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Ultrastructural aspects of the embryo and different endosperm compartments, in *Eruca sativa* Hill cv. Nemat (Brassicaceae) during Heart and Torpedo stages

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Abstract — These observations are the first on the ultrastructure of endosperm and embryo of *Eruca sativa* Hill. We investigated the cv. Nemat, which is characterized by a particularly high amount of lipids and glucosinolates.
Our observations suggested that the thick and abundant micropylar endosperm, completely surrounding the suspensor, may be the main active source of nutrients for the embryo. This endosperm, like the central chamber endosperm, is particularly rich in functional chloroplasts and cellularizes later with respect to the other previously investigated Brassicaceae. The last (distal with respect to the embryo) suspensor cell exhibits important features related to the passage of nutrients, such as wall ingrowths. In fact these ingrowths appear as highly convoluted labyrinthine wall projections. Such ultrastructural features are typical of transfer cells. The accumulation stage in *E. sativa* cv. Nemat appears to occur early (Heart stage of embryo development, as *Brassica napus*). The endosperm compartment called Chalazal Endosperm Cyst (CEC), contributes actively to the embryo trophism during the Heart and Torpedo stages. This function is evident because of the high number of chloroplasts in the cyst and for the observed continuity between the CEC and the other endosperm compartments (CC endosperm and micropylar endosperm) in cv. Nemat. The morphology of the CEC appeared to be more similar to the pyriform shape sensu Brown et al., but with a more flattened base with respect to the proposed examples, and without labyrinthine wall. The Chalazal Chamber appeared to be more similar to the Brown's type B in *E. sativa*. The presence of chloroplasts with a well developed thylakoid system indicates an active photosynthetic activity by the majority of the seed tissues. *E. sativa* leaves are normally harvested for food, while the seeds of cv. Nemat appear to be particularly rich in oil. The premature independence of seeds and fruits from the necessity of absorbing nutrients from the rest of the plant, could indicate the possibility of harvesting both leaves (earlier) and seeds (later) in this plant without compromising a full seed maturation.

Keywords Biofuels, Endosperm, *Eruca sativa*, Lipids, Seed development

Abbreviations: CC: central chamber; ChC: chalazal chamber; CEC: chalazal endosperm cyst; CPT: chalazal proliferative tissue; MC: micropylar chamber; NL: nucellar lysate; RER: Rough Endoplasmic Reticulum; SER: Smooth Endoplasmic Reticulum; TEM: Transmission Electron Microscope.

INTRODUCTION

The Brassicaceae are a family having been extensively investigated with morphological, ultrastructural and genetic techniques. It has a great importance as a source of food and, in relation to the high lipid seed content, typical of

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many species as source of biomass, also for biofuels production.

Eruca sativa Hill belongs to tribe Brassiceae, subtribe Brassicinae (NAGPAL *et al.* 2008). It is an economically remarkable species, since it is normally cultivated because of its edible leaves, but owns seeds with a quite high lipid content, that increases in some cultivars. Other representatives of subtribe Brassicinae, as *Brassica carinata*, own seeds with high oil content.

E. sativa Hill cv. Nemat was also called *E. sativa* Hill ssp. *oleifera* or *E. sativa* Hill ssp. *oleifera* cv. Nemat (LAZZERI *et al.* 2004) without formal description of the taxa. This *cv.* is characterized by a higher oil seed and glucosinolate content with respect to the normally grown *E. sativa* (LAZZERI *et al.* 2004; PAPINI *et al.* 2009).

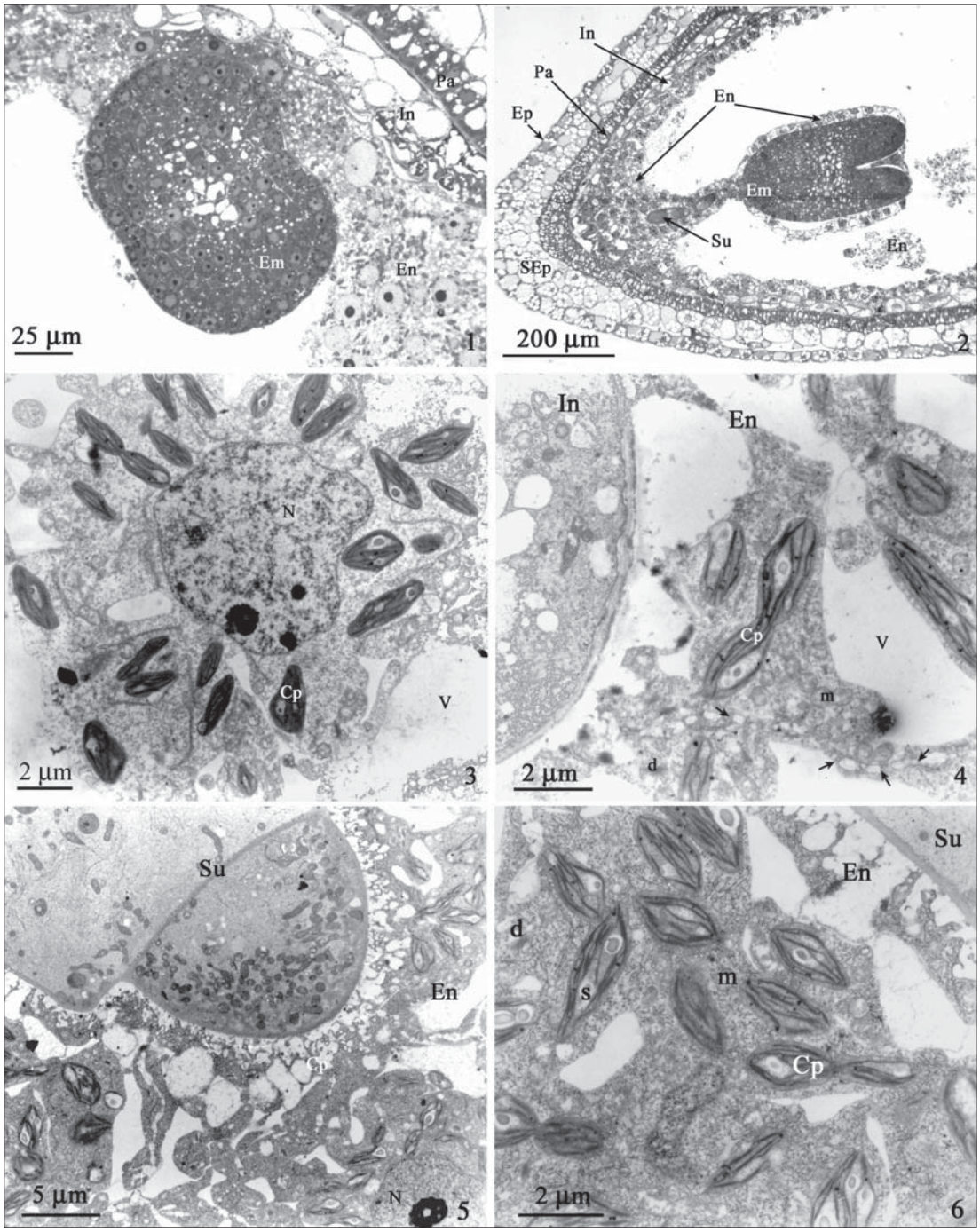
Lipid-derived biofuels belong to first generation biofuels and as such raise questions about the possible conflict with agriculture for food and the general economic sustainability of the production (PAPINI and SIMEONE, in press). The possibility of cultivating a plant that can be used both for food (leaves) and biofuel production (seeds) may help to overcome some of the weak points of first generation biofuels. Studies on seed development, lipid synthesis and storage, are of interest in breeding projects and in development of new cultivars for food and industrial applications.

