contain partially methyl-esterified pectin with up to 40% of de-esterified residues, while the JIM7 antibody recognizes

proliferating cells containing more than 80% of methyl-esterified pectin residues (Bárány et al., 2010; Solís et al., 2016; Willats et al., 2006; Xu et al., 2011). Therefore, these antibodies can detect the degree of pectin methyl-esterification, correlating it with cell wall stiffness or loosening, which occurs during the development of the seed from the ovule, and more importantly, the degree of cell differentiation in each stage (Rodríguez-Sanz et al., 2015). An understanding of the dynamic changes in pectin methyl-esterification and remodeling of cell wall will enable us to gain more knowledge of the megasporogenesis, megagametogenesis, and embryogenesis processes (Rafińska and Bednarska, 2011).

Anatomical and biochemical aspects relating to the development of the ovule and embryo in *Capsicum* spp. are poorly understood (Dharamadhaj and Prakash, 1978; Lengel, 1960). *C. chinense* is a pungent chili pepper and, to date, research on this species is limited. Currently, cellular differentiation during megasporogenesis, megagametogenesis, and embryogenesis is mainly unknown. In this work, we report a detailed morpho-histological and immunocytochemical characterization of the dynamics of cell changes and pectin methyl-esterification in cell walls of the different cell lineages that accompany the ovule and seed development in habanero chili pepper (*C. chinense* Jacq.).

2. Materials and Methods

2.1 Crop nutrition condition

Seeds of the Habanero chili pepper var. Mayan Ba'alché (*C. chinense* Jacq.) were obtained from the germplasm bank of the Scientific Research Center of Yucatan. Twenty plants were cultivated under hydroponic conditions in a greenhouse with average temperatures of 33°/21°C (day/night), during the winter of 2015-2016. A nutrient solution was prepared using the following nutrient concentration (mg.L⁻¹): Total-Nitrogen 159.1, Phosphorus 38.1; Potassium 175.2; Calcium 98.8; Magnesium 28.8; Sulfur 38.4; Boron 0.4; Copper 0.4; Iron 1.7; Manganese 0.4; Molybdenum 0.1; Nickel 0.1; Zinc 0.2. with an EC of 1.6 dS.m⁻¹, pH of 6.0 (Furlani, 1997).

2.2 Biological material

Two hundred flower buds with < 1 mm length were tagged and monitored; ten of them were harvested each day over a period of 10 days, comprising the pre-anthesis (PreA) and anthesis days, following the methodology reported by Aleemullah (Aleemullah et al., 2000). Plant flowering began 35 days after seedling transplant. One hundred and twenty closed flower buds, just prior to opening, were selected and tagged using paper stickers. After flower opening and self-pollination, the development of the fruit was characterized. Ten fruits were harvested at 3, 5, 10, 20, 30 and 40 days post-anthesis (DPA). Pericarps from these fruits were removed with a sterile scalpel; the seeds were then carefully detached from the placental tissues and placed in fixing solution.

2.3 Fixing and staining of the tissue sections

The vegetal tissue samples were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C. After fixing, the samples were washed in PBS, then dehydrated in acetone (30, 50, 70, 90 and 100%) and embedded in resin (Technovit 8100) at low temperature (Rodríguez-Sanz et al., 2015). Thin sections of 3 to 5 µm were prepared using a microtome (Microm GmbH, mod. HM340E), stained with 0.05% (50 mg/ 100 mL) Toluidine blue in distilled water and mounted with Mowiol (Alfred et al., 1982). Structural analysis was carried out under brightfield using a Zeiss Axioplan 2 microscope.

2.4 Immunofluorescence on tissue sections

Tissue sections (2 µm thickness) from flower buds or developing seeds were hydrated three times, 5 minutes each time, in PBS 1X (NaCl 138 mM, KCl 3 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 1.5 mM, pH 7.4). Tissue sections were blocked for 10 minutes with 5% bovine serum albumin (BSA), washed again with PBS buffer 1X, and then incubated for 1 hour with the JIM5 (undiluted) or JIM7 (1:5 dilution) monoclonal antibodies, the former against low-esterified pectin and the latter against methylated pectin (Solís et al., 2016). Samples were washed five times for 5 minutes each with PBS buffer 1X and then incubated for 45 minutes in the dark with 1:25 dilution of anti-rat secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes, Cat. no. A-11001). After washing 5 times for 5 minutes each time with PBS 1X, 1 mg/ml DAPI (4', 6-diamidino-2-phenylindole; Fluka) was added to the tissue sections, and these were incubated for 10 min and analyzed with a confocal microscope (Leica TCS-SP5). As a control, samples were prepared as above but without primary antibody.

