BIOLOGIA PLANTARUM 51 (3): 421-429, 2007

Structural and chemical study of callus formation from leaves of *Rubia tinctorum*

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Abstract

Dedifferentiation was monitored in *Rubia tinctorum* L. leaves over a 14-d period after callus induction using transmission electron microscope (TEM), high performance liquid chromatography (HPLC), spectroscopy and thin layer chromatography (TLC). Photosynthetic pigment loss of leaves took 3 - 5 d coinciding with the first period of anthraquinone accumulation. Callus cells were discernible in the region of the vascular bundles and wounded edges of leaves after 10 - 14 d. Characteristic ultrastructural alterations were manifested in vacuolization, appearance of mitochondria, amount of smooth endoplasmatic reticulum and cytoplasm, caryolympha density of nuclei and cytoplasm content of cells. There were special events in the transfer cells: unequal divisions of dedifferentiated plastids and lytic activity in the cell wall. Our results show that mesophyll cells seem to be stopped at a particular level of dedifferentiation, while transfer cells embodied in veins of leaves pass through further alterations and lead to callus formation. Findings suggest that a sort of dedifferentiation drift manifests in the various cells of *R. tinctorum* leaves during callus induction and depending on their specialized status they achieve different levels of dedifferentiation. Approximately 4 weeks after callus induction, root growth has started from the young calli.

Additional key words: anthraquinone synthesis, madder, mesophyll cells, photosynthetic pigments, transfer cells, ultrastructure.

Introduction

Plant cell suspension cultures are used to produce several compounds for medical and industrial purposes. The first step in this process is callus induction from different tissues of the intact plants. During this callus formation differentiated cells of explants dedifferentiate and become able to go through cell divisions again. This dedifferentiation process is influenced by several endogenous and exogenous factors, for instance gene expression (Jamet *et al*. 1990), secondary metabolism regulation (Komamine *et al*. 1989), developmental stage of the plant organ used as explant (Kintzios *et al*. 1996), composition and hormone content of culture medium (Roy and Banerjee 2003), as well as macronutrients and sugars present during callus induction (Kintzios *et al*. 2004). Individual steps of developmental changes can be followed with light microscope, transmission electron microscope (TEM), scanning electron microscope, and analytical methods.

Madder (*Rubia tinctorum* L.) is a perennial herb containing several anthraquinone derivates, concentrated mainly in the roots and the rhizome, while leaves contain negligible quantities. These compounds are known as natural yellowish-reddish dyes, with pharmacological effects and genotoxicity (Itokawa *et al*. 1983, Krizsán *et al*. 1996, Schneider *et al*. 2004). *R. tinctorum* cell suspension cultures are sufficient to produce dyes mentioned above, either naturally or after facilitation with elicitor treatments (László *et al*. 1992, Bóka *et al*. 2002).

The aim of this work was to investigate some aspects of dedifferentiation during callus formation from intact leaves of *R. tinctorum* L. We have followed ultrastructural changes in mesophyll and vascular bundle regions. At the same time, alterations in anthraquinone contents and composition were measured by high performance liquid chromatography (HPLC). We have also investigated the loss of photosynthetic pigments in the measured samples.

Received 22 August 2005, *accepted* 28 April 2006.

Abbreviations: ACI - after callus induction; HPLC - high performance liquid chromatography; TEM - transmission electron microscope; TLC - thin layer chromatography.

Acknowledgements: We thank to Dr. M.C.F. Proctor and Dr. M. Marschall for critically reading the manuscript. Thanks are due to Cs. Jónás, K. Gergely and I. Kasnya for their technical assistance. This research was supported by the Hungarian Research Found (OTKA T 038003).

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Materials and methods

Callus induction: Fully developed shoots of madder (*Rubia tinctorum* L.; family *Rubiaceae*) were harvested from the Botanical Garden of Research Institute of Medical Plants (Budapest, Hungary) in May 2004. Freshly detached leaves from the middle region of stems were surface-sterilized for 3 min in a 1 % (v/v) sodium hypochlorite solution, rinsed twice in sterilized distilled water (3 min each), and washed with 40 % (v/v) ethanol twice for 1 min. Leaves were rinsed twice in sterilized distilled water and large ones were cut into 1 - 2 cm^2 pieces with a sterilized scalpel while smaller leaves were placed on the medium intact. Callus induction was carried out on MS medium containing 0.6 % (m/v) agar in Petri dishes, at room temperature (24 - 26 °C) and dim natural light. Callus development was followed with a *Technival* stereomicroscope (*Zeiss*, Jena, Germany).

Suspension culture: Suspension cultures were initiated from callus maintained for years (originated from rhizome) and grown on MS liquid medium at room temperature and dim natural light. They were shaken on a rotary shaker (125 rpm) and subcultured every 14 d.