The Brassicaceae have, at least in the initial developmental stages, a nuclear type of endosperm (BAUD *et al.* 2008; BERGER *et al.* 2006). In this endosperm, mitosis occurs without cytokinesis. The result is a multinucleate syncytium (NGUYEN *et al.* 2000). The nuclear endosperm is an unusual and poorly understood tissue (NGUYEN *et al.* 2000) and its cytoplasm is characterized by nuclear-radial arrays of microtubules (OLSEN *et al.* 1995; BROWN *et al.* 1999; PICKETT-HEAPS *et al.* 1999; BROWN *et al.* 2004). Generally in Brassicaceae the developing seed is subdivided in three chambers: the micropylar chamber (MC), the chalazal chamber (ChC) and the central chamber (CC), each with a peculiar pattern of endosperm development. The cellularization of the syncytial endosperm begins in the micropylar chamber, when the embryo is at the heart stage of development and spreads through the central chamber (SCHULZ and JENSEN 1974; PACINI *et al.* 1975; VIEGI *et al.* 1976; VIJAYRAGHAVAN and PRAHABKAR 1984; VIJAYRAGHAVAN *et. al.* 1984; MANSFIELD 1994; VAN LAMMEREN *et al.*1996; BROWN *et al.* 1999; NGUYEN *et. al.* 2000). The chalazal endosperm can be distinguished from the other endosperm compartments because of the high density of its cell mass (VINKENOOG *et al.* 2002) and is the last endosperm compartment to cellularize (MANSFIELD and BRIARTY 1990; BERGER 1999). It can remain syncityal even in the mature seed (VIJAYRAGHAVAN and PRAHABKAR 1984). In this case this endosperm, is also named chalazal endosperm cyst (CEC). The chalazal cyst is a syncitial mass representing a peculiar specialization of the endosperm that begins early in development (BROWN *et al.* 1999; 2002; 2003; 2004; NGUYEN *et al.* 2000). The CEC differs from the organization of the adjacent CC endosperm, where nuclei are associated with the radial microtubule system (BROWN *et al.* 2002; 2003). Moreover the CEC, shows different morphologies between the main tribes of Brassicaceae and this variation is of potential taxonomic value (BROWN *et al.* 2004).

Detailed ultrastructural studies on this type of endosperm are not common. BROWN *et al.* (2004) distinguished three main zones in the CEC of Brassicaceae: an apical one, containing nuclei, plastids and mitochondria; a mid-zone, rich in endomembranes but with rare large organelles and a basal portion bordered by a labyrinthine wall. This last zone, was found in some species belonging to tribes Alysseae, Arabideae, Lepidieae, Sisymbrieae and Thelypodieae. Moreover BROWN *et al.* (2004) identified different cysts (CEC) shapes (pyriform, ovoid, wide ovoid and filiform) and four types of chalazal chamber (ChC) based on their shape and dimension (types: A, B, C, D).

The chalazal proliferative tissue (CPT) is formed by the enlargement of several layers of nucellar cells at the chalazal end of the embryo sac. When the embryo reaches the early globular stage these enlarged cells start to disintegrate (SCHULZ and JENSEN 1971). These cells of the CPT degenerate leaving an extensive region of lysate between the CEC and the remaining cells of the CPT (BROWN *et al.* 2004). The CPT is a maternal tissue that lies close to the site of nutrient unloading from the vascular system, hence it is probably involved in translocation of nutrients into the endosperm (VINKENOOG *et al.* 2002).

During the embryo development of Brassicaceae the following stages can be recognized (SIMONCIOLI 1974; MANSFIEL and BRIARTY 1991): Zigote, Single-terminal cell, Two-terminal cell, Quadrant, Octant, Dermatogen, Globular (early globular, midglobular, late globular), Heart (early heart, midheart, late heart), Torpedo and Cotyledons bend. In the passage from the Globular stage to the Heart stage, cell divisions are very frequent and the embryo assumes a triangular profile. Once the Heart shape is completed the cells begin to



Figs. 1-6 — (1) Light Microscope image. Developing seed at the embryo Heart stage in the micropylar chamber. (2) Light Microscope image. Developing seed at the embryo Torpedo stage in the micropylar chamber. (3) Endosperm of the central chamber during the Heart and Torpedo stages. The cytoplasm is rarefied and vacuolated, with big nuclei, normally roundish and euchromatic, chloroplasts containing starch and solitary RER cisternae. (4) Endosperm of the central chamber during the Heart and Torpedo stages: cytoplasm detail, where chloroplasts, RER, mitochondria and some dictyosomes are visible. Below a tract of wall in formation is visible. The wall is crossed by plasmodesmata (arrows). On the left a portion of a cell belonging to the internal integumental layer is visible. (5) Portions of the last and second-last distal suspensor cells at the Torpedo stage, included in the micropylar endosperm tissue. (6) Cytoplasm of the micropylar endosperm tissue at the Torpedo stage with chloroplasts and mitochondria. The image shows also chloroplasts containing starch and plastoglobules.

differentiate, and in the torpedo stage the central cells of the hypocotyl form the primary vascular tissue (MANSFIELD and BRIARTY 1991).

The embryo suspensor has both the mechanical function to push the embryo towards the Central Chamber and a role in the transport of nutrients towards the embryo. The nutrients come in part from the maternal conductive tissues and in part from the tissues of the developing seed (integuments, endosperm). The suspensor, with its haustorial function, assumes the role of a temporary embryonic root (MAHESHAWARI 1950; AVERY *et al.* 1959; SCHULTZ and JENSEN 1969; VIEGI *et al.* 1976). In *E. sativa* the suspensor is of the long filament type, typical of Brassicaceae (NATESCH and RAU 1984), formed by 9-13 cells and with an endopolyploidy degree increasing in the cells in distal position with respect to the embryo and reaching its highest degree in the second-last distal cell, remaining high also in the last one (CORSI 1972; VIEGI *et al.* 1976). This latter is elongated and not vacuolated (VIEGI *et al.* 1976). Moreover plasmodesmata occur between the suspensor cells and from the suspensor to the embryo (CORSI 1972).

The aim of this work was to investigate thoroughly the micromorphological aspects of the different types of endosperm observed in the seed of *E. sativa* cv. Nemat during the intermediate phase of embryo development (Heart and Torpedo stages), and the relationship beetween the endosperm and the adjacent tissues. We were particularly interested in the relationship with the embryo tissues, the embryo suspensor cells and the nucellus (CPT). Other considered aspects are the cytoplasm details of these last tissues. In fact, despite many important studies about the embryogenesis of Brassicaceae, there is a lack of detailed description of the ultrastructural aspects in genus *Eruca*. In particular a better knowledge of the ultrastructural aspects of the endosperm of this species could provide insights about the storage mechanisms in the embryo. Such a study could assume further importance due the potential use of *E. sativa* seeds (particularly the cotyledons) as lipid source. A better knowledge about the embryo development may suggest biotechnological genetic targets in order to improve productivity and quality, particularly in cv. Nemat. Finally more data about some micromorphological aspects of the endosperm of Brassicaceae could be useful also from a taxonomical point of view. Indeed, this morphological data have already been used with success for genera delimitation (BROWN *et al.* 2004).