3. Results

3.1 Development of the floral buds

From the observations carried out during the developmental period of the floral buds, starting from buttons of 1 mm in length up to the occurrence of anthesis, it was possible to determine that the Habanero chili pepper requires 9 PreA, and this permitted us to classify the floral buds according to the PreA day (Fig. 1 A), following the methodology of Aleemullah (Aleemullah et al., 2000). During the first days of floral bud development (9 to 6 PreA), the sepals cover all the floral bud structures, and from day 5 to 1 PreA the petals emerge and enlarge more than the sepals, at this point the petals cover most of the internal floral structure. Prior to the anthesis day, all the internal floral organs have almost completed their development, including the ovules in the ovary (Fig. 1 B, C), while on the anthesis day, the petals open, revealing the pistil which includes the stigma, style, the ovary and the ovules, and the processes of anther dehiscence begin (Fig. 1 A). In angiosperm plants, flower and fruit development is tightly interrelated because flower development is the stage that precedes pollination and further development of fruit and seed (Primack, 1987).

3.2 Anatomical characterization of the ovule

The histological analysis of the sections of ovary samples during the different PreA days showed that at day 9 PreA the ovule is visible, showing an inclination in its growth. This inclination shows an outstanding growth of the integument on the convex side (Fig. 2 A). Most of the ovules in angiosperms present a curvature; this characteristic allows the micropyle to remain close to the placenta. The micropyle is the structure through which the pollen tube passes during fertilization (Endress, 2011).

In the ovules pertaining to the 9 days (PreA), three main zones can be seen during their emergence. The first zone corresponds to the outer layer which gives origin to the dermal layer, while the second zone corresponds to the subdermal layer which presents anticlinal and periclinal divisions, and the underlying tissue from which the procambium will originate represents the third zone (Fig. 2 A'). Subsequently, the nucellus tissue and the megaspore mother cell (MMC) differentiate from the first two zones. At 8 PreA (Fig. 2 B), the archesporium cell is observed, and it is surrounded by a layer of nucellus cells. Both the archesporium and the nucellus cells are being invaginated by the integument, which is composed of at least four layers of cells. A close-up of the nucellus cells showed that the tissue oriented to the micropylar axes is formed by a single layer of cells, while in the chalazal axis the nucellus is composed of three layers (Fig. 2 A'). The ovules of 7 PreA analyzed, showed the integument completely surrounding the nucellus and the archesporium cells. The integument showed at least 13 layers of cells in the widest area oriented to the micropyle axis (Fig. 2 C). In the integument tissues next to the nucellus, the two most internal cell layers acquire an elongated form and are orientated tangentially in a palisade-like cell structure, which

209 further differentiates in the endothelium; these cells stain stronger than the rest of the integument
210 cells (Fig. 2 C). The chalazal axis shows one cell which stains less than the rest of the integument
211 cells, with a greenish tone. A close-up of the archesporium and nucellus cells showed that the
212 former is a binucleate cell that stains stronger than nucellus cells at this stage, while the nucellus
213 still has one cell layer in the micropyle axis (Fig. 2 C').

214 At 6 PreA, the ovules have the anatropous shape with a significant differentiation in the integument
215 tissues where at the base of the funiculus, a group of procambial cells starts to differentiate to
216 extend the chalazal axis. Additionally, the integument shows the micropyle channel (Fig. 2 D). The
217 close up of the area corresponding to the chalaza and megasporial cells shows the presence of four
218 megasporial cells, and the nucellus cells are still present (Fig. 2 D'). The ovules of 5 PreA display
219 two layers of endothelial cells that stain stronger than the rest of the integument cells, while the
220 nucellus encloses a group of cells, two of which show the strongest staining in the megasporial cells
221 (Fig. 2 E). The megasporial cell adjacent to the chalazal axis is larger than the megasporial cell
222 oriented to the micropyle axis. At this stage, the bigger cell might be initiating the differentiation of
223 functional megaspore cell (FM) and the degradation of the rest of megasporium cells (Fig. 2 E'). At
224 3 PreA, the endothelium still stains stronger than the rest of the cells. Two cells are also present
225 inside the endothelium (Fig. 2 F). At this stage, the nucellus cells are disorganized and start to
226 disappear. In contrast, the functional megaspore divided into two cells that are polarized at the
227 chalazal and micropyle axis (Fig. 2 F'). At 1 PreA, the endothelium cells of the ovule maintain the
228 palisade-like arrangement and still stain stronger than the chalazal area and the rest of the
229 integument. At this moment, the chalazal area contains more cells than in previous stages (Fig. 2
230 G). A close up of the endothelium area shows that the nucellus is absent and four nucleated cells
231 are visible (Fig. 2 G').

