Running title: Ultrastructural analysis in marigold

PLASTID ANALYSIS OF PIGMENTED UNDIFFERENTIATED CELLS OF *Tagetes erecta* L. BY TRANSMISSION ELECTRON MICROSCOPY

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Abstract

Marigold (*Tagetes erecta*) flower has been used in industry because of its high pigment content. Flower color development implies chloroplast-chromoplast transition associated to carotenoids biosynthesis. In this work, undifferentiated pigmented marigold cells were recovered by somaclonal variation and analyzed by transmission electron microscopy in order to observe the pigment accumulating structures. Callus were achieved from leaf explants and developed on MS medium added with 2, 4-D and BA. After several rounds of recurrent selection for pigmented calli, green, yellow and brown (oxidized) calli were obtained. The carotenoids profile changed in the obtained calli, at green material violaxanthin, lutein, zeaxanthin and β-carotene were produced, while yellow calli produced mainly lutein (80%) as well as the brown calli (30%). Chloroplast-chromoplast transition was followed by measuring plastids size and shape of undifferentiated marigold cells development by digital image analysis. Cellular alterations were evident in brown callus. Chloroplasts were the main structure in green callus, and in yellow calli were clearly observed the formation of plastoglobules, correlating with a chloroplast-chromoplast transition. The high number of plastoglobulli observed in yellow callus, might be directly related to pigment synthesis and accumulation. Morphometrical analysis showed that chromoplasts of *T. erecta* cells are a combination of the globular and reticulotubular type.

Key words: calli, chromoplast, marigold, TEM

Introduction

Plant tissue culture has been used as an important technique for the study of different cell processes contributing to simplify the breeding procedures and overcome some agronomic and environmental problems (Rout et al. 2006; Vanegas et al. 2002). Added to these important facts, these tools have not been exploited for studies at subcellular level. In specialized tissues, it has been reported the differentiation of plastids during plant development, also was found a correlationship between pigmentation and chloroplast to chromoplast differentiation (Suzuki et al. 2005; Vázquez-Caicedo et al. 2006). This process is associated with chlorophyll degradation and synthesis of new pigments; carotenoids accumulation is carried out in chloroplastic specialized structures named plastoglobules and the development of new pigment-bearing structures was observed (Bréhélin et al. 2007; Vothknecht and Soll, 2005).

In terms of subcellular analysis, development of plastids in specialized tissues of some species has been reported. In these studies, during cell development the growth phase is accompanied with plastid division, as well as structural and biochemical changes yielding several interconvertible plastid types of specialized functions (Bonora et al. 2000; Bréhélin et al. 2007; Vásquez-Caicedo et al. 2006). Chromoplasts mostly occur in mature tissues and are generally classified as globular, tubular, reticulotubular, membranous, and crystalline types (Vásquez-Caicedo et al. 2006). The carotenoids may differ in their physical properties and bioavailability according to the underlying chromoplast structure. For instance, the crystalline structure of the chromoplast is enhancing the stability of the all-trans configuration (Welsch et al. 2000).

Marigold (*Tagetes erecta*) is appreciated because of its pigmented flowers, they are traditionally used in folk ceremonies and traditional medicine; moreover, they have been

linked to some health benefits, such as prevention of cancer, cardiovascular diseases and they are an important source of carotenoids for the food industry. In marigold, the main pigment that accumulates in flowers is lutein, and the color differences between varieties are the result of differences in lutein content (Delgado-Vargas and Paredes-López 2003; Del Villar-Martínez et al., 2005). In subcellular analysis, changes that occurred during plastid differentiation in flower morphogenesis have been analyzed. Also it was demonstrated pigment deposition in specific structures (lipidic vesicles) during flower development (Del Villar-Martínez et al., 2005). Although tissue culture systems for *Tagetes* have been established, no studies in undifferentiated pigmented cells or subcellular analysis have been conducted so far. It would be important to generate, trough somaclonal variation or genetic manipulation, pigmented undifferentiated cells in order to propose the use of marigold as a bioreactor. However, to validate this assumption, the chromoplast ultrastructure in *Tagetes erecta* undifferenciated cells remained to be elucidated. The structure and changes that are generated in chloroplast-chromoplast transition in these cells have not been deeply analyzed yet. In this work, undifferentiated pigmented marigold cells were analyzed by transmission electron microscopy in order to observe the pigment accumulating structures and morphometrical characteristics.