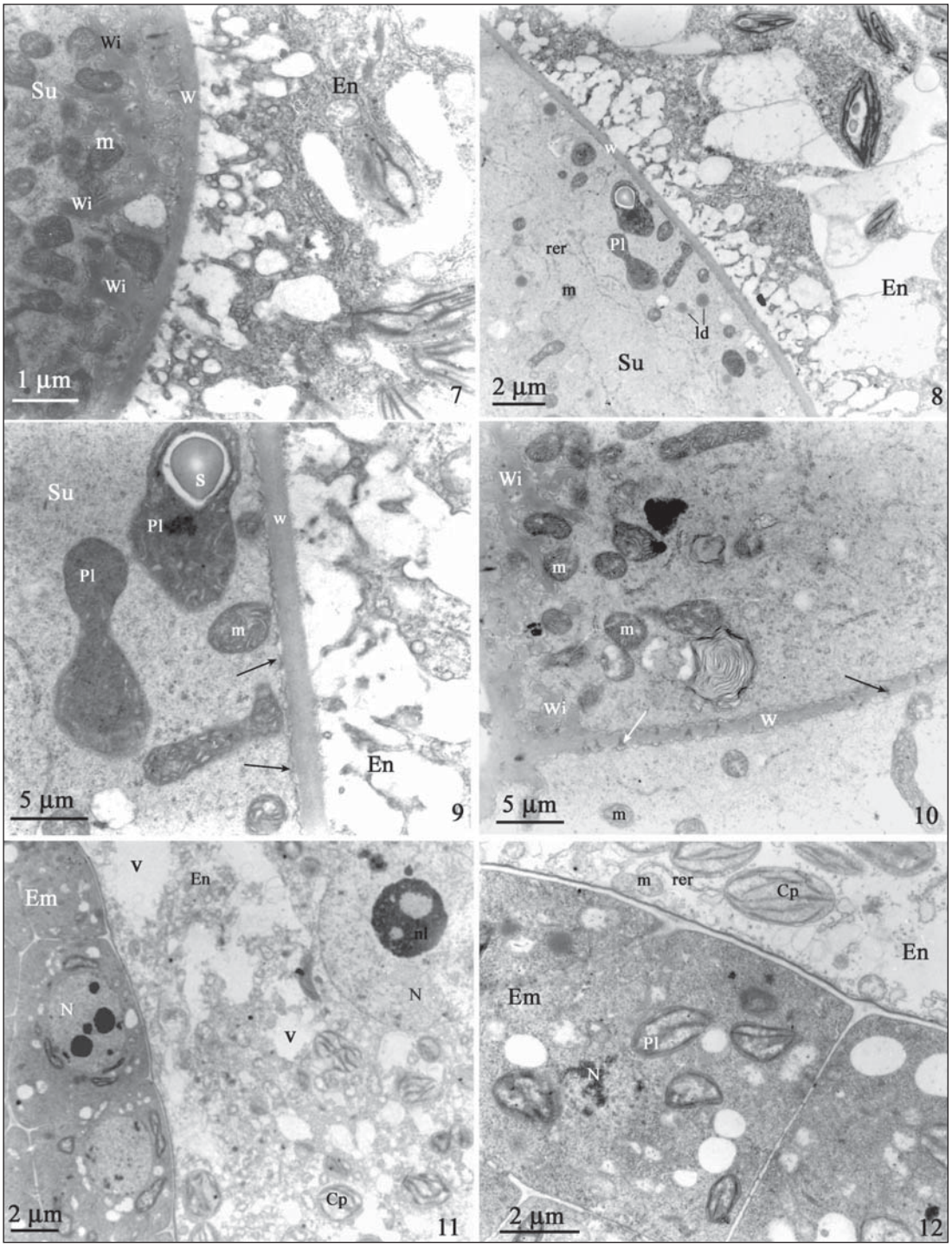
MATERIALS AND METHODS

Anatomy and ultrastructure - Plants of *Eruca sativa* Hill cv. Nemat were grown in the Botanical Garden of the University of Florence (Italy). Not yet mature fruits (siliquas) were collected at different developmental stages: 1.5cm x 1mm; 1.8 x 1.9mm; 2.2cm x 2.1mm; 2.5cm x 2.6mm. Images were obtained prevailingly from the third and fourth stages, corresponding to the Heart and Torpedo embryo stages in the developing seed. Developing seeds were prefixed overnight in 1.25 % glutaraldehyde, at 4° C in 0.1 M phosphate buffer (pH 6.8). The samples were fixed in 1% OsO4 in the same buffer for 1 hr. After dehydration in an ethanol series and a propylene oxide step the samples were embedded in Spurr's epoxy resin (SPURR 1969). Transverse sections approximately 80 nm thick were cut with a diamond knife, stained with uranyl acetate (GIBBONS and GRIMSTONE 1960) and lead citrate (REYNOLDS 1963), then examined with a Philips EM300 TEM at 80 kV. Semi thin sections (1-5µm) obtained using glass knives, were stained with Toluidine blue, 0,1 %, observed and photographed with a light microscope.

RESULTS

The Light Microscope images 1 and 2 show the general aspect of the embryo at the Heart (Fig. 1) and Torpedo (Fig. 2) stages and its relationship with the micropylar and the CC endosperms and with the tegumental tissues.

Endosperm of the Central chamber (CC) during Heart and Torpedo stages - During these two stages, the endosperm of the central chamber (Figs. 3, 4) did not change its morphology. We observed some small differences between the endosperm of the CC adjacently to the internal teguments and that located more centrally in the CC lumen. In general it was a nuclear endosperm (syncytial), characterized by big more or less roundish nuclei, usually euchromatic and with a rarefied and vacuolated cytoplasm (Figs. 3, 4). In the cytoplasm (Figs. 3, 4) we distinguished chloroplasts with a well developed thylakoid system and containing starch. The chloroplasts (Fig. 3) showed often a typical radial disposition around the nucleus. The ribosomes (Fig. 3) were not frequent, while some RER cisternae could be observed in the cytoplasm (Fig. 3). We observed a quite high number of mitochondria. At the Torpedo stage, in the endosperm of the CC situated close to the teguments, we began to observe tracts of forming wall. The wall was



Figs. 7-12 — (7) In the last distal suspensor cell at the Torpedo stage, extensive labyrinthine wall ingrowths are evident. Many mitochondria are present among the ingrowths. (8) Portion of the second-last distal suspensor cell and highly vacuolated micropylar endosperm at the Torpedo stage. The walls of the second-last suspensor cell do not have labyrinthine ingrowths. The cytoplasm of the second-last suspensor cells contains plastids, some of them with starch, mitochondria, RER cisternae and lipid droplets. (9) Detail of the more external zone of the cytoplasm in the second-last suspensor cell at the Torpedo stage. Plastids with tubular membranes are evident. The arrows indicate the plasma membrane ingrowths. (10) Many plasmodesmata (arrows) cross the transverse walls of the last and second-last suspensor cells. The first can be recognized because of the labyrinthine wall ingrowths and for the numerous mitochondria. (11) Heart stage. The micropylar endosperm around the embryo shows a highly vacuolated syncytial cytoplasm with roundish nuclei and huge nucleoli. The cytoplasm appears apparently disrupted in some points. (12) Cells of the embryo protoderm at the Heart stage. In the syncytial endosperm chloroplasts lacking in starch, swelling RER cisternae and mitochondria are shown.

crossed by initial plasmodesmata (Fig. 4).