Structural investigations: Samples were collected from immediately before (control) and 1, 3, 5, 7, 9, 11 and 13 d after callus induction (ACI). As control leaves both intact and sterilized materials were investigated. Fixation, dehydration and embedding steps were carried out using the methods of Bóka *et al.* (2002). For microscopy, the samples were fixed for 2 h in 2 % glutarealdehyde dissolved in 0.1 M phosphate buffer (pH 7.2), and postfixed in 1 % OsO_4. After fixative steps, the samples were rinsed in the same phosphate buffer. The dehydration with ethanol was followed by embedding in Durcupan resin. Toluidine blue-stained 1 μm thick semithin sections were observed with an *Olympus BH2 DIC* light microscope (Tokyo, Japan). Ultrathin sections were prepared by a diamond knife using *Reichert Jung UltracutE* ultramicrotome (Vienna, Austria). These sections were stained with 5 % uranyl acetate for 4 min and led citrate for 6 min (Reynolds 1963) and studied by *Hitachi 7100* TEM (Tokyo, Japan) at 75 kV accelerating voltage.

Determination of pigment content: For analytical work, 0.2 g fresh mass of leaves were taken at the sampling times indicated above, frozen and lyophilized (*Freeze Dryer Modul YO, Jecons Ltd*., Bedfordshire, UK) immediately to avoid degradation. From each sample 20 mg of lyophilized leaf was extracted with 2 cm^3 of 80 % (v/v) methanol for 3 h at room temperature. After centrifugation 0.025 and 0.05 cm^3 of supernatant were applied to *Merck* $60F_{254}$ silica layer (20 × 20 cm aluminum sheet), and were developed over 10 cm with diethyl ether-acetone-hexane (20:20:60 v/v) based on the method described by Isaksen (1991). The separated pigment patches were measured with *Desaga CD-60* densitometer (*Desaga GmbH*, Heidelberg, Germany), at λ = 425 nm detection wavelength. All extractions and developing processes were made in triplicate, and identification of chlorophyll *a+b* was carried out using its R_f value. Densitometric measurements of methanolic extracts were verified by spectrophotometry (Porra 2002).

Determination of anthraquinone composition of leaf, callus, suspension culture and intact rhizome: Five anthraquinone derivatives (lucidin, alizarin, purpurin, anthraquinone, and lucidinethylether) were identified by HPLC and measured in control leaves, leaves after callus induction, intact rhizome, maintained callus and suspension culture. Leaf samples were collected at 0 (control), 2, 4, 6, 8, 10, and 13 d after callus induction, frozen and lyophilized. Dry samples were powdered. Subsequently, 5 cm^3 of 80 % (v/v) ethanol was added to 20 mg of each sample. Extraction took place for 24 h at room temperature. Supernatants were separated and the extraction procedure was repeated two times. The unified supernatants were evaporated on a vacuum evaporator at 35 °C to dryness. The dehydrated residues were hydrolysed with 5 cm^3 of 2 M trifluoroacetic acid at 100 °C for 2.5 h under reflux condenser. Thereafter, supernatants were evaporated to dryness with Rotadest at 35 °C, and resolved in 0.5 cm^3 80 % (v/v) ethanol. This solution was injected into the HPLC system. The system consisted of two *Pharmacia LKB* HPLC pumps and *VWM 2141* UV-VIS detector (*Pharmacia LKB Biochrom Ltd.*, Cambridge UK), *MOS Hypersil BDS RP-C18* 150 × 4 mm column (*Shandon Southern Products*, Runcorn, UK) with 5 μm particles, and *Rheodyne* manual injector with 0.02 cm^3 loop. For identification purposes diode-array, and mass selective detectors were applied according to Boldizsár *et al.* (2004). Separation of anthraquinones was accomplished with a linear gradient program from 20 % B eluent (v/v) to 90 % B (v/v) during 40 min at room temperature. A eluent consisted of acetonitrile : 0.02 M Na-acetate buffer 15:85 % (v/v), B eluent consisted of acetonitrile : 0.02 M Na-acetate buffer 85:15 % (v/v) (Krizsán *et al.* 1996). Detection wavelength was 254 nm, flow rate was 1 cm^3 min^{-1}. In order to get standard curve we have injected alizarin (*Reanal Ltd*., Budapest, Hungary) solutions (in 80 % v/v ethanol) in different concentrations into HPLC system, and the calibration curve was linear with r^2 = 0.9993 in the measured concentration range. Values of anthraquinone derivatives were expressed in alizarin equivalency. All extractions and injections were made in triplicate and average peak areas were applied to calculate the concentrations of different derivatives.

All calculations on chlorophyll and anthraquinone contents were repeated three times on samples that derived from independent experiments.

Results

Unequal growth resulted in curls and undulations on the originally flat leaves. After a few days the intensive green colour of explants faded but remained green during the whole time of experiments (2 weeks). Meantime, from the end of the first week, colour of veins turned to yellow from their light greenish, almost white tinge. At the same time callus formation has started at these areas (Fig. 1*A*). Small groups of callus cells ripped the epidermis over the vascular bundles at certain points of venation and in a short time roots were initiated from calli appearing (Fig. 1*B*). Steady decrease of pigments and the foreseen degradation process was obvious. Chlorophyll *a*+*b* (Chl) strongly decreased during the first 3 days, subsequently chlorophyll content decreased slowly, however, around the 5 - 7th day a small and transient increase was detectable. At the 13th day Chl content was 4.3-fold reduced (Fig. 2).