Materials and Methods

Plant material and pigmented calli selection

Undifferentiated marigold (*Tagetes erecta*) cells were obtained from leaf explants cultivated in MS medium (Murashige and Skoog 1962) added with 2,4-D (2.0 mg/L) and BA (2.0 mg/L**),** and incubated in **a** dark growth chamber (25±2° C). In order to identify pigmented cells, the culture was separated in small fractions and subcultured several times

in the same medium (Figure 1). During calli culture (from seventh round), differentially pigmented portions were observed (yellow, green, and brown). Stable pigmented cell lines were obtained, and re-growth in a fresh medium. For HPLC analysis, samples of the selected lines were frozen in liquid nitrogen and store at -70ºC until use. For ultrastructure analyses of each cell type, fresh samples were collected for the treatment.

Carotenoid determination by HPLC

Carotenoids were extracted from calli with different pigmentation, according to Delgado-Vargas and Paredes-López (1996). Fresh tissue (2g) samples were extracted with HEAT (hexane:absolute ethanol:acetone:toluene, 10:6:7:7 v/v/v/v) and 40% methanolic KOH solution. The HPLC analysis was carried out on an Agilent 1100 system consisting of a quaternary pump, a photodiode array detector, a column temperature control module and an autosampler, which was set to draw 20 µL from the samples (Agilent, Palo alto, California) The pigments were separated on a YMC 30 column (5 µm, 250 x 4.6 mm) (YMC, Wilmington, NC) kept at 17 ºC. Methanol (MeOH), methyl-*ter*-butil ether (MTBE), and water were used in the mobile phase. The linear gradient elution was the same as described by Meléndez-Martínez et al (2007): 0 min. 90% MeOH + 5% MTBE + 5% water; 12 min, 95% MeOH + 5% MTBE; 25 min, 89% MeOH + 11% MTBE; 40 min, 75% MeOH + 25% MTBE; 60 min, 50% MeOH + 50% MTBE; 62 min, 90% MeOH + 5% MTBE + 5% water. MeOH and MTBE contained small portions of butylated hydroxytoluene and trietylamine (0.1% and 0.05%, respectively) to protect the carotenoids over the HPLC analysis (Hart and Scott, 1995). The mobile phase was pumped at 1 ml / min, and the chromatograms were monitored at 430 nm. Pigments were identified by analyzing their chromatographic and UV/vis spectroscopic characteristics with standards isolated from appropriated sources as described by Meléndez-Martínez et al (2007)

Tissue sampling and microscopy (TEM)

Small pieces of marigold callus were cut and fixed for 1 h with 2.5% glutaraldehyde in sodium cacodylate buffer (0.05 M). After fixation, samples were washed 5 min in washing solution (sucrose 0.25 M in the same buffer). Post-fixation was carried out in OsO_4:bidestiled water solution (1:50 w/v) for 1 h. Tissue samples were washed and dehydrated through graded (10%, 10 min steps) ethanol series from 50% to absolute; propylene oxide was the intermediate solvent for infiltration for 20 min. The first infiltration step was carried out with propylene oxide and EPON^{TM} resin (2:1 v/v) for 1 h. A second infiltration step was carried out with propylene oxide and resin (1:1 v/v) for 1 h. Finally, propylene oxide and resin (1:3 v/v) for 1 h. After infiltration steps, embedded samples were transferred to embedding moulds filled with fresh undiluted resin and polymerized in a convection oven at 60º C for 24 h. To select samples for TEM observation, 200-500 nm thick sections were obtained with ultramicrotome. Thin sections were attached to a 200-mesh copper grid, contrasted with lead citrate and uranyl acetate, and observed at 60 kV in a JEOL 1010 transmission electron microscope (JEOL, Inc., Boston, USA). Each image was recorded in Tiff format with size of 640 X 480 pixels.