Micropylar Endosperm and cells of the suspensor at the Torpedo stage - The micropylar endosperm around the suspensor cells was syncytial, as that lining the lumen and free in the Central Chamber. The endosperm embedded completely the suspensor cells (Fig. 2). Also this endosperm compartment showed roundish nuclei (Fig. 5) and chloroplasts radially disposed around the nuclei as in the Central Chamber. The chloroplasts (Figs. 5, 6) showed a well developed thylakoid system and contained starch granules and plastoglobules. The endosperm cytoplasm (Figs. 5, 6, 7), in contact with the walls of the last and the second-last (distal) suspensor cells showed big vacuoles with irregular profile and medium electron density content, dilated SER and RER cisternae, many ribosomes, free or associated in polysomes, some mitochondria and dictyosomes. The last distal suspensor cell (Figs. 6, 7) showed a wall with remarkable ingrowths followed by the plasma membrane. This disposition led to a labyrinthine profile (Fig. 7). The Transfer Cell aspect was confirmed by the presence, around the ingrowths, of many large mitochondria (Fig. 7).

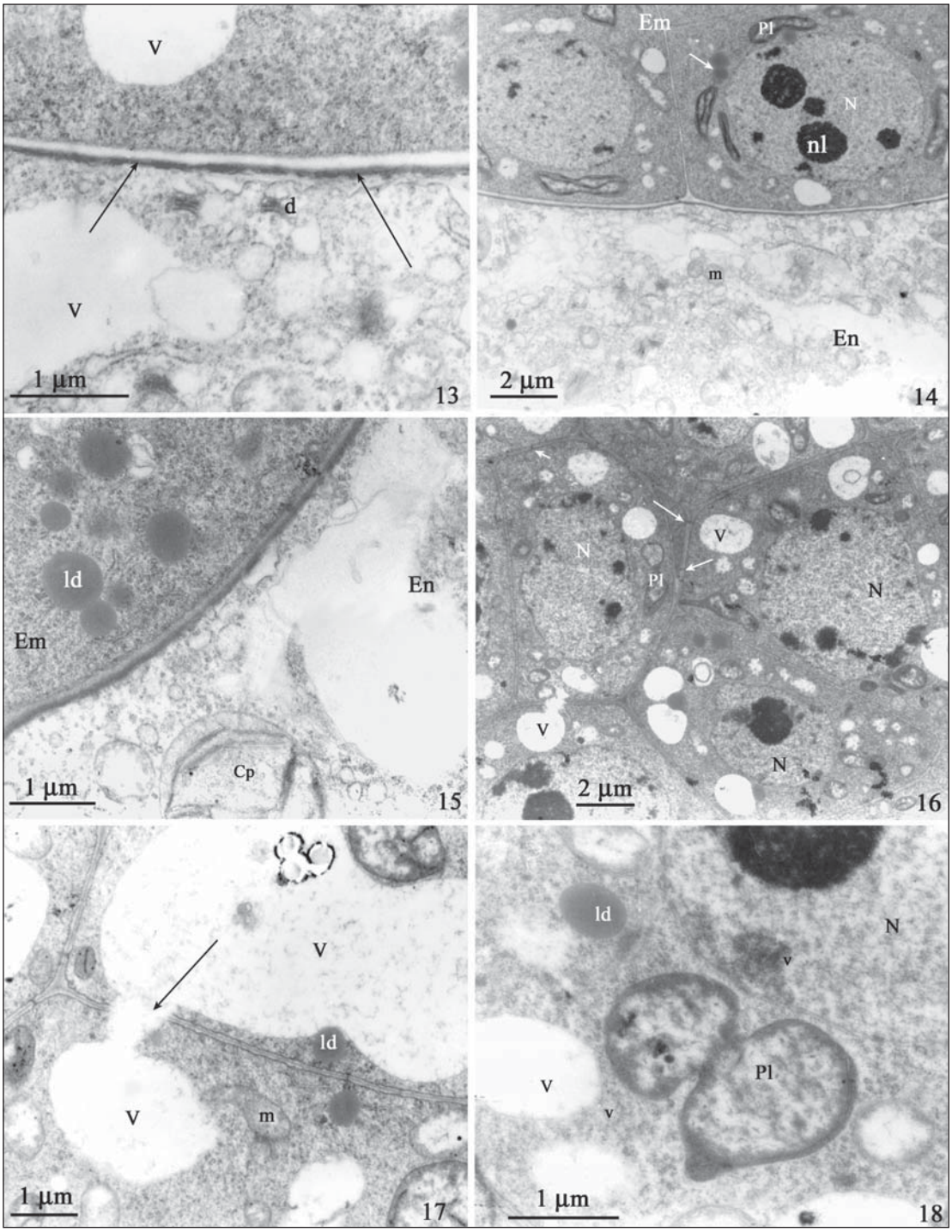
The walls of the second-last distal suspensor cells (Figs. 6, 8) were not labyrinthine and apparently not cutinized, except for a short tract of the tangential walls close to the last distal cell. Nevertheless the plasma membrane showed small ingrowths along the tangential walls (Fig. 9). We observed big vesicles in the endosperm adjacent to the suspensor wall. The vesicles content was very similar to that of the more internal vacuoles (Fig. 8).

The cytoplasm of the second-last distal cell had less mitochondria with respect to the last one (Fig. 10), but it showed long RER cisternae and plastids, (Figs. 8, 9) some of them in division and containing distinctive tubular membranes. The wall between the last and the second-last suspensor cells were crossed by numerous plasmodesmata (Fig. 10).

Micropylar Endosperm adjacent to the embryo at Heart and Torpedo stage - The endosperm around the embryo at the Heart stage was still nuclear (syncytial) (Fig. 1). At the Torpedo stage the endosperm had already begun to cellularize (Fig. 2), but only around the embryo itself. This was the area of the developing seed, where a cellularized endosperm was first visible. Nevertheless the endosperm around the embryo suspensor still maintained its nuclear condition (see above). The micropylar endosperm around the embryo at the Heart stage showed a syncytial cytoplasm with many vacuoles (Fig. 11). The cytoplasm appeared apparently disrupted in some points (Fig. 11). No plasmamembrane ingrowths were visible in the endosperm around the embryo protoderm. In the rarefied cytoplasm matrix, big roundish nuclei with evident nucleoli containing electron transparent elliptical areas were present (Fig. 11). Moreover in the cytoplasm we observed rare ribosomes, mitochondria with few cristae and dilated RER cisternae (Figs. 12, 13, 14). We observed even big chloroplasts (Figs. 11, 12) with a well developed thylakoid system, but without starch. Many vacuoles and many budding dictyosomes were present (Fig. 13).