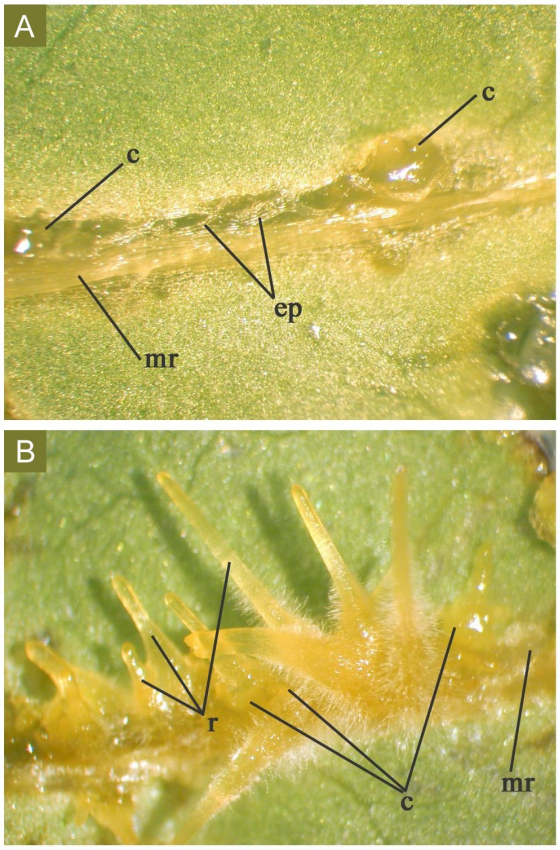

Fig. 1. Stereomicroscopic view of callus initiation and root formation in explants from *R. tinctorum* leaves. *A* - midrib region of the explant 1 week after callus induction (ACI). *B* - roots differentiated from the callus at the midrib 4 weeks ACI (c - callus, ep - remnants of epidermis, mr - midrib, r - root). Magnification 30×.

First cell divisions appeared after 3 d in certain cells of veins (Fig. 3*A*) while in the mesophyll cells there were no signs of structural changes (Fig. 3*B*). After 7 d, daughter cells originating from divided vascular elements were regularly visible in the longitudinal sections of the veins (Fig. 3*C*). Cytoplasm content of mesophyll cells was higher and their vacuolization had changed characteristically. The large central vacuole was reduced to several smaller compartments and groups of small vacuoles arose in the cytoplasm, mainly near the nucleus (Fig. 3*D*, 4*F-H*).

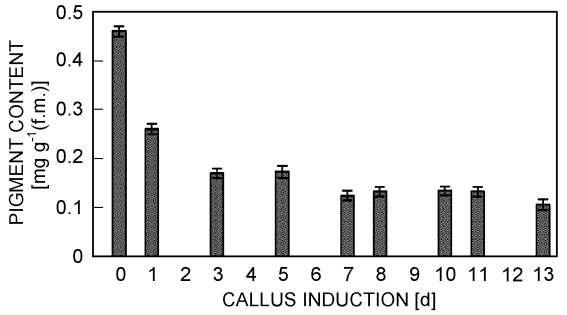

Fig. 2. Changes of chlorophyll content in leaves during callus formation (*error bars* represent standard deviations, $n = 3$).

Ultrastructural changes were conspicuous and characteristic during the investigated period; numerous differences were observed in the mesophyll tissue and vascular bundles. Mesophyll cells of control samples showed normal ultrastructure of plastids with small and electron-dense plastoglobuli. Starch was not detectable. The inner membrane system of chloroplasts showed normal structural features. The large, centrally-positioned vacuole contained sparse precipitates, the tonoplast was undamaged. The plasma membrane of the mesophyll cells showed moderate waving (Fig. 4*A*). In the vascular bundles typical transfer cells were visible. Transfer cells showed characteristic cell-wall protrusions deep into the cell lumen. These local thickenings were more or less the same in density and structure as the rest of the cell wall. The central part of these cells contained one or few vacuoles, plastids were medium sized and only few grana were observable inside them. Grana consisted only a few thylakoids (Fig. 5*A*).

After one day, the endoplasmic reticulum began to disintegrate in the mesophyll cells, prevalent inner membranes twists (myelin bodies) appeared in the cytoplasm. Several vesicules developed near to plasma membrane, the vacuoles did not show differences. Compared to control cells, the plastids of mesophyll cells seemed to be normal; however, considerable quantity of starch appeared in them (1 - 3 medium sized grains). Plastoglobuli of these chloroplasts became larger and their number increased (Fig. 4*B*,*C*). Ultrastructure of transfer cells had already changed after one day. Some of these cells showed symptoms of lytic activity in the cell wall. As a consequence of this process, the middle lamella became loose, in some regions vesiculated. The inner cell wall layer bearing protrusions and the

thickened regions seemed to be electron transparent and fibrillar. As a morphological symptom of autolysis, deliminating membrane isolated vacuole(s) with the central part of cytoplasm and myelic structures appeared (Fig. 5*B*,*C*).