Digital image analysis and morphometrical parameters evaluation

The captured images were processed according the method proposed by Chanona et al. (2003). Corel Photo Paint software (Corel Draw V11.0, Corel Corporation, USA) was used for this purpose. First, images were transformed to a gray scale of 8 bits in a bit map format (*.bpm extension). Intensity, brightness and contrast of transformed images were adjusted in order to achieve a precise definition of the border of plastids (perimeter). Afterwards,

plastids images were collected as individual objects, copied and transfer to a new template of 640 x 480 pixels. Images were binarized (2 bits) as shows in Figure 2. Individual plastids were analyzed with the Sigma Scan Pro software (V 5.0, SPSS Inc., USA), the morphometrical parameters evaluated were: a) perimeter (P), which is the longest distance between the pixels that delimitate the border of the shape; b) area (A), it corresponds to pixels contained by the object; c) shape factor (Sf), given by $(4\pi A)/(P^2)$ in which Sf = 1 corresponds to a circle or round shape; d) compacity factor (Cf), defined as P^2/A, which correspond to a circle formed by pixels given as 4π; this factor gives useful information on the rugosity of the perimeter, and therefore on the irregularity of an object; and, e) elliptic factor, determined by the maximal and minimal length relationship of the object (SSPS 1999**).**

Results and Discussion

Somaclonal variation and pigment production

Somaclonal variation is an interesting toll used for the recovery of material with desired characteristics, such as biotic and abiotic stress resistance or metabolite production. In the technique is used the natural cell response and adaptation to different factors. We screened the cultures for pigment production using a repetitive growth of the calli trying to expose the natural variation of the cultures. After seven rounds of cultivation early differentiated cells were observed. Then small sections of calli were isolated and established as differentiated cultures. The stability of the selected cells was maintained after several rounds of growth. At this time the analysis for pigment content and subcellular structures were done.

Pigment production

In order to explore the changes in carotenoids content at the undifferentiated pigmented lines (Table 1,), the first step was to analyze the carotenoid profile of each tipe of calli (Figure 2). The analysis was established with standards, violaxantin, lutein, zeaxantin and β-carotene were eluted at 19. 52, 23.49, 25.77 and 48.01 minutes.

The HPLC profile of an extract from the green calli is shown in the Figure 2A; two mayor peaks eluted early; a peak was seen to elute at 19 minutes similarly to violaxantin, and a second peak eluted to 23.4 minutes, similar to the lutein. Two other peaks with a smaller area elute later at 26.5 and 47.9, corresponding to zeaxantin and β-carotene**.** The HPLC profile for the yellow calli (Figure 2B) shown the presence of a main peak at 23.6 min and the small one at 26.7 minutes of elution, corresponding to lutein and zeaxantin. For the brown material (oxidized) only a small peak near to 23 min was observed, which could correspond to lutein (data not shown). The advanced developmental stage and cell disorganization of the cells may contribute to the degradation of the carotenoids.

Table 1 resumes the results for carotenoids analysis, as seen, some important differences are notorious in the carotenoids profiles in the different selected calli, at green four different carotenoids, at yellow just two carotenoids. A notorious change in lutein content is evident between different lines, in green calli a 0.17 ug/g FW concentration was determined, while in the yellow line 0.64 ug/g FW was found. It represents a 350% in the lutein concentration (increase of 3.5 times**).** In brown calli (oxidized) the lutein content diminished to 0.05 ug/g FW, that correspond to 20% (a decrese of 5 times) of the lutein content at green calli. In green calli, violaxantin and lutein are the predominant carotenoids while in the yellow selected materials, the main carotenoid was lutein. In marigold flowers

similar behavior was found, the overproducer variety increase the lutein synthesis, while the white flower almost abolishes the lutein production (Del Villar-Martinez et al., 2005)

Transmission electron microscopy (TEM)

Sections of the selected marigold calli were analyzed and different kind of plastids were observed (Figure 3): proplastids (cytoplasmic organelle from which a plastid develops); chloroplasts (which contain internal membranes and a characteristic prolamellar body); and, chromoplasts (responsible for pigment synthesis and storage). Dramatic changes in chloroplast ultrastructure including reorganization or disassembling of internal membranes were observed, as reported for broccoli floret sepals, postharvested ripening mango and carrot roots (Susuki et al. 2005; Vázquez-Caicedo et al. 2006; Vothknecht and Soll 2005); vacuole was frequently found since these cells have high water content. A close relationship between callus friability and intercellular spaces was observed (Figure 3).