The embryo cells at the Heart and Torpedo stages did not show particular ultrastructural differences: in both cases they appeared as meristematic cells in division or, sometimes, in preliminary differentiation stages (Figs. 11, 12, 14, 16). The most striking feature was the beginning of the storage of reserve substances (lipids and protein bodies) in some vacuoles (Figs. 15, 18). The most external cells (protoderm) had a cutinized wall (Figs. 12, 13, 14, 15). The nuclei of the protoderm (Fig. 14) were roundish, sometimes with more than one nucleolus. In the cytoplasm some big lipid droplets were evident (Fig. 15). The cytoplasm contained numerous ribosomes and tracts of RER cisternae (Fig. 15). In the same cells electron transparent vacuoles, big mitochondria and quite large plastids were observed (Figs. 12, 14). The cells belonging to the more internal layers (ground meristem or even procambium) showed a similar cytoplasm situation (Figs. 16, 17), but the plastids were more irregular in shape, often in division, (Fig. 18). The main difference between the cells of the protoderm and those of the internal layers was that the vacuoles in the internal cells were larger and were starting to accumulate granular osmiophilic materials of probable protein nature and some crystalloids of medium electron density surrounded by strongly osmiophilic material (Fig. 17). In more internal areas of the embryo we observed intercellular vacuoles crossing the wall between two different cells (Figs 16, 17).

Torpedo stage: appearance of the Chalazal Endosperm Cyst - When the embryo was at the Torpedo stage, a Chalazal Endosperm Cyst (CEC) was evident at the chalazal pole (Fig. 19). This endosperm compartment appeared different from the micropylar and the central chamber ones, since it showed a more compact and less vacuolated cytoplasm. The endosperm syncytial mass of the CEC in the central zone of the chalazal side tended to get narrower but maintained its continuity with



Figs. 13-18 — (13) Contact point between the syncytial endosperm and the protoderm cells at the Heart stage. The embryo cells have a thick cuticle (arrows). In the endosperm some dictyosomes are present. (14) Cells of the embryo protoderm at the Heart stage. The roundish nuclei have sometimes more than one nucleolus. In the embryo cytoplasm some lipid droplets are present. (15) Many lipid droplets are present in the cytoplasm of the protoderm cells at the Heart stage. (16) Embryo cells at the Heart stage. The walls are crossed by plasmodesmata (arrows). The roundish nuclei show sometimes more than one nucleolus. The vacuoles sometimes contain osmiophilic material. (17) Embryo cells at the Heart stage. Intercellular vacuoles (arrows). In the vacuole some crystalloid structures surrounded by osmiophilic protein material are evident. Plasmodemata cross the walls. (18) Plastid of a embryo cell at the Heart stage during the division process.

the Central Chamber Endosperm (Fig. 19). The change from one endosperm to the other could be identified for the more evident radial disposition of chloroplasts around the nuclei in the central chamber with respect to the more unordered situation of the CEC chloroplast (Figs. 25, 26). At the center, the CEC showed a more rarefied and vacuolated cytoplasm (Fig. 19). In the basal zone, close to the Chalazal Proliferative Tissue (CPT), the CEC appeared to be more compact (Figs. 19, 25). It was surrounded by the integument cells and by the nucellar cells of the CPT (Fig. 19). In the CEC we observed a central area just above the basal zone, where the syncytial cytoplasm was more vacuolated and the endomembrane system widely developed (Fig. 20). Some clusters of vacuoles with electron transparent content were evident. The nuclei (Fig. 21) were often lobated and heterochromatic. RER cisternae were sometimes close to the nuclear membrane. In some tracts the RER and SER cisternae swelled and produced vesicles (Fig. 22). The plastids, as in the other endosperm compartments, were real chloroplasts with a well developed thylakoid system (Fig. 22). Frequently swollen SER cisternae (Fig. 23) surrounded cytoplasm portions, finally producing endophagocytosis vacuoles containing cytoplasm and organules remnants (Fig. 24). RER cisternae were often present around the endophagocytosis vacuoles (Figs. 23, 24).

In the basal zone of the chalazal endosperm cyst, the cytoplasm was less vacuolated and the endomembrane system was less developed (Fig. 25). The nuclei (Figs 25, 26) could show both amoeboid and roundish shapes and were euchromatic with evident nucleoli. Chloroplasts (Figs. 25, 26) were more numerous with respect to the cytoplasm of the central zone of the CEC. These chloroplasts were elliptic in shape, with small and very osmiophilic plastoglobules and few starch granules (Figs. 25, 26). Some lipid droplets were present (Figs. 25, 26).

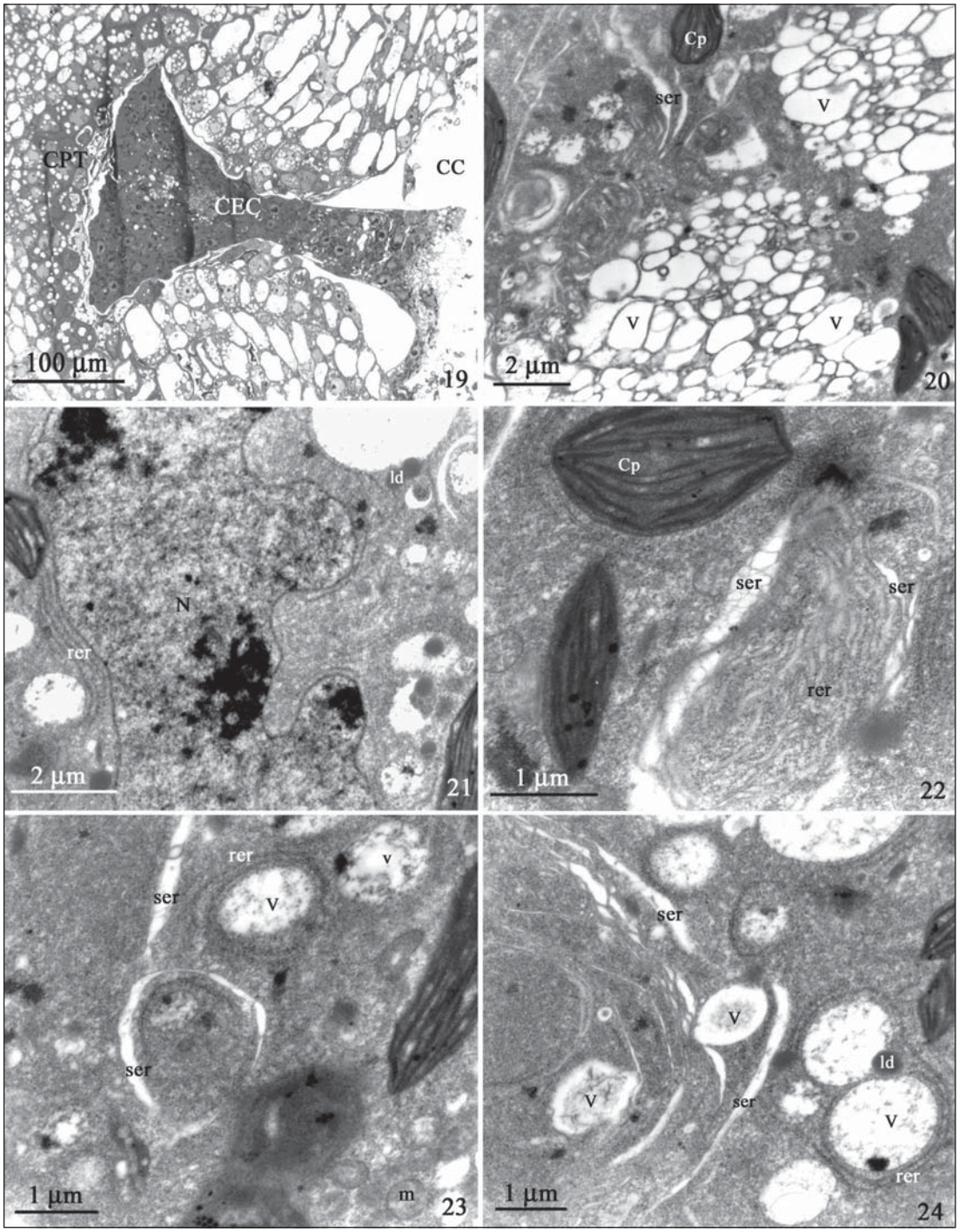
The few layers of nucellar cells of the CPT (Fig. 19) were located underneath and sometimes laterally to the CEC and could be distinguished by the cells of the internal integumental tissue for having a more compact cytoplasm and less vacuoles.