232 At anthesis day, the ovule is wholly developed and is formed by the integument (Int) and the
233 embryo sac (ES); at this stage, the ovule is mature (Fig. 2 H). The embryo sac shows two nucleated
234 cells close to the center; these cells become the polar cell, while at the micropyle axis, two strongly
235 staining elongated cells are present (Fig. 2 H'). The last cells become the synergids. On the
236 chalazal axis, inside the embryo sac, the parental cells for antipodals were not clearly distinguished,
237 but in multiple tissue sections, they were observed.

238 239 3.3 Immunohistochemical detection during the development of the ovule 240

241 The immunodetection of the pectin with JIM5 and JIM7 antibodies showed that at 9 PreA day the
242 low-esterified pectin (JIM5) was preferentially located in the outer layer of the ovule (Fig. 3 A, A'),
243 while JIM7 cross-reacted homogeneously with all cell layers of the ovule (Fig. 3 B, B'). These
244 results indicate that the integument cells are actively dividing and that they have a high content of
245 esterified pectin. Immunodetection of esterified and low-esterified pectin at the archesporial cell in
246 the ovule produced similar intense signals among the nucellus cells and the outer layer of the

integument (Fig. 3 C, C', D, D'), suggesting similar content of low-esterified and highly esterified pectin in these cells. However, in the internal cells of the integument, the JIM7 antibody produced a stronger signal in comparison with that of JIM5 (Fig. 3 C, D), indicating more highly methyl-esterified pectin. Similar fluorescent signals for JIM5 and JIM7 were observed among the cell walls of the cells in the layers corresponding to the integument and the four megasporial cells. Interestingly, the JIM5 antibody produced a stronger signal in the cell wall of nucellus cells (Fig. 3 E, E') compared to that of JIM7 (Fig. 3 F and F'). These findings support the possibility that nucellus cells are quickly demethylating and differentiating in the ovule.

The JIM5 antibody produced a heterogeneous signal in the floral bud at the day of anthesis; in the innermost layers of the integument cells, corresponding to the endothelium, the antibody did not produce fluorescence, but in the middle and outer layers, the signal increased towards the external cell layer. The layer delimiting the embryo sac also produced a strong signal (Fig. 3 G, G'). In contrast, JIM7 produced a low signal in the layer delimiting the embryo sac and at the inner and middle cell layers of the integument (Fig. 3 H, H'). Both antibodies produced similar signals in the outer cell layer, but in the chalazal cells, JIM5 immunoreacted with a group of cells (Fig. 3 G, G'), while JIM7 gave a strong signal only in a single cell (Fig. 3 H, H'). Taken together, these results show that when the ovule has reached maturity, most cell walls have little highly methyl-esterified pectin.

3.4 Fruit developmental stages

As a result of the monitoring of fruit development at 3, 5, 10, 20, 30 and 40 DPA (Fig. 4 A), an increase in whole fruit size can be observed over time. The fruits of 3 DPA were < 4 mm and had a small pericarp, shorter than the sepals. During the following days, the pericarp increased its size while the sepals stopped their growth. At 10 DPA the pericarp had enlarged, reaching a length between 1.2 and 1.5 cm and a width between 0.8 and 1.2 cm, while maintaining a smooth surface. At 40 DPA, the length of the pericarp (fruit) increased to 4.5-5.0 cm and the width to 3.0-3.5 cm. At this stage, the pericarp showed some clefts on its surface, and a change in the color from green to greenish-orange was observed; this transition indicated that the chili pepper was physiologically mature. Parallel with the growth of the chili pericarp, the development of all the fruit tissues progressed (Fig. 4 B, C, D). Fruits at 10 DPA had seeds with a faint green hue (Fig. 4 B); longitudinal and transversal sections of fruits at 30 DPA showed the seeds were attached to the base and middle part of the placental tissue (Fig. 4 C). At this stage, the seeds have reached their maximum size, although they have not reached maturity. Mature fruits of 40 DPA become orange and have creamy yellow seeds joined to placental tissues; the seed color and hardening of the testa indicate that the seeds are mature at this point (Fig. 4 D).