Differentially pigmented calli (yellow, green, and brown) were obtained (Figure 4) and cultured in complete darkness, in order to avoid *in vitro* oxidation. Seeman and Barriga (1983) reported that light is a determinant factor on *in vitro* oxidation. Yellow and green calli were friable and stable for several periods of sub-culture, and conserved their characteristic pigmentation (Figure 4). Cells from green callus shown chloroplasts as main feature (Figure 4a), organelles provided with thylakoid membrane system fully developed (López-Juez and Pyke 2005; Vothknecht and Soll, 2005). Yellow callus was characterized by the presence of abundant mitochondria; spherical and oval shaped chromoplasts filled with well defined plastoglobuli (Figure 4b), these plastids have capacity to accumulate pigments as it has been reported by Vázquez-Caicedo (2006) where numerous large

plastoglobules (discrete plastid substructures**)** were present in the chromoplasts of mango fruit mesophyll cells. Some structural disorganization and cell collapse were observed in brown callus (Figure 4c). Nevertheless, analyzed callus presented some dehydration degree; microscopic observations showed cell damage and a clear membrane disruption. Shrunk and condensed membranes were observed separated from the cell wall being impossible to observe well defined plastids (Figure 4c).

Figure 5 shows the possible transition proplastid-chromoplast and concomitant formation of plastoglobules. This is evident in tissues undergoing color changes associated with flower or fruit development (Deuère et al. 1994); interestingly, this was also observed in marigold callus. All plastids regardless of their status, retain their ability to become into each other; it should be noted that is a likely route and, it may be done directly from proplastid to chromoplast. All plastids regardless of their status, they retain their ability to become into each other; it should be noted that a likely route may be directly proplastid to chromoplast (Vothknecht and Westhoff 2001). Chromoplast biogenesis involves highly intricate, developmentally controlled processes, consisting of both structures degradation and creation of new ones, process is accompanied by internal changes in chloroplasts, followed by thyllacoidal membranes disintegration, which are replaced by simple non-chlorophyllic thylakoids derived in part from the internal membrane of plastids (Camara and Brangeon 1981; López-Juez 2007; Vishnevetsky et al. 1999; Vothknecht and Westhoff 2001). Up to now, the descriptions have been done in a extensive way in specialized tissues, such as flowers and fruits, while in undifferentiated tissues the reports are scarce or absent. In our work we did not know how the over producing cells store the pigments, and how this process was associated to an specific pigment (lutein). This is the first report for a

relationship between lutein production and cell structure development in undifrentiated cells.

Assessing the ultrastructural morphometry

For morphometric analysis, chloroplasts and chromoplasts were subdivided in two categories according to their area, above 1.0 μm^2 and below 1.0 μm^2 (Table 2). Yellow calli chloroplasts were larger than green calli chloroplasts (1.73 and 0.24 μm^2 respectively) probably due to chloroplasts from yellow calli that undergo transition to chromoplasts. Therefore, the differentiation into chromoplasts had occurred at an earlier stage of cell development, whereas numerous large plastoglobuli were present in the chromoplasts. The size of the plastoglobuli varied with diameters from 0.1 to 0.5 µm. In addition to the plastoglobuli, tubular membranes with and without electrondense contents were observed as single tubules (Figure 4).

Results for adimensional descriptors are shown in Table 2 for green and yellow calli, for different plastids classes (chloroplasts, chromoplasts). It was found that chromoplasts had the highest elliptical factor (EF). These photosyntetically active plastids are more elongated than chromoplasts. According to Vishnevestky et al. (1999), Vázquez-Caicedo et al. (2006), chromoplasts are grouped in four categories: globular, tubular, membranous and crystalline. Elongated forms of plastoglobuli (high EF) and their connection to membranes could be assumed as developing stages. Single stroma thylakoids, individual prolamellar-like bodies and crystalline structures (Figure 5), which might represent phytoferritin, could be detected. The results showed that these structures were similar in shape to those observed in yellow calli. On the basis of the latter observations and the suggested classification of chromoplasts (Vishnevestky et al. 1999; Vázquez-Caicedo et al. 2006), the chromoplasts of *T. erecta* cells are a combination of the globular (shape factor close to 1.0) and