The integumental cells (Fig. 19) were similar to those of the CPT for their roundish shape and for the presence of amyloplasts. They were often characterized by only one big central vacuole or by few big vacuoles. In the cytoplasm of the CPT cells, the nuclei were roundish and quite euchromatic (Fig. 27). Big multilobated amyloplasts were present, together with a high number of mitochondria (Fig. 28). Nevertheless, around the CEC the nucellar cells began to undergo auto-degeneration processes. The first signals were the partial dissolution and detachment of wall layers adjacent to the endosperm cyst and the autophagocytosis vacuoles (Figs. 27, 28) that contained parts of the cytoplasm with organelles, mainly mitochondria. Afterwards (Fig. 29) we observed the detachment of the plasma membrane from the wall. At this point the nucellar cells of the CPT began to show an amorphous and osmiophilic cytoplasm, still containing huge starch crystals (Fig. 29). Finally (Fig. 30) these cells lost their individuality producing a cellular lysate, where dead cells images, empty and flattened, could be tentatively identified only because of the wall residuals.

DISCUSSION

The endosperm at the micropylar pole of *Eruca sativa,* particularly that around the suspensor cells, has a very active role with highly stainable nuclei (VIEGI *et al*. 1976). Our ultrastructural observation on *Eruca sativa* Hill cv. Nemat showed that the last distal (with respect to the embryo) suspensor cells at the Torpedo stage, is in contact with the micropylar endosperm. This fundamental suspensor cell has wall ingrowths and plasma membrane following the contour of the wall ingrowths with associated mitochondria that are typical of Transfer Cells (YEUNG and CLUTTER 1979). Moreover, the confirmed presence of many plasmodemata crossing the transverse walls of the suspensor cells indicates a passage of nutrients from the surrounding tissues towards the embryo through the suspensor via the Transfer Cell (the last suspensor cell) and not vice versa (GUNNING and PATE 1974). From the last suspensor cell, the nutrients continue their way through the other suspensor cells eventually reaching the cells of the developing embryo. Moreover the transfer between the outer integument and the inner integument, between the inner integument and the endosperm, and between the endosperm and the embryo might be also apoplastic (KIM and ZAMBRYSKI 2005).

The importance of the suspensor in *E. sativa* in the first stages of embryogeny was demonstrated by CORSI (1972). Also VIEGI *et al*. (1976) showed that the suspensor of *E. sativa* is very active for absorption and transport towards the embryo, of nutrients coming from the micropylar endosperm and other ovule tissues. VIEGI *et al*. (1976) proposed for the suspensor, also the function of growth factors production. The same situation of labyrinthine wall



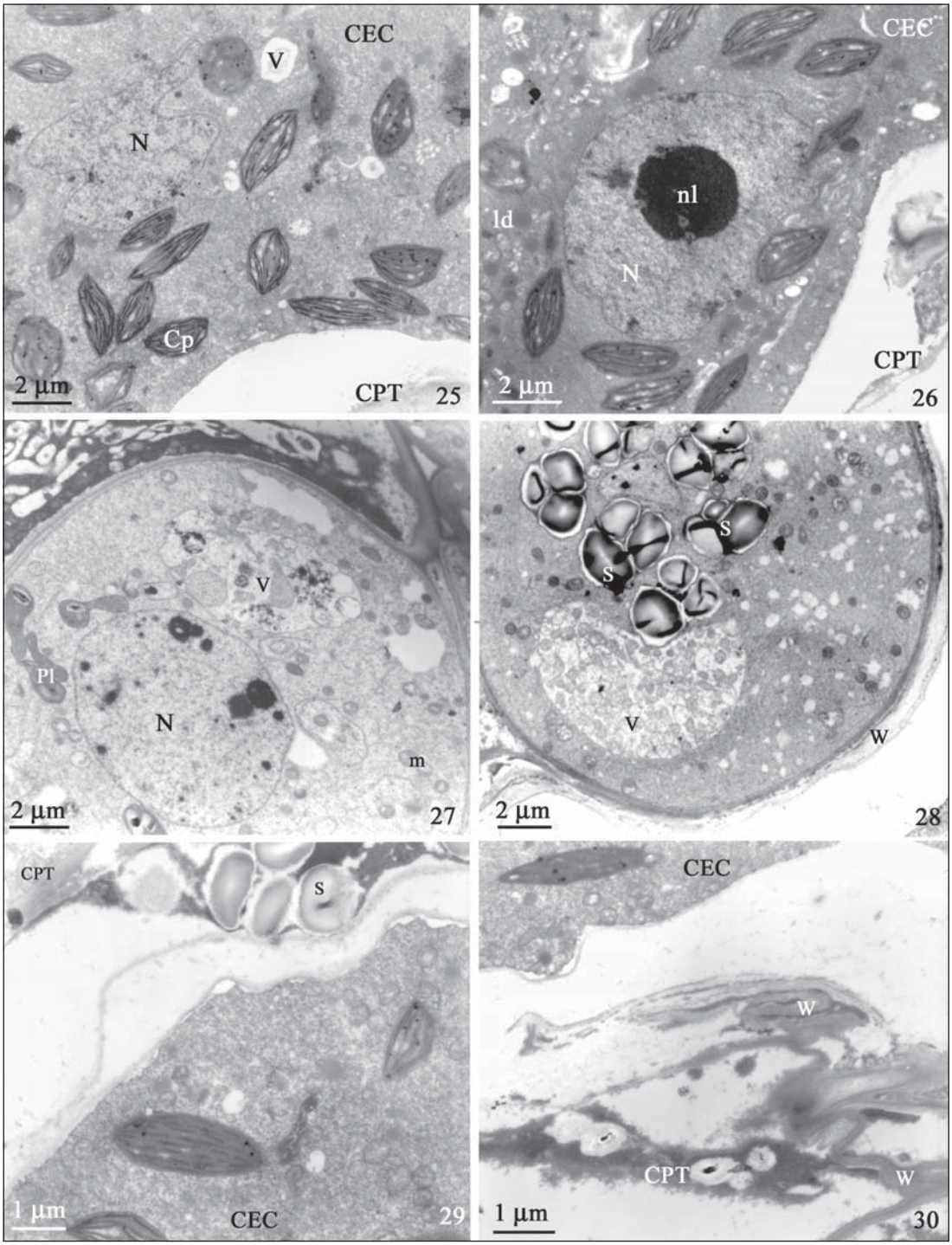
Figs. 19-24 — (19) Light Microscope Image of developing seed at the Torpedo stage in the chalazal chamber (ChC). The chalazal endosperm cyst (CEC) is surrounded basally by the internal integumental tissues and by the chalazal proliferative tissue (CPT). The CEC is in contact, apically, with the endosperm of the central chember (CC). (20) Chalazal endosperm cyst (CEC), central zone. Detail of the cytoplasm. (21) Chalazal endosperm cyst (CEC), central zone. Detail of a multilobated nucleus (N). (22) Chalazal endosperm cyst (CEC), central zone. Group of RER and SER cisternae, in some point swelling and budding vesicles. (23) Chalazal endosperm cyst (CEC), central zone. SER cisternae are surrounding some cytoplasm portions. Some RER cisternae are surrounding endophagocytosis vacuoles. RER cisternae are surrounding some vacuoles. (24) Chalazal endosperm cyst (CEC), central zone. Detail of the cytoplasm with SER cisternae, some free, some apparently forming endophagocytosis vacuoles. RER cisternae are surrounding these vacuoles.