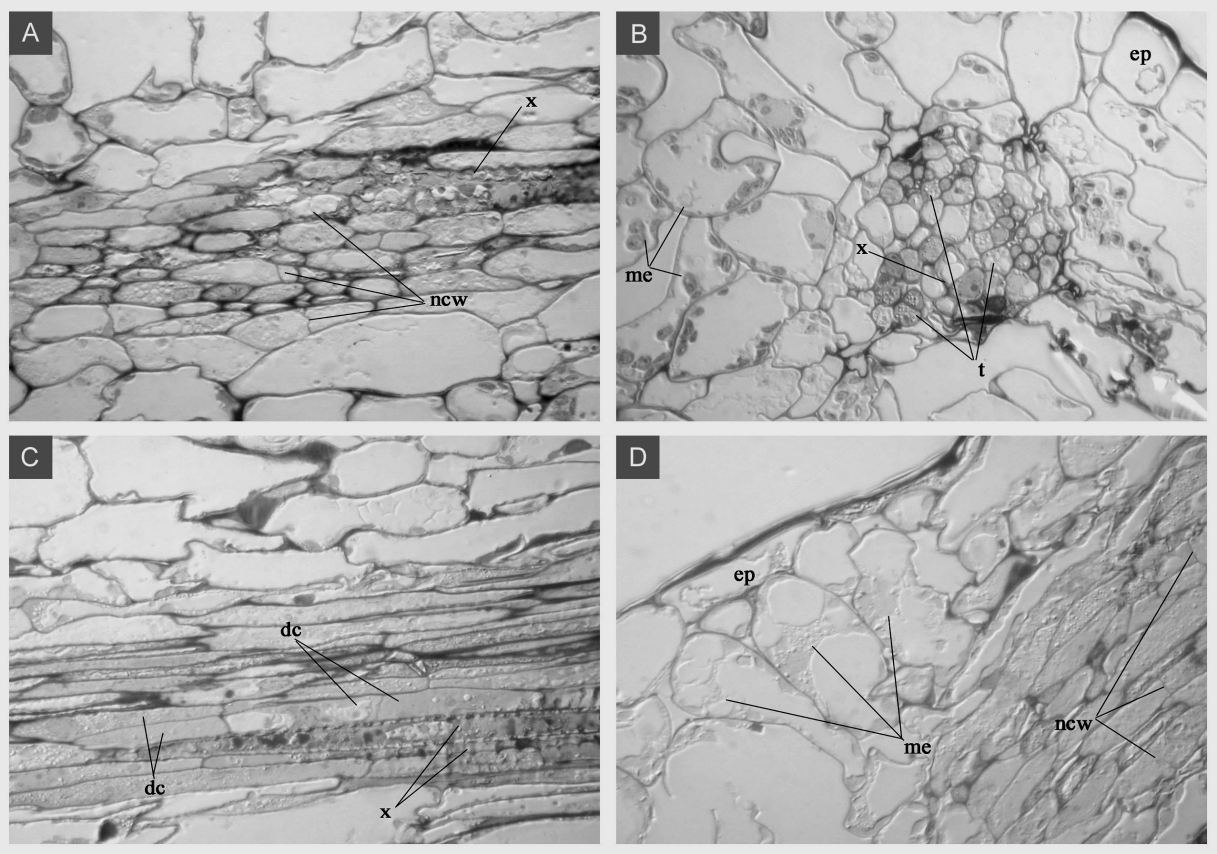

Fig. 3. Cross-section of *R. tinctorum* leaves 3 d (*A*,*B*) and 7 d (*C*,*D*) ACI. *A* - vascular bundle almost in longitudinal section (x - xylem, ncw - newly formed cell walls). *B* - vascular bundle in cross-section; position of transfer cells is clearly visible (x - xylem, me - mesophyll cells, ep - epidermis, t - transfer cells). *C* - longitudinal section of vascular bundle with divided cells (x - xylem, dc - daughter cells). *D* - mesophyll region of leaf and vascular bundle almost in longitudinal section with newly formed cells (ep - epidermis, me - mesophyll cells with enriched, highly vacuolized cytoplasm, ncw - newly formed cell walls). Magnification 425×.

In the 3-d-old samples, grana of chloroplasts in the mesophyll cells were slightly higher, starch content decreased. A number of small vacuoles appeared in the cytoplasm near to nucleus, which probably derived from vesicules of smooth endoplasmic reticulum. The cytoplasm became lightly tenuous, and the organelles began to aggregate (Fig. 4*D*). In some transfer cells the delimited central part of plasma looks to disappear autolytically while in others only smaller areas show similar feature. Plastid division seemed to be in several cases unequal. The cell wall next to the plasmalemma turned into a light but more compact layer without protrusions (Fig. 5*D*). However, in some cases, the cell wall remained structurally almost unchanged.

Five days after callus induction, plastids of mesophyll cells contained starch occasionally, while grana were enlarged. Plastoglobuli were numerous and enlarged. High numbers of mitochondria were visible in the cytoplasm and many vesicules and small vacuoles were discernible. Apart from the central vacuole and cytoplasmic vacuolar structures some myelin bodies were present indicating continued autolytic activity. The plasma membrane appeared less wavy (Fig. 4*E*). The cytoplasm of transfer cells was dense, and size of plastids varied strongly, probably as a result of further unequal division of these organelles. The small plastids had weakly developed thylakoids (Fig. 5*E*).

On the 7^{th} - 9^{th} day, the number of plastoglobuli significantly increased, and they formed clusters. Starch content in plastids of mesophyll cells disappeared. Grana were noticeable but deteriorating. The aspect of the cytoplasm was brighter, less organelles and ribosomes were observable. Flocks of small cytoplasmic vacuoles became slightly larger; there were still autolytic areas in the cytoplasm (Fig. 4*F*). At this stage of callus induction, cytoplasm of transfer cells was dense and compact, in some cells almost without vacuoles. The inner cell wall layer became denser but it could be distinguished. Mitochondria were numerous but hardly visible because of the low contrast between them and cytoplasm (Fig. 5*F*).