3.5 Anatomical characterization of zygotic embryo

The zygote with the first cell division and initial development of endosperm (end) is observed in seeds with 3 DPA; the integument (Int) covers most of the seed. The zygote has a suspensor, and below the suspensor, the micropyle tissue (Mi) was located (Fig. 5 A, A'). In this stage, predecessor cells of the testa seed tissue (Te) are observed (Fig. 5 A). The endosperm is covered by the endothelium, and all structures are surrounded by the integument (Fig. 5A'). The embryo at the pre-globular stage was completely surrounded by vacuolated-nucleated endosperm cells (Fig. 5 B, B'). At 5 DPA, the seeds continue their development with the endosperm (End), embryo (Emb) and integument (Int) increasing their size, and the chalaza cells (Ch) start to scroll from the upper to the lowest position of the seed, close to the micropyle (Fig. 5 C, D). The embryo is in progress between the globular and heart stages, with a well-defined protodermis (Pd), while the suspensor cells are not observed (Fig. 5 C', D').

Histological analysis of seeds collected from fruits at 10 DPA shows that the seeds have almost completed their growth, the testa delimited the size of the seeds; nevertheless, the embryo is still in the torpedo, and cotyledonary stage with a suspensor joined to placental tissue (Fig. 5 E, E', F, F'). At this point, the presence of a corrosion cavity in the endosperm can be clearly observed; these cavities become evident at the globular stage. At 20 DPA, the embryo has a semi-conical shape, with nucleated periderm cells and two cotyledons, one of them larger than the other (Fig. 5 G). In seeds collected at 30 DPA, the embryo is increasing in size, and one cotyledon is larger than the other (Fig. 5 H). Histological analysis showed that the embryo had not completed its maturity since the integument is still a wide area of the seed. It is well known that in mature seeds from *Capsicum* spp., the integument is a single layer of cells or it is absent in some cases (Watkins and Cantliffe, 1983). The corrosion cavity (cc) remains present at this stage, and the cotyledonal embryo is filling it (Fig. 5 H). Histology of seeds collected at 40 DPA shows that they have a lignified testa, with an integument presenting fewer cell layers in comparison with previous stages, but which is more abundant in the area where the seed attaches with the placenta; at this point the embryo has reached maturity, filling the corrosion cavity and together with the endosperm, occupying most of the seed structure (Fig. 5 I).

3.6 Immunohistochemical detection of pectin with JIM5 and JIM7 antibodies in zygotic embryo

The immunoanalysis with JIM5 and JIM7 antibodies shows similar staining of seeds at 3 DPA compared to those observed on the cell walls of the ovule at anthesis day. Although the JIM5 antibody produced a single visible signal in the endosperm (Fig. 6 A, A'), the JIM7 produced a low signal in all of the integument cells which also shows a large nucleus (Fig. 6 B, B'). When the developing seeds showed embryos at an early globular stage, JIM5 produced a stronger signal in the endosperm in comparison with that of JIM7. It was remarkable that the signal in the endosperm was detectable for the first time with both antibodies, deeper in the area surrounded by the integument (Fig. 6 C, C', D, D'). JIM5 and JIM7 produced a strong signal in two or three narrow cell layers of the internal face in the integument (Fig. 6 C, C', D, D'). A closer observation of the embryo

at an early globular stage shows that JIM7 immunoreacts with the external cell layers of the suspensor and cell walls inside the embryo cells (Fig. 6 E, E'). The signal produced by the binding of JIM5 is similar to that of JIM7, but JIM5 does not bind to the internal cell layers of the embryo or suspensor cells (Fig. 6 F, F'). These results support the recognition of cell walls only in differentiated cells by JIM5, while JIM7 recognizes cell walls in cells transiting to differentiation. Moreover, a closer inspection of embryo cells tested with JIM7 revealed dividing cells with the signal in the cytoplasm and around the nucleus (Supplementary Fig. S1). The result is consistent with that found by Baluska et al. (2002) in the cells of maize root meristem treated with Brefeldin A (BFA), an inhibitor of cell exocytosis, and where JIM7 immunoreacted into the compartment produced by BFA treatment.

In the mature embryo (20 DPA), JIM5 produced a strong cross-reaction with the cells that originate from the pro-vascular tissues and less binding in the area that surrounds the apical meristem (Fig. 7 A, A'). In contrast, the JIM7 antibody produced a very low signal in both areas (Fig. 7 B, B'). The root apical meristem area (RAM) immunoreacted with JIM5, while the JIM7 did not bind with any cells of the RAM area (Fig. 7 D, D'). Additionally, the JIM5 immunoreacted more strongly with cell layers that might differentiate into vascular tissues than with cells close to the RAM. The calyptra emitted a very low fluorescent signal in cell layers that delimits the protoderm (Fig. 7 C, C'). JIM5 binds homogeneously and strongly with all cotyledonal cells (Fig. 7 E, E'), while JIM7 immunoreacted poorly with cell walls of these cells (Fig. 7 F, F'). In the hypocotyl hook area, the JIM5 antibody binds heterogeneously with the embryo tissues; cells close to the endosperm cross-reacted more strongly than the cells close to the integument, showing a gradual attaching of JIM5 antibody (Fig. 7 G, G'). JIM7 antibody produced a weak signal mainly in the provascular area, while the rest of the embryo tissue did not show cross-reaction (Fig. 7 H, H').