ingrowths in *E. sativa* last distal suspensor cell, has been observed in the corresponding suspensor cell of *Stellaria media* (NEWCOMB and FOWKE 1974). In this species the last cell of the suspensor is elongated and with a huge vacuole. SCHULZ and JENSEN (1969) observed similar morphological features with wall ingrowths, in *Capsella.* In this case, the last distal suspensor cell (here defined basal) is not in contact with the endosperm, but completely inserted in the integument. Apparently in *Capsella* the nutrients reach the suspensor from the integument itself and not through the endosperm. SIMONCIOLI (1974) reported abundant and large wall ingrowths in the micropylar part of the basal cell of the suspensor of *Diplotaxis erucoides* during the Heart and Torpedo stages. During these stages, in *D. erucoides*, the last suspensor cell is surrounded by a layer of endosperm that is not particularly thick, but shows wall ingrowths in the contact zone with the teguments, starting from the Globular stage until the Torpedo stage (SIMONCIOLI 1974). Apparently in *D. erucoides*, both the endosperm and the internal teguments collaborate to the embryo development. This last situation is an intermediate between that of *Capsella* and that of *E. sativa* cv. Nemat studied in this investigation. Our observation shows that the active role of source of nutrients for the embryo, is carried out mainly by the thick and abundant micropylar endosperm that surrounds completely the last distal suspensor cell. This last cell is hence completely separated by the integumental tissues and exhibits important features related to the passage of nutrients such as wall ingrowths. In fact the last suspensor cell shows highly convoluted labyrinthine wall projections, that are typical of transfer cells, involved in short-distance traslocation (GUNNING and PATE 1974; NATESCH and RAU 1984). The release of nutrients to the embryo in general can be attributed to the endosperm, to the nucellus, to the internal teguments, to the placenta or combinations of more than one of these tissues (NATESCH and RAU 1984). In *E. sativa* cv. Nemat no other tissue appears to be involved than the endosperm, since the other tissues are too far away from the suspensor and not in direct contact with it. Moreover, no wall modification showing short distance nutrient transmission between the endosperm and other tissues was observed.

In *Capsella* some of the suspensor cells more proximal with respect to the last distal one show wall expansions that appear to be turned outside, towards the micropylar endosperm (SCHULZ and JENSEN 1969). Our ultrastructural data suggest that, in this species also a passage of solutes from the suspensor towards the endosperm occurs. In *E. sativa* cv. Nemat the suspensor cells above the last one do not show wall ingrowths, but neither are they cutinized. Hence an exchange suspensor-endosperm is still possible. Nevertheless TEM observations show small plasma membrane ingrowths in the suspensor cells, while on the endosperm side big vesicles, with a content very similar to that of the vacuoles present more internally inside the endosperm itself, are present. These data, together with the apparent loss of cutinization of the suspensor wall, suggest that the passage of substance is towards the suspensor cells also at this level, even if in a less intense way with respect to the last suspensor cell.

The embryo protoderm cells, both at the Heart and at the Torpedo stage, have cutinized walls, and hence the direct nutrient passage from the endosperm to the embryo would be quite inefficient, while the main way would be through the suspensor. The presence of plastids with tubular membranes system, somehow resembling those of mitochondria in the suspensor second-last cell (but not in the last one) was already observed in the suspensor cells of *Pisum* (MARINOS 1970), *Phaseolus vulgaris* (SCHNEPF and NAGL, 1970), *Ipomoea* (PONZI and PIZZOLONGO 1972), *Stellaria* (NEWCOMB and FOWKE 1974), and *Tropaeolum* (NAGL and KUHNER 1976).

In *E. sativa* cv. Nemat the embryo cells start the accumulation of lipid and protein substances already at the Heart stage, when we already observed vacuoles containing structures of medium electron density surrounded by strongly osmiophilic material of protein nature. These vacuoles appeared to be probably the precursors of the protein bodies later observed in the cotyledon cells. This accumulation stage appears to occur earlier with respect to what happens in *Arabidopsis thaliana,* where electron-dense spherical bodies begin to appear in the embryo cells only at the Torpedo stage (MANSFIELD and BRIARTY 1991). As in *Arabidopsis* the highest beginning concentration of lipid droplets occurs in the protoderm cells, rather than in the more internal layers in *E. sativa* cv. Nemat. In *Capsella* the lipid droplets begin to appear still later than in *Arabidopsis*, that is at the late Torpedo stage (SCHULZ and JENSEN 1968). More similarly to *E. sativa* cv. Nemat, the lipid bodies begin to appear in the embryo at the Heart stage in *Brassica napus* cv. Topas (HE and WU 2009). Such an early start of lipid accumulation could be related to the fact that seeds of both *E. sativa* cv. Nemat and *Brassica napus* (particularly their cotyledons) are particularly rich in lipids and hence the differenti-



Figs. 25-30 — (25) Chalazal endosperm cyst (CEC), basal zone. Detail of the cytoplasm, rich in chloroplasts, ribosomes and with an amoeboid nucleus. Also endophagocytosis vacuoles are present. (26) Chalazal endosperm cyst (CEC), basal zone. A roundish euchromatic nucleus with a huge nucleolus is visible. (27) Nucellus cell of the CPT. A huge roundish nucleus with endophagocytosis vacuoles enclosing cytoplasm portions are remarkable. The plastids have an irregular shape. (28) Nucellus cell of the CPT. Multilobated amyloplasts are evident in the cytoplasm. A big endophagocytosis vacuole contains mitochondria. (29) Nucellus cell of the CPT in advanced degeneration stage. The quite electron dense cytoplasm is still showing partially dissolved starch granules. (30) Detail of CPT cells in the final degenerative phase producing a nucellar lysate and stacked wall residuals.

ation of the meristematic cells in order to accumulate lipids begins in advance with respect to other species. *Arabidopsis* e *Capsella*, in fact, belong to family Brassicaceae too and show lipid accumulation in the cotyledons, but in less quantity. In fact these last two species are not currently used for lipid production neither for food nor industrial purpose.