reticulotubular type (elliptic factor values around 1.5). Nevertheless it is important to note that deeper research could correlate elliptic and shape morphometric factors, with shape classification proposed by different authors (Sitte et al. 1980). We can say in general that chromoplasts with sizes over 1.0 μm^2 are the most irregular plastids showing high EF and CF, and low SF values (Table 2). In other words, they are ovoid, rough and slightly rounded. This peculiarity coincides with the results reported by Diaz-Polanco (1996) who pointed out that chloroplasts in general have a rather elliptical shape, while López-Juez and Pyke (2005) indicated that chromoplasts have very different forms, for example ovoid and spherical. In the other hand, the compacity factor (CF) gives an idea of border rugosity degree, and it is directly related to perimeter. CF showed that chloroplasts are more rugged than proplastids in both green and yellow calli, although chromoplasts from cells of yellow callus presented more smooth edges than chloroplasts probably by the accumulation of pigments in the specialized structures which makes the organelle fits to its internal content. Then, the value of CF could confirm that overaccumulation of carotenoids decreased the rugosity of the membranes, inducing the formation of vesicles which then separate from the membrane to form plastoglobuli; in concordance, it was suggested that plastoglobuli originate from the thylakoids (Deuère, et al. 1994). Hence, the accumulation of lutein in *T. erecta* chromoplasts is related to the carotenes affinity to a lipophilic medium, which is provided by the plastoglobuli. This particularity was observed in mango cells (Vázquez-Caicedo et al. 2006), as well as in *Arum italicum* (Borona et al. 2000).

The present study confirmed that carotenoids (mainly lutein) of *T. erecta* cells were deposited in the plastoglobular substructures of the chromoplasts (Table 2, Figure 4). Apparently, plastoglobuli are formed due to lipid overproduction, whereas membranous structures are induced by a higher portion of proteins (Suzzuki et al. 2005). Although it has

been established that plastoglobuli are composed of a neutral lipid core surrounded by a monolayer of galactolipids (Bréhélin et al. 2007), the exact composition and function of plastoglobuli in *T. erecta* are unknown and show some variation between chromoplasts and chloroplasts. There are no records of this kind of studies in *Tagetes erecta* cells cultivated *in vitro*, the only available work (Del Villar-Martinez et al. 2005) was made with plant material *in vivo* (ligules**),** it makes the plastid morphometrical description an important contribution to understand pigment accumulation and cell shape relationship.

Although is has been considered that change from chloroplast to chromoplast is a complex process occurring in highly specialized tissues, our ultrastructural studies reveal it may happen in undifferentiated cells which are not specialized**.** As it can be seen it is interesting that is not necessary a complex system either for differentiation of plastid nor for the plastoglobules development.

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Table 1. Lutein content in *Tagetes erecta* organogenic calli

Sample	Carotenoids detected (% Abundance)	Retention time minutes	Lutein content mg/g FW
Control (green)	Violaxanthin (45) Lutein (35) Zeaxanthin (12) β-carotene (5)	19.0 23.4 26.5 47.9	0.17
Selected (yellow)	Lutein (85) Zeaxanthin (4)	23.6 26.7	0.64
Oxidized (brown)	Lutein (30)	23.4	0.05

Table 2. Morphometric characteristics of plastid observed in differential pigmented calli of marigold (*Tagetes erecta* L.).