4 Discussion

This report makes a significant contribution to the fundamental knowledge of the morpho-histological changes and the status of pectin esterification in the different cell lineages in the megasporogenesis, megagametogenesis, and zygotic embryogenesis processes in *Capsicum chinense* Jacq, a domesticated species with increasing commercial interest (Raveendar et al., 2017). Other descriptions in *Capsicum* species have been partial, focusing on some specific developmental stages (Dharamadhaj and Prakash, 1978; Filippa and Bernardello, 1992; Ghimire and Heo, 2012); this is the first report addressing the study of all steps in these processes, as a whole. The monitoring of nine days before flowering (Fig. 1) and from the ovule-pollination to 40 DPA, enabled us to identify each stage of cell differentiation from somatic cells to female gamete and embryo development (Figs. 2, 5). Additionally, the immunodetection with the JIM5 and JIM7 antibodies in tissue sections of the primary developmental stages revealed the dynamic changes in the degree of methyl-esterification of pectin in cell walls of the different cell lineages during the ovule differentiation to mature zygotic embryo (Figs. 3, 6, 7). The information obtained in this work

can be used in future genetic improvement programs and will allow us to learn from zygotic embryogenesis to further identify the bottlenecks in other embryogenic processes (Winkelmann, 2016).

4.1 Megasporogenesis and megagametogenesis

The main histological findings during the ovule formation in *C. chinense* are described below. The observation of the progress of the ovule from 9 PreA to anthesis shows that the archesporial cell follows a polygonum-type pattern division until the formation of the embryo sac is complete, and the ovules adopt their anatropous shape. Polygonum-type pattern division and the anatropous shape of the ovule are characteristic of the Solanaceae family, e.g., *Withania somnifera* (Bittencourt and Mariath, 2002; Dharamadhaj and Prakash, 1978; Ghimire and Heo, 2012). Toluidine staining produced acceptable contrast among the different cell types, allowing the distinction between the chalaza cells, the micropyle cell layer, the integument and most importantly, the cell differentiation of the embryo sac (Fig. 2). At the early and middle stages (9 to 6 PreA), the ovule is covered by the integument (Fig. 3 C, C', D, D', E, E'), which is probably involved in the protection of the embryo sac development and nutrition of the embryo, as described in *Taraxacum* genus in the Asteraceae family (Musiał et al., 2013). The ovule of *C. chinense* shows one integument, which is characteristic of more evolved plants (Endress and Igersheim, 2000; Wang and Ren, 2008). This is consistent with descriptions in other Solanaceae (Ghimire and Heo, 2012). However, the histological study could be improved by using additional dyes to stain starch, proteins, lignin, and lipids in order to obtain an integral picture of histological and metabolic changes. Regarding the 5 to 1 PreA and the anthesis day, they contained all the structures of mature flowers, including a high number of ovules in the ovary (Fig. 1 B). At the anthesis day, the flower contains mature ovules as revealed by their structural organization (Fig. 1 C), showing two synergid cells, one egg cell located in the micropyle sac and one polar nucleated cell positioned at the center of the embryo sac, together with vestiges of the antipode cells in the chalazal side (Fig. 2 H, H'). These findings were in concordance with an earlier description of *Capsicum annuum* (Dnyansagar and Cooper, 1960). However, after ovule fertilization a lower number of seeds are usually observed in *C. chinense* fruits, thus suggesting a high ratio of ovule abortion. This phenomenon has been described in *Solanum phureja* where approximately half of their ovules degenerate at pollination (Dnyansagar and Cooper, 1960). After pollination, the fertilized fruits remain joined to the plant, growing in size, while non-pollinated fruits become yellow and are aborted. The degree of development of pollinated fruit and the maturity correlate with the seed development, which is why this is a simple criterion suitable to study the embryogenic process (Darnell et al., 2012; Munting, 1974).