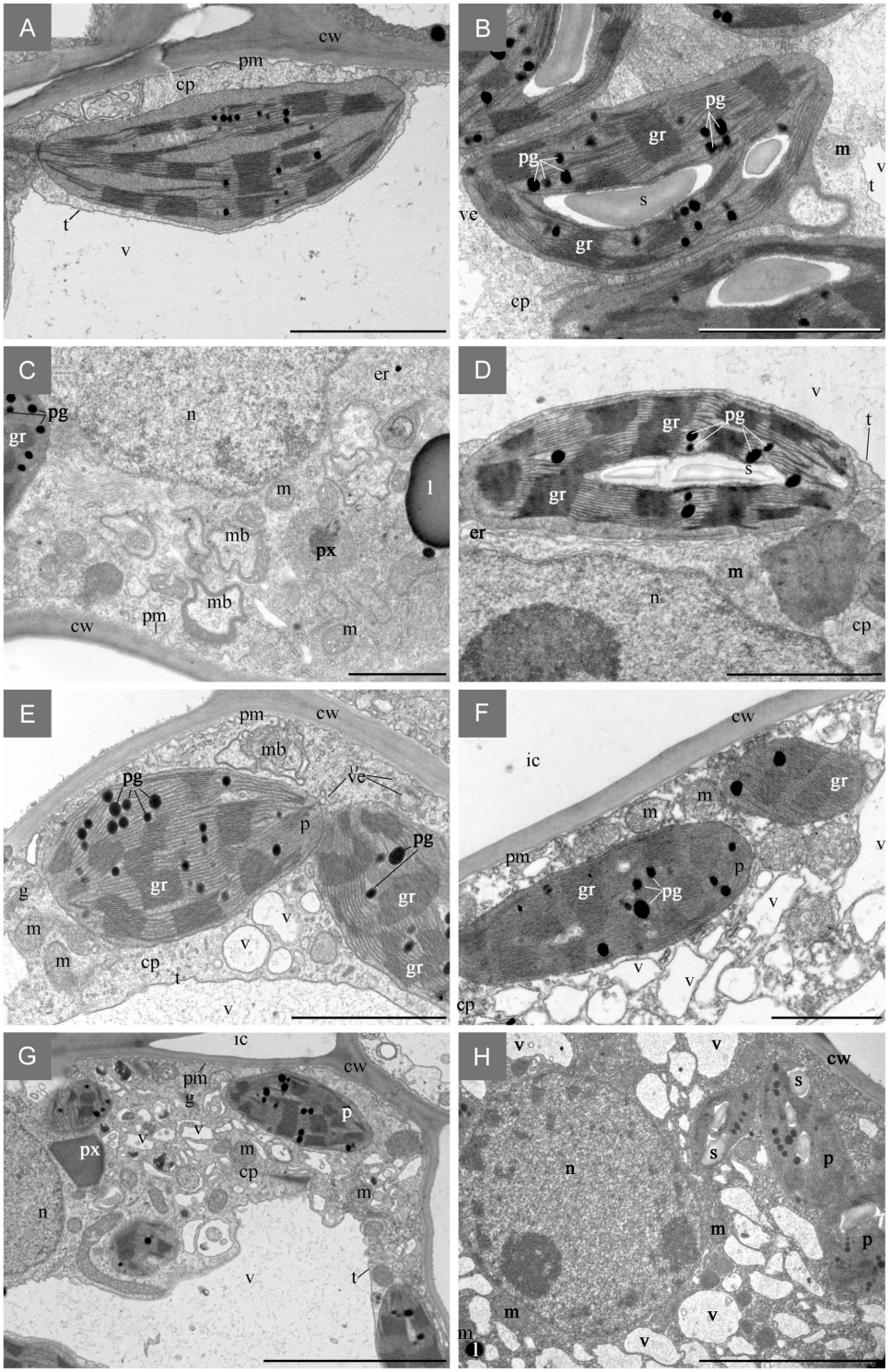

Fig. 4. Electron micrographs of mesophyll cells in *R. tinctorum* leaves. *A* - control mesophyll cell, *bar* = 2 µm. *B* and *C* - mesophyll cell 1 d ACI, *bar* = 2 µm. *D* - mesophyll cell 3 d ACI, *bar* = 2 µm. *E* - mesophyll cell 5 d ACI, *bar* = 2 µm. *F* - mesophyll cell 7 d ACI, *bar* = 2 µm. *G* - mesophyll cell 11 d ACI, *bar* = 5 µm. *H* - mesophyll cell 13 d ACI, *bar* = 5 µm (cp - cytoplasm, cw - cell wall, dm - delimiting membrane, er - endoplasmic reticulum, f - fibrillar region of cell wall, g - Golgi stack, gr - granum, i - invagination, ic - intercellular space, l - lipid droplet, m - mitochondrium, mb - myelin body, md - middle lamella, n - nucleus, p - chloroplast, pg - plastoglobuli, pm - plasma membrane, px - peroxisome, s - starch, t - tonoplast, v - vacuole, ve - vesicle).