The cellularization of the micropylar endosperm and that in the Central Chamber in *E. sativa* cv. Nemat starts at the Heart stage and, at the Torpedo stage is completed only as a single layer around the embryo (Fig. 2). The first evidence of a passage from the nuclear to the cellular phase is the presence in the endosperm cytoplasm of dictyosomes involved in walls formation, preceding the appearance of wall-plasmamembrane-plasmodesmata tracts. It occurs first in the CC endosperm, in that adjacent to the internal teguments, and only lastly in the central part of the lumen. In *E. sativa* the passage from nuclear endosperm to cellular endosperm occurs in average later with respect to *Alyssum argenteum*, *Brassica nigra*, *Diplotaxis erucoides*, *Capsella bursa-pastoris*, *Matthiola tricuspidata* (VIEGI *et al.* 1976) and *Alyssum maritimum* (VIJAYARAGHAVAN *et al.* 1984). In these other Brassicaceae, the cellularization of the endosperm is completed already at the Torpedo stage or even earlier, in the Heart stage. Even in the spontaneous *E. sativa,* VIEGI *et al.* (1976) showed that the endosperm cellularization is completed already at the end of the Heart stage, that is much earlier than in the cv. Nemat. The ultrastructural results suggest that the nuclear stage allows a more efficient trophic activity of the endosperm towards the embryo cells. Since in *E. sativa* cv. Nemat the nuclear stage lasts until the Heart and Torpedo stages, we can suppose a link between this longer nuclear phase, with the consequent lack of walls, and the higher quantity of lipids stored in the embryo, with respect to the normal *E. sativa*. Moreover the presence of big chloroplasts apparently active in photosynthesis, in the micropylar endosperm and in that of the central chamber until late Torpedo stage, demonstrates that this tissue is capable of autonomous photosynthesis until advanced embryo developmental stages. In the intermediate and intermediate-advanced developmental stages the seed is still green, and the photosynthetic activity appears to be carried out mainly by the endosperm, since the other tissues of the developing seed do not have chloroplasts with a well developed thylakoid system. Even in the chalazal endosperm cyst (CEC) of *E. sativa* cv. Nemat the number of chloroplasts is high, even with respect to the same type of endosperm in other Brassicaceae (see the figures of the CEC in BROWN *et al.* 2004). Even these plastids have a well developed thylakoid system, even if not so well developed as in the other two endosperm compartments. ROLLETSCHEK *et al.* (2003) and LIDDIARD and CARMAN (2010) suggested that O2 production by photosynthetic activity in the seed could be necessary to provide O2 to the developing embryo, particularly if the seed coat acts as a barrier against the diffusion of this gas. Moreover the amount of functional chloroplasts in the seed of *Eruca* suggests that the photosynthetic product could be a relevant part of the carbohydrates necessary for the anabolic activity in the developing embryo.

The cyst in *E. sativa* appeared to be more similar to the pyriform shape sensu BROWN *et al.* (2004), but with a more flattened base with respect to the proposed examples. The chalazal chamber appeared to be more similar to the type B by BROWN *et al.* (2004). Moreover, differently from other Brassicaceae (BROWN *et al.* 2004), the CEC in *E. sativa* cv. Nemat is in a situation of uninterrupted continuum with the central chamber endosperm and no wall with labyrinthine ingrowths is present between the chalazal endosperm and the CPT, nor such ingrowths were present in the CEC of *Brassica oleracea* (BROWN *et al.* 2004), species belonging to the same tribe (Brassiceae) as *Eruca*. Instead, wall infoldings were described in some representatives of Alysseae, Arabideae, Lepidieae, Sisymbrieae and Thelypodieae by BROWN *et al.* (2004).

During the Torpedo stage the CEC appears to be in a phase of dynamic equilibrium between autophagy and the building of new cytoplasmic components, polysaccharides and proteins. These substances are necessary to carry out the function of storage and processing of nutrients coming from the maternal tissues. When this equilibrium changes towards the autophagy, the endosperm is destined to undergo Programmed Cell Death. An evidence of autophagic activity was the abundance of autophagic vacuoles originating from the SER and by the strict contact of them with RER cisternae, probably involved in lytic enzymes production. The autophagic vacuoles included cytoplasm and organelles, particularly ribosomes and plastids, all of them observed at different stages of degeneration. Nevertheless an anabolic activity by the CEC is demonstrated by the abundance of ribosomes and the huge nucleoli. The fast ribosomes turn-over shows a high protein synthesis activity, while the demolition of plastids would provide a high quantity of lipidic substances, as shown by

the presence of lipid droplets in the cyst. Protein and lipid storage will be transferred to the embryo in its last developmental phase, that is during its bending, that will end in strict contact to the CEC. The abundant chloroplasts in the CEC suggest the hypothesis that also this endosperm compartment contribute extensively to the production of O2 and carbohydrates, that will be necessary for the development of the embryo during the late stages of its ontogeny. The passage of carbohydrates from the CEC to the embryo occurs however indirectly, through the endosperm of the central chamber, with which the CEC is in continuity. The few layers of nucellar cells in the CPT, the poor thickness of the nucellar lysate and, particularly, the lack of transfer modifications between CEC and CPT, indicate that the contribution of the nucellar tissues and, in general, of the seed maternal tissues tends to be reduced during the medium-late embryo development.

Conclusion - the importance of all the three endosperm compartments for the embryo ontogeny in *E. sativa* cv. Nemat is clearly highlighted on micromorphological basis, particularly for the mechanism of reserve storages, eventually accumulated in the embryo. Moreover the high photosynthetic capability, shown by the numerous active chloroplasts in all the endosperm compartments, demonstrates a partial independence of the seeds from the maternal tissues in this species. *E. sativa* leaves are normally harvested for food, while the seeds of cv. Nemat appear to be particularly rich in oil. The premature independence of seeds and/or fruits from the necessity of absorbing nutrients from the rest of the plant could indicate the possibility of harvesting both leaves (earlier) and seeds (later) in this plant without compromising a full seed maturation.

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FIGURE LEGENDS

Key to labelling: Cp: chloroplast; d: dictyosomes; Em: embryo En: endosperm; Ep: epidermic layer; In: inner integument; ld: lipid droplets; m: mitochondrion; N: nucleus; nl: nucleolus; Pa: palisade layer; Pl: plastid; rer: rough endoplasmic reticulum s: starch; Su: suspensor; SEp: sub-epidermic layers; ser: smooth endoplasmic reticulum; V: vacuole; W: wall; Wi: wall ingrowths.