4.2 High methyl esterification of pectin is associated with cell proliferation during megasporogenesis and megagametogenesis

During cell differentiation in megasporogenesis and megagametogenesis, the cell walls are subjected to dynamic chemical modifications. The degree of pectin methyl-esterification in cell walls is one of the critical factors that govern the fate of cells during differentiation, and currently it is used as a marker of the primary morphogenic events (Bidhendi and Geitmann, 2016; McCann and Roberts, 1994; Solís et al., 2016). The dynamic degree of methyl-esterification pectin detected with JIM5 and JIM7 antibodies in the earlier stages of megasporogenesis in *C. chinense*, revealed a higher proportion of highly methylated pectin which is consistent with a large number of highly dividing cells (Fig. 3 A, B). As megasporogenesis progressed, the immunorecognition with JIM5 of low-esterified pectin in archesporial and nucellus cell walls was abundant in the tissues. It is interesting to note that the integument area adjacent to the nucellus did not immunoreact with JIM5, while the JIM7 immunoreaction was similar in the integument, archesporial cell, and nucellus cells (Fig. 3 C, C'). The labeling pattern suggests that the integument grows actively from the adjacent area of nucellus cells to the outside and that the most external cell layer is more differentiated than the rest of the integument cells.

The degree of pectin esterification in the integumentary cell walls and the four megasporial cells was similar when it was detected with JIM5 and JIM7 (Fig. 3 E, F), which suggests a high presence of both types of pectin in cell walls at this stage. It is remarkable that the labeling with JIM5 increases in the cell wall of nucellus cells as the maturity of the ovule increases. The increase in the de-esterified pectin in the cell wall of the nucellus cells could be related with priming of the tissue for degradation or absorption (Fig. 3 E). The patterns of immunolabelling of the JIM5 and JIM7 antibodies, during the MMC formation and the four megaspore differentiations after meiosis in *Anona cherimola* and *Persea americana* (Lora et al., 2017) were similar to the results found in this study. These results indicate that the dynamics change in methyl-esterification of pectin in cell walls during megasporogenesis and megagametogenesis in angiosperms is mainly conserved, and our results are consistent with current knowledge. Curiously, the MMC and the four megaspore cells in *Arabidopsis thaliana* show different immunological patterns in comparison with those found in *A. cherimola* and *P. americana* (Lora et al., 2017) and the results observed here; however, they were similar to the pattern observed in late stages of the megagametogenesis in *C. chinense*; according to Lora et al. (2017), these differences can be related to the position of the plant on the evolutionary scale.

4.3 Zygotic embryogenesis

Histological analyses of the early development of the fruit show the zygote and all other tissues that will give rise to the mature embryo and seeds (Fig. 5 A, A', B, B'). As observed during megagametogenesis, the integument increases the number of cell layers in young seeds until the full size of the seed is reached, as described by Filippa and Bernardello (1992) in *Athenaea*, *Aureliana* and *Capsicum* genera. Inside the integument, the micropyle and chalaza with vascular

bundles expand towards the placental tissue. At early stages of embryo development, the endosperm has a “cellular type” division which is common in the Solanaceae family (Fig. 5 B, C). The integument accumulates nutrients to support embryo nutrition in further stages (Ghimire and Heo, 2012); when the seed reached maturity, the integument was reduced to only one or two cell layers.

The first histological report on *Capsicum* histology by Cochran (1936) was beneficial for the present work because he described in detail the complete ontogenic process, from the megasporogenesis to the embryogenesis (Cochran, 1938). This report enables us to analyze and classify all the stages studied here. However, one limitation of Cochran’s work is that the results were recorded in drawings and many cell details are missing. Modern studies use photographic records, where resolution and structural details are better seen, but most of them focus on the subcellular localization of particular RNAs or proteins in specific stages and do not describe in detail the anatomic structures. In this report, a photographic record was carried out for all the stages of ovule and seed development in *C. chinense*, from megasporogenesis to mature embryo. Some anatomical structures, previously not described in *Capsicum*, were clearly observed, for example, the hypostases (Fig. 5 B’), which is probably a source of plant growth regulators or nutrients as was suggested in *Theobroma cacao* (Hasenstein and Zavada, 2001), and “the corrosion cavity”, an empty space that enables the embryo to grow (Fig. 5 C-I) (Buchholz, 1918). It is important to emphasize that this is the first report in *Capsicum* where the full embryogenesis is histologically analyzed and can serve as a reference for further studies, in this and other genera. It is surprising that “the corrosion cavity” has not been described in previous reports of *Capsicum* (Cochran, 1936; Filippa and Bernardello, 1992) because it is clearly evident.