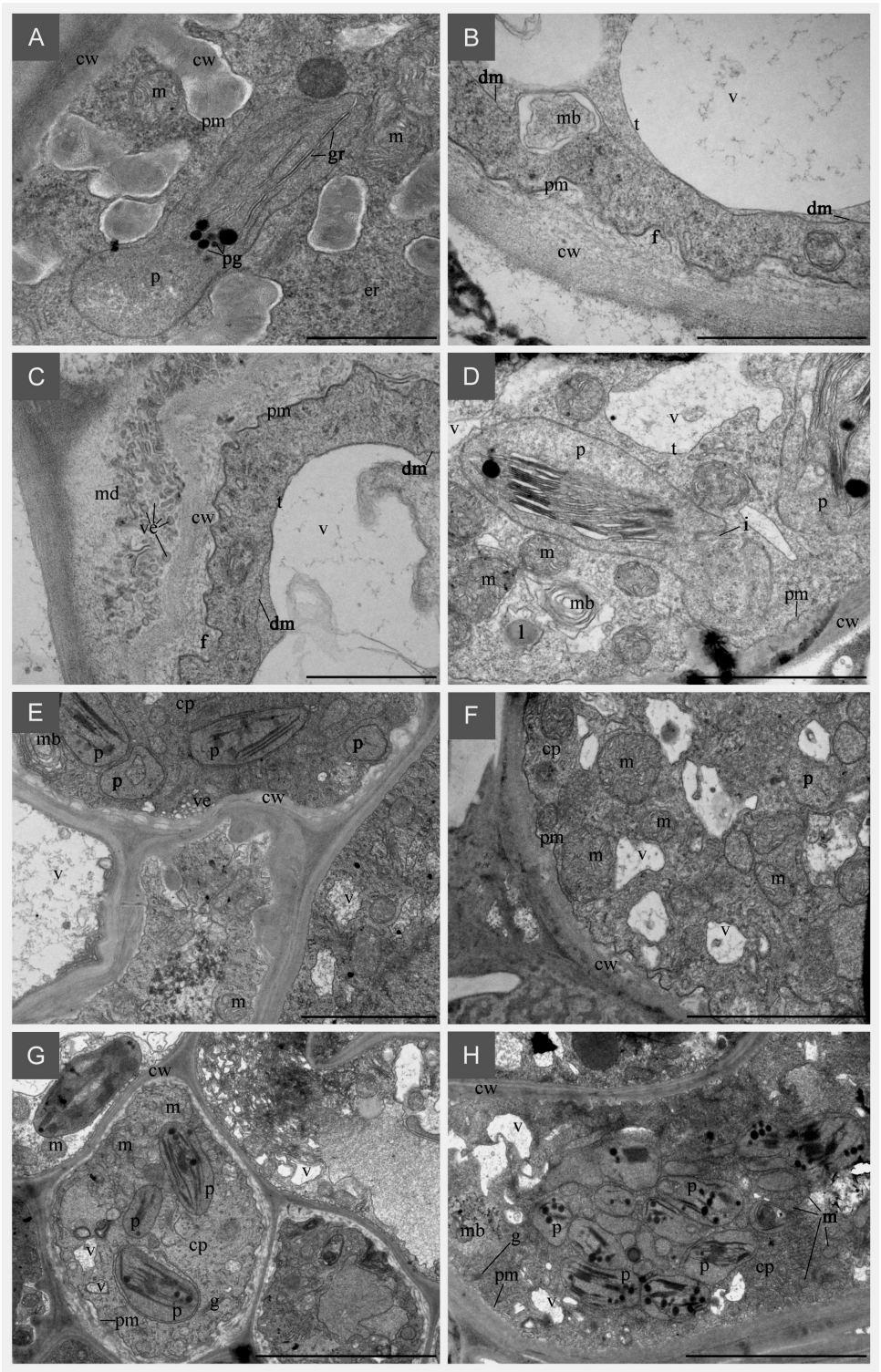

Fig. 5. Electron micrographs of transfer cells in *R. tinctorum* leaves. *A* - control transfer cell, *bar* = 1 µm. *B* and *C* - transfer cell 1 d ACI, *bar* = 1 µm. *D* - transfer cell 3 d ACI, *bar* = 2 µm. *E* - transfer cell 5 d ACI, *bar* = 2 µm. *F* - transfer cell 7 d ACI, *bar* = 2 µm. *G* - transfer cell 11 d ACI, *bar* = 5 µm. *H* - transfer cell 13 d ACI, *bar* = 5 µm. Legend see as for Fig. 4.

In the vacuoles of mesophyll cells next to vascular bundles fine precipitate was detectable after 11 - 13 d. The small cytoplasmic vacuoles arranged often around nucleus or fused together and with the extant central vacuole. Chloroplasts had well-developed thylakoids, high grana. Starch did not appear in plastids on the 11th day but it was visible in small quantities after 13 d. Number of plastoglobuli decreased, their cluster

formation left off (Fig. 4*G*,*H*). Cytoplasm of transfer cells was dense near nucleus and contained several organelles. The high amount of smooth endoplasmic reticulum, number of Golgi and numerous mitochondria and plastids various in size were characteristic. Nuclei were enlarged, often with dense caryolympha. In particular regions of the dedifferentiated transfer cells groups of small vacuoles were visible. At these areas high number of mitochondria were detectable (Fig. 5*G*,*H*).