Callus	Plastid (μm^2)	Area (μm^2)	Perimeter (μm)	Eliptic factor	Compacity factor	Shape factor
Green	Chloroplasts >1	----	----	----	----	----
	Chloroplasts <1	0.24 ± 0.01	2.51 ± 0.07	2.35 ± 0.43	18.57 ± 1.90	0.68 ± 0.07
Yellow	Chloroplasts >1	1.73 ± 0.18	6.81 ± 0.40	2.04 ± 0.54	19.23 ± 4.21	0.68 ± 0.13
	Chloroplasts <1	0.72 ± 0.08	4.20 ± 0.29	1.83 ± 0.41	16.54 ± 1.17	0.76 ± 0.07
	Chromoplasts >1	1.41 ± 0.10	5.94 ± 0.23	1.52 ± 0.32	16.10 ± 1.47	0.78 ± 0.06
	Chromoplasts <1	0.48 ± 0.10	3.19 ± 0.38	1.51 ± 0.23	15.50 ± 0.42	0.81 ± 0.02

Values marked with ± correspond to standard error

Figure 1. Undifferentiation process on leaf explant of *Tagetes erecta* a) leaf cutting, b) Leaf explant on culture media. Inset: undifferentiating explant, c) Undifferentiated tissue.

Figure 2: Reversed-phase C_{30} of undiferentiated marigold cells. A) Profile from an extract of green calli; 1: violaxanthin, 2: lutein, 3: zeaxanthin, 4: b-carotene, 5: esterified carotenoids. B) Profile from an extract of yellow calli; 1:lutein, 2: esterified carotenoids. Detection was carried at 450 nm

Figure 3. Plastids found in different pigmented marigold calli . a) Green callus, (cloroplasts) b) Yellow callus (chromoplasts) c) Brown callus (No plastid found). ic: intercellular space, chl: cloroplast, chr: chromoplast, pg: plastoglobuli, mi: mitocondrium. bar represents 500 nm.

Figure 4. TEM pictures of marigold undifferentiated cells. a) panoramic view of whole cell, b) panoramic view of three neigborg cells, c) proplastid appereance, d) chloroplast presence, e) chromoplast and motochondirium view. v: vacuole, n: nucleous, ic: intercellular space, pp: proplastid, cl: cloroplast, cr: chromoplast, pg: plastoglobuli, mi: mitochondrium (bar a:10 µm; b: 2 µm, c, d, e: 500 nm).

Figure 5. Morphological changes occurring along plastid development in marigold (*Tagetes erecta*) calli. a) proplastid; b) chloroplast; c) intermediate stage; Proplastid-chromoplast; d) intermediate stage, chloroplast.-chromoplast; e) chromoplasts. (bar = 500 nm). ic: intercellular space, pp: proplastid, chl: cloroplast, chr: chromoplast, pg: plastoglobuli, mi: mitocondrium.