At 10 DPA, all the embryo cell tissues were defined as a torpedo stage, and from there, the embryo begins maturation by enlarging and increasing cell characteristics that are observable at 20, 30 and 40 DPA (Fig. 5 F-I). During these maturation stages, the embryo changes its shape, showing an evident hook, which is probably a result of the differences in cell number and cell wall composition between the internal and external cell layers of the embryo surface. As a result of these differences, the cotyledon from the external face is the largest during maturation. Similarly, it may be possible that close communication between the embryo and endosperm is occurring to control the volume of “the corrosion cavity” thereby facilitating embryonic growth.

4.4 De-methyl esterification of pectins during zygotic embryogenesis in relation to embryo differentiation

Analysis of the dynamics of methyl-esterification pectins in the cell wall of embryo cells showed that pre-globular embryos contain a high level of methyl-esterified pectins in the inner and outer cell

walls, while low-esterified pectins are very scarce. The high content of methyl-esterified pectins is congruent with the high number of cells in division in this stage. Similar labeling patterns with JIM5 and JIM7 antibodies were observed in zygotic embryos of *Capsicum annuum* and in young haploid embryos derived from pollen (Bárány et al., 2010; Bárány et al., 2005; Bueno et al., 2005; Raghavan, 1986). The results support the proposal that high levels of methyl-esterified pectin mark morphogenic cells, as in the case of the apical cells of the globular zygotic embryo in this study. The high level of methyl-esterification in the cell walls of first stage zygotic embryos can be extrapolated to somatic and microspore embryogenesis because similar patterns were observed during the transition from somatic proembryos to early globular embryos in banana (*Musa* spp. AAA, cultivar 'Yuveyoukang 1') and in microspore embryos of *Capsicum annuum* (Bárány et al., 2010; Xu et al., 2011). All these findings, together with the observation that actively dividing embryo cells of *Capsicum chinense* produce a strong signal with JIM7 in the cytoplasm surrounding the nucleus, give support to the recycling of pectins by exocytosis / endocytosis and represent a significant mechanism of pectin turnover during morphogenetic processes, as proposed by Baluska et al. 2002.

At advanced stages, low-methyl esterification increases gradually through the whole process of embryo maturation (Fig. 7 B, D, F, H). In *C. annuum*, the JIM5 antibody preferentially labels differentiated cells in mature embryos (Bárány et al., 2010; Bárány et al., 2005). Low-methyl-esterified pectin was detected in *C. chinense* cells near the shoot apical meristem (SAM), root apical meristem (RAM), the cotyledon, provascular tissues and parenchymal cells of the hypocotyl (Fig. 7 A-D). It is known that de-esterification produces negative charges (Wolf et al., 2009) and improves the capacity of ion exchange and water-binding (Chen et al., 2015; Kausz and Z. Hassid, 1967) if de-methyl-esterified pectate cross-links with Ca^{2+} it becomes rigid "hard" pectin which receives the name of "egg boxes" (Peaucelle et al., 2012). De-methyl esterification of cell wall pectin also plays a role in seed germination (Müller et al., 2013). Many reports have clearly established that pectin esterification/de-esterification plays a key role in differentiation during plant megasporogenesis and embryogenesis. Our findings in *C. chinense*, showing the changes in pectin esterification/de-esterification patterns suggest that this plays a vital role in governing the coordination of cell division and differentiation in embryogenesis. The complete histological characterization from megasporogenesis to embryogenesis carried out in this study has established a reference for decision-making in further studies on *C. chinense*, i.e., a comparison between zygotic and somatic embryogenesis will display which stages prevent the latter from achieving an efficient production of healthy embryos.

5 Conclusion

The information obtained from the critical steps during the ovule and zygotic embryo formation can be used in future genetic improvement programs or can help to design strategies to manipulate and

eventually prevent the generation of aberrant embryos in somatic and microspore embryogenesis in *C. chinense*. This study is part of the research we are developing aimed at elucidating the biochemical and molecular bases of the recalcitrance of the genus *Capsicum*.

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646

647 **Conflicts of interest:**

648 The authors declare that they have no conflict of interest.

649

Figure Legends

Figure 1. Flower development in *Capsicum chinense* at PreA and anthesis. (A) PreA stage takes typically 9 days in this plant, shown from left to right in the figure. (B) Longitudinal section of the floral bud at 2 days PreA showing the ovary with ovules, stigma and style, anthers, sepals and petals. (C) Transversal histological section of the ovary showing the ovules joined to the septum, and the ovary wall surrounding loculi and ovules. Histological preparations were stained with toluidine blue.