During callus formation in the measured leaves, amounts of anthraquinones became higher, however, the derivatives did not change equally. Alizarin showed transient accumulation, its maximum was observable at 4th day. Anthraquinone was detectable in leaves only from the 8th day with a 10th day maximum, and it was not detected in the maintained callus and intact rhizome. Dramatic changes were measured in values of purpurin, lucidin, and lucidinethylether. After an early and quick accumulation, increase of them became slower around the 5th day, furthermore after the 8th day synthesis of these compounds increased again which lasted until the 10th day, and after it slow reduction was noticeable (except in purpurin values). The greatest increase was measured in lucidin concentration. The sum values of the investigated compounds showed similar two-stage accumulation (Fig. 6*A*,*B*).

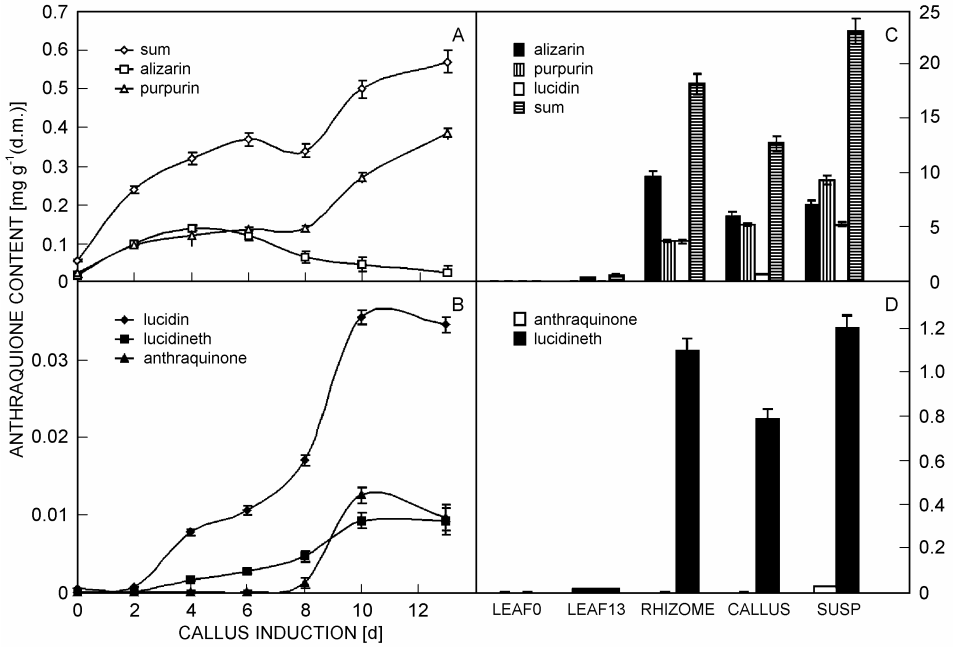

Fig. 6. Changes of anthraquinone contents in measured leaves during callus formation, and comparison of the chemical characters in different organs, callus and suspension culture: *A* - alizarin, purpurin, and sum (sum of all measured anthraquinones) during callus formation; *B* - lucidin, lucidinethylether, anthraquinone during callus formation; *C* - alizarin, purpurin, lucidin, and sum in different organs, callus and suspension culture.; *D* - anthraquinone and lucidinethylether in different organs, callus and suspension culture (Leaf0 - control leaf, leaf13 - leaf 13 d ACI, rhizome - intact rhizome, callus - maintained callus, susp - suspension culture). *Error bars* represent standard deviations (n = 3).

We compared the anthraquinone content in control and callus forming leaves, also in stable callus maintained for years, suspension culture and intact rhizome. Considerable differences can be found in the concentrations of anthraquinone derivatives in control leaves and stable callus (Fig. 6*C*,*D*). For instance, lucidin quantity in the callus is 1320-fold higher than in the leaves. Lucidinethylether was not detected in control leaves (it appears in calli). The sum amount of anthraquinones was 223-fold higher in the stable callus than in control leaves. There was also a huge contrast between the anthraquinone content of 13-d-old leaves after callus induction and maintained callus (sum value was 22-fold times elevated; alizarin value was 200-fold higher in the maintained callus). Smaller differences were determinable in the compared chemical parameters of rhizome, callus, and suspension culture (Fig. 6*C*,*D*). Total anthraquinone content was the highest in the suspension culture (2-fold higher then in maintained callus). Then again, the genotoxic lucidin content of stable callus was the lowest (5.5-fold lower than in rhizome and 8-fold lower then in suspension culture). There were no crucial differences in lucidinethylether amounts but anthraquinone was only detectable in the suspension culture. It is important to mention that rhizome contained the highest concentration of alizarin, however the purpurin had its lowest value in it.

Discussion

Occasional appearance, cryptic expression and the complex interaction of different cell types make dedifferentiation difficult to observe under natural conditions (Harikrishna *et al.* 1989). *In vitro* cultures offer a regulated and simplified model system to investigate basic events of dedifferentiation. An additional benefit of this type of experimental work is the possibility of manipulating some casual factors, which makes the observed process clearer (Preininger *et al.* 2003, Nath and Buragohain 2005). We used *R. tinctorum* in our experiments because it provides an exceptionally nice example to illustrate that origin of explants determines the cytological characteristics, organogenetic capacity and ability to cell divisions of obtained cultures. Callus and suspension cultures induced from rhizome of *R. tinctorum* have been maintained for years showing low organogenetic tendency (László *et al*. 1992, Bóka *et al.* 2002). Yellow cells contain large amount of anthraquinones and they are morphologically uniform; tracheid-like structures are almost lacking. By contrast, cultures started from *R. tinctorum* leaves differed from them fundamentally: different leaf cell types attained various dedifferentiation stages and were able to follow new developmental orientations.