Figure1

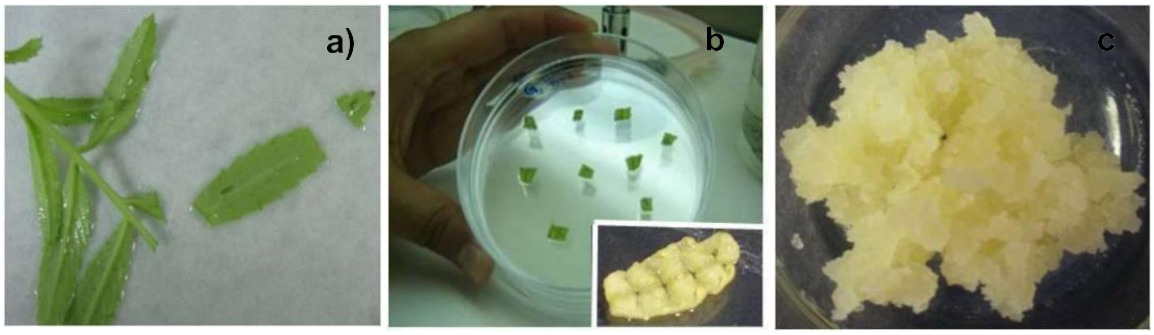
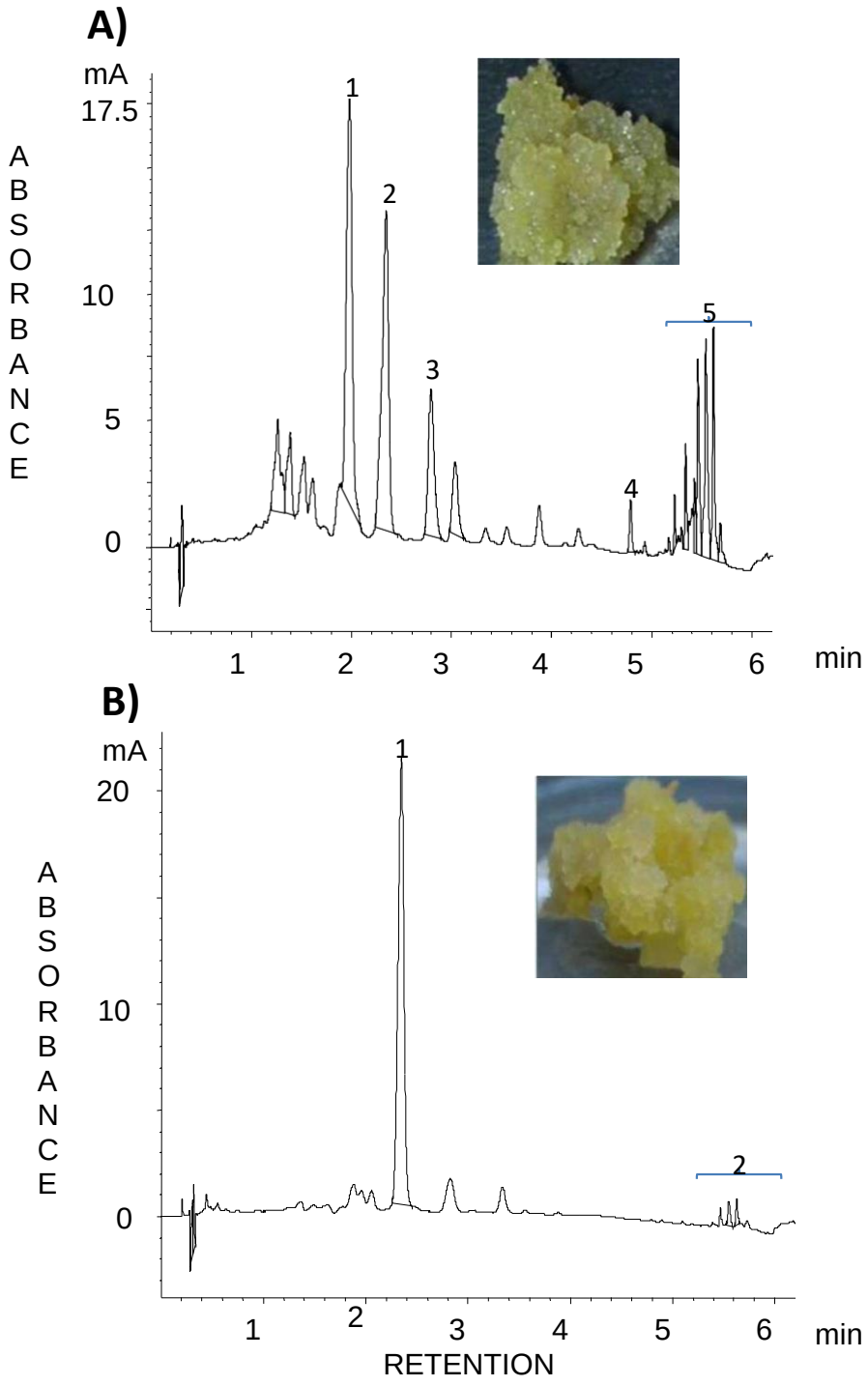