Figure 2. Histology of the ovules during the floral bud development. Observations were carried out at 9 days (A and A'), 8 days (B and B'), 7 days (C and C'), 6 days (D and D'), 5 days (E and E'), 3 days (F and F'), 1 day (G and G') at PreA stage, and at anthesis (H and H'). Tissue sections were of 3 -5 µm thin, stained with toluidine blue, and observed at 40 X and 200 X (these last labeled with apostrophe). Arrows indicate in A the inner epidermis of the ovary wall, in A' they show the three zones of the ovule. In B' indicating the nucellus and the megaspore mother cell (MMC). In C the arrows point to the chalaza and micropyle. In C' indicating the megaspore dyad, endothelium and micropyle channel. In D the arrow highlights the micropyle and in D' the nucellus and the megaspore in tetrad. In E' indicating the fundamental megaspore. In F the arrow shows the endothelium and in F' the nucellus and embryo sac in two nucleated stages. In G arrows indicate the chalaza and embryonic sac, and in G' the embryonic sac at four nucleated stages. In H' the mature embryonic sac, polar nuclei, the egg cell and synergids are indicated. In the pictures, inner epidermis (in. epi), cell zones (I, II, III), integument (Int), funiculus (Fu), megaspore mother cell (MMC), chalaza (Ch), micropyle (Mi), endothelium (Ent), fundamental megaspore (FM), embryonic sac (ES). The size of the bars inside the images are 40 µm (A, B, C, D, E and F), 20 µm (A', B', C', D', E', F'), 75 µm (G and H), and 30 µm (G' and H').

Figure 3. Immunolocalization during ovule development of highly methyl-esterified and low-methyl-esterified pectin with Alexa Fluor 488-conjugated JIM7 and JIM5 antibodies. Stages of 9, 8 and 6 days PreA and anthesis were analyzed. After immunoreaction with JIM5 or JIM7 (green signal), the preparations were stained with DAPI to see the nuclei. Figures labeled with apostrophes are a superposition of images for immunoreaction and DAPI staining (blue signal). The size of the bar inside the images is 50 µm.

Figure 4. Development of *Capsicum chinense* Jacq., fruit at different DPA. (A) Maturation progress is shown from left to right. (B-D) Observation of longitudinal and transversal dissections of *Capsicum chinense* fruits. Important components of the fruit are indicated in fruits of 5 DPA (B), 30 DPA (C) and 40 DPA (D); figures show the loculi and seeds with different degrees of development.

Figure 5. Micrographs from *Capsicum chinense* seeds at different stages of development. Testa (Te), integument (Int), endosperm (End), embryo (Emb), chalaza (Ch), hypostases (Hyp), micropyle (Mi), suspensor (Su), endothelium (Ent), cotyledons (Co), shoot apical meristem (SAM), radicle apical meristem (RAM), procambium (Pc) ground meristem (Gm). The size of the bars inside the images are 250 µm (A and B), 50 µm (A'), 75 µm (B'), 450 µm (C and D), 20 µm (C' and D'), 450 µm (E and F), 50 µm (E' and F'), and 500 µm (G, H and I).

Figure 6. Immunolocalization in ovule and embryo at 3 DPA of methyl-esterified and low-methyl-esterified pectin. Samples at 3 DPA were processed as described in Fig. 3, and images labeled with apostrophes are a superposition of images for immunoreaction and DAPI staining. Green signal (JIM5 or JIM7 antibody recognition), blue signal, DAPI staining of nuclei. The size of the bars inside the images are 50 µm (A, B, E, and F) and 75 µm (C, C'; D and D').

Figure 7. Immunolocalization in embryo at 20 DPA of methyl-esterified and low-methyl-esterified pectin. Samples were processed as described in Fig. 3, and images labeled with apostrophes are a

701 superposition of images for methylated or low-methylated pectin immunoreaction (green) and DAPI
702 staining (blue). The size of the bars is 50 μm , except in G and G' where it is 75 μm .

703
704 **Supplementary Fig. S1.** Immunodetection with JIM7 of the high-methyl-esterified pectin on actively
705 dividing embryo cells at 3 DPA. Merging the images shows the nucleus stained with DAPI (blue)
706 and the high-methyl-esterified pectin immunostained with JIM7 (green). The white arrow in the
707 figure indicated a strong signal of high-methyl-esterified pectin in the cytoplasm surrounding the
708 nucleus of actively dividing embryo cells. The size of the bars is 50 μm .

3 DPA

5 DPA

10 DPA

20 DPA

30 DPA

40 DPA

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