Initiation of callus started at regions of the vascular bundles and wounded edges of leaves and was discernible after 10 - 14 d. Mesophyll cells of unwounded leaf-blade regions dedifferentiated to a certain level but cells could not divide. Epidermal cells were almost unchanged. The colour change of veins from greenish to yellow predicted the appearance of new cells, whereas the other parts of the leaves only lost their green colour slightly. After 3 - 5 d the photosynthetic pigment content did not altered further and accumulation of the anthraquinone derivatives began.

Based on HPLC analysis of the anthraquinone components, a two-step process of their synthesis was realized, after an early biosynthetic activity of cells a new active period has begun. The background of accumulation of anthraquinone derivatives is not fully understood, cells may not be able to open all of biosynthetic pathways in same time (Sjolund and Weier 1971). Besides, the quantity of different anthraquinone derivatives did not rise equally.

Interestingly, earlier findings showed that dedifferentiation of nuclei, chromatin decondensation and changes in density of nucleus staining took 72 - 96 h, also in two distinct steps (Harikrishna *et al.* 1989, Zhao *et al.* 2001). In line with these steps the degradation of photosynthetic pigments and ultrastructural changes were observable, as well as the first accumulation steps of anthraquinone derivatives. The early ultrastructural changes differed in mesophyll and transfer cells. These changes originated partly from the difference of specialized states of these cells, however, lytic activities and vacuolization were observable at each cell types.

In mesophyll cells, a transient starch accumulation was obvious with a maximum on the 1^{st} - 2^{nd} days, while the amount of this storage material was strongly decreased later. Higher starch content might be resulted by the high sucrose concentration of medium but similar transient starch accumulation was observed *in planta* too as a consequence of stress factors (Nyitrai *et al.* 2004).

After 3 - 5 d transfer cells were under unequal-like divisions, daughter cells were different in size and structure. Similar dedifferentiation events have been described from other species (Sjolund and Weier 1971, Harikrishna *et al.* 1989, Hou and Han 1994, Mikuła *et al.* 2004). In the control leaves, plastids of transfer cells were undifferentiated (poor inner membrane system, low number of plastoglobuli). During callus induction plastids of these cells looked to undergo unequal divisions and dedifferentiated cells contained plastids differing in size and structure. Dramatic changes detected in the cell wall were characteristic of this cell type. The inner layer of the wall tailed away, its material became fibrillar and electron transparent. Middle lamella around the transfer cells turned into vesiculated and shiny coat. These structural changes might be related with the loss of original function and the isolation of cells entering cell cycle (Zhao *et al.* 2001).

These findings are in good accordance with the earlier published results: starch appearance in chloroplasts, vesiculation of cytoplasm, more organelles, plastid division (Sjolund and Weier 1971, Nishitani *et al.* 1979, Fransz and Schel 1991, Hou and Han 1994, Mikuła *et al.* 2004). However, membrane formation, cell wall tailing away, lytic activity in the cell wall and some vesiculated regions near to middle lamella seem to be specific dedifferentiation steps in transfer cells. Intensive synthesis and secretion of hydrolytic enzymes, biochemical changes in the cell wall and unusual division of plastids indicate serious changes in the function of apoplast and symplast of transfer cells.

Further steps of dedifferentiation also differed in the two investigated cell types. In mesophyll cells, the autolytic processes progressed further, number of mitochondria increased and higher rate of smooth endoplasmic reticulum, as well as nuclei with rich caryolympha imply high levels of protein and RNA synthesis and amplified metabolic activity (Johnson 1969, Sjolund and Weier 1971, Harikrishna *et al.* 1989). In the transfer cells density of organelles increased, cytoplasm became compact, smooth endoplasmic reticulum was larger in size, while the central vacuole disappeared. The enriched cytoplasm contained plastids of various size showing features of dedifferentiating chloroplasts (Glick and Sears 1994). However, amoeboid plastids did not appear and membrane invaginations indicate protein loss of membranes (Sjolund and Weier 1971). The described dedifferentiation events prove that the majority of alterations found in transfer cells led to callus formation near to veins of leaves, while mesophyll cells seemed to stop at a particular level of dedifferentiation with higher rates of biosynthetic

activity. Aoki and Hase (1964) stated that plastid dedifferentiation might be induced by presence of high glucose level. In *R. tinctorum* leaves we have found that plastids of mesophyll cells and transfer cells behaved unlikely, however, both of them were exposed to the same sugar and hormone concentrations of the medium. It points to the fact that endogenous effectors also had important regulative role.

These findings suggest that a sort of dedifferentiation drift manifests in the different cells of *R. tinctorum* leaves after callus induction, but transfer cells could only form callus in the unwounded parts. An additional attribute of this callus was the high organogenetic capacity. Shortly after callus production roots were initiated on the callus and this feature was maintained for a longer period.

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