Figure2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RETENTION

Figure 3

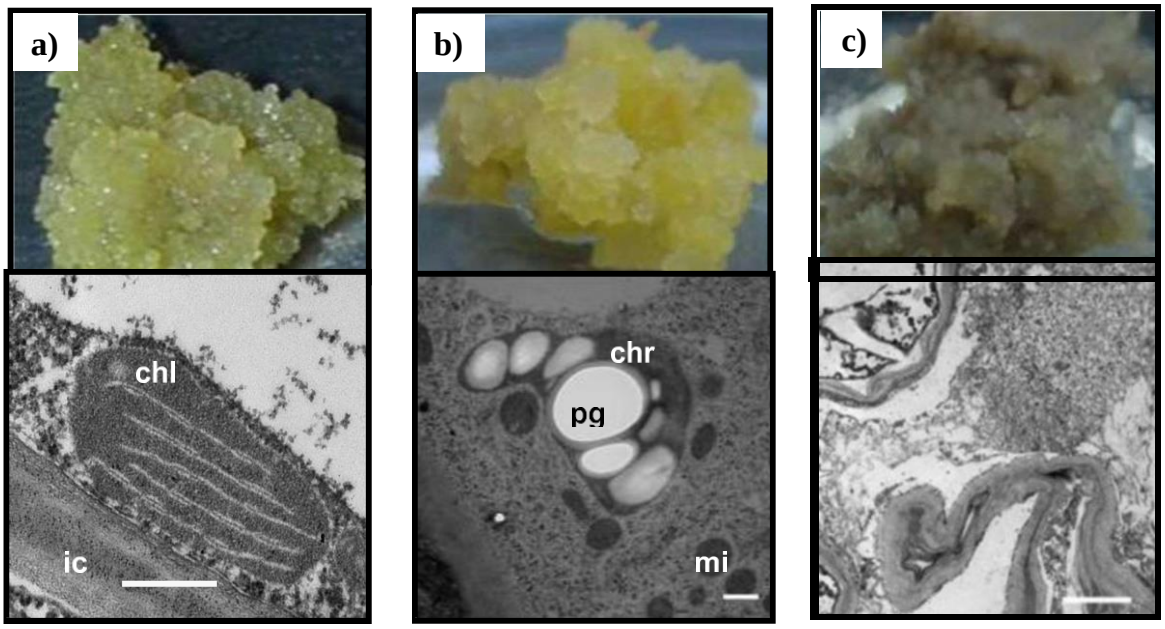

Figure 4

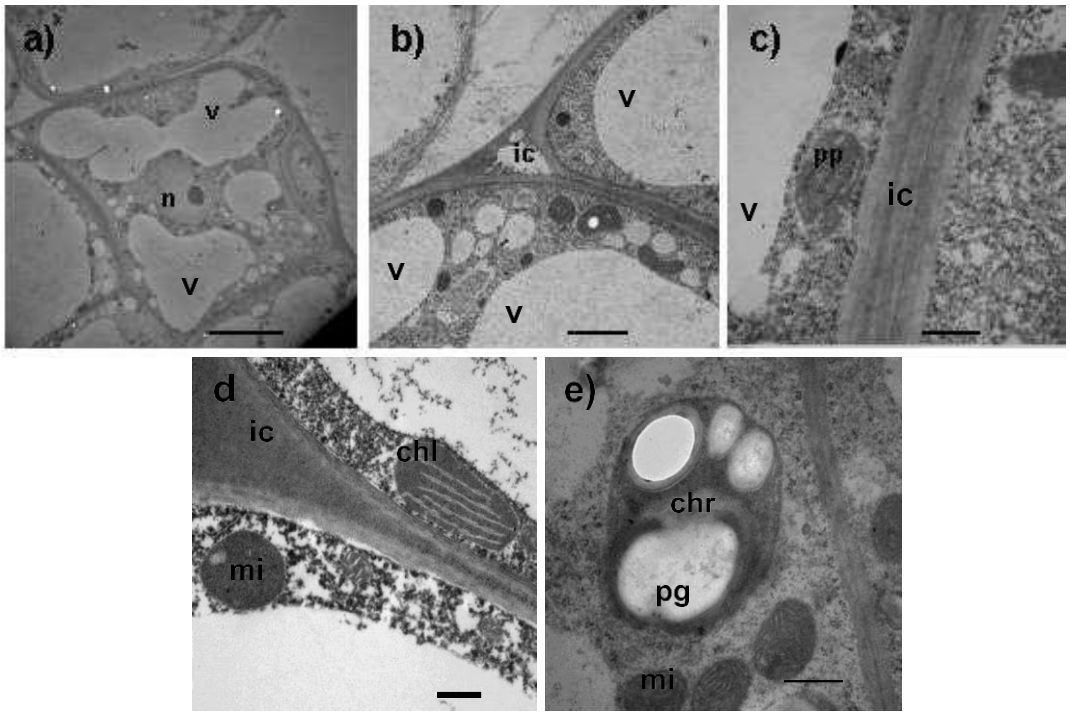

Figure 5