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Original Scientific Paper

In vitro morphogenesis and secretion of secondary metabolites of *Nicotiana tabacum* tall glandular trichomes

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ABSTRACT: The tall glandular trichomes occurring on young expanding leaves of *Nicotiana tabacum* grown *in vitro* were investigated using light and electron microscopy. Previous studies reported that the tall glandular trichomes of tobacco produce large quantities of various compounds including diterpenes. To explore the cellular structures required and putatively involved in diterpene biosynthesis and secretion, the present study focused on the development of the tall glandular trichomes, and on histochemical and ultrastructural analysis of their secretory cells under *in vitro* conditions. Electron microscopy confirmed earlier light microscopy observations of the glandular nature of the tall leaf trichomes. Their head cells, which secrete resinous material, exhibited characteristics common to gland cells: a dense cytoplasm, numerous mitochondria and little vacuolation. They contained structurally well-developed chloroplasts and an elaborate network of endoplasmic reticulum, as well as electron dense inclusions in chloroplasts and in small vacuoles. The dominance of smooth endoplasmic reticulum suggests its involvement in diterpene biosynthesis. Histochemical tests were positive for both lipophilic and hydrophilic secretions, which included terpenoids (essential oils and resins) and acidic/neutral lipids, as well as phenolic compounds (tannins), alkaloids, mucilage/pectin and polysaccharides.

Key words: glandular trichomes, histochemistry, morphology, tobacco, ultrastructure

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INTRODUCTION

Plant trichomes are uni- or multicellular epidermal appendages of diverse form, structure and functions represented by protective, supporting, and glandular hairs, which cover the surfaces of most plants (Esau 1953). They frequently function as the first line of defense against biotic and abiotic stresses by space hindrance or increased light reflectance (Wagner 1991). Glandular secreting trichomes found in some plant species are specialized structures, whose principal function(s) may be to produce pest- or pollinator-interactive chemicals that are stored or volatilized at the plant surface. Depending on the species, trichomes can produce a variety of metabolites, among which terpenoids are particularly well represented and have been used by humans in a variety of industries (Tissier *et al.* 2013).

Leaf trichomes of the plant species *Nicotiana tabacum* contribute to plant defense response against biotic and abiotic stress and also influence leaf aroma and smoke flavor. They are distinguished by their large size, high density and superior secretion ability. Tobacco leaf glandular trichomes (GT), covering the entire leaf surface throughout development, were classified by Tanaka

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(1955) into two groups: tall GTs with spindle shaped glands and short GTs with spherical glands.

Tobacco trichomes produce diterpenoids in large amounts, which renders them a platform for terpenoid biosynthesis engineering (Tissier *et al.* 2013). Head cells of glandular trichomes of *N. tabacum* are known to be the only site for the biosynthesis of certain exudate compounds including diterpenes and sucrose esters (Keene & Wagner 1985; Kandra & Wagner 1988). Croteau & Johnson (1984) suggested subcellular compartmentalization to be likely for the biosynthesis of terpene compounds. Nielsen *et al.* (1991) hypothesized that chloroplasts in *N. tabacum* trichomes are necessary for the synthesis of certain exudate compounds. This study aimed at providing data on the ontogenetical development and localization and composition of the secreted material of tall leaf GTs of tobacco plants grown *in vitro*.

MATERIAL AND METHODS

Plant material and culture conditions. *Nicotiana tabacum* L. cv. Wisconsin 38 was used as plant material. Plants were grown *in vitro* on half-strength Murashige & Skoog (1962) culture medium at 25 ± 2 °C and a 16-h photoperiod with a photon flux density of 45 μmol m^{-2} s^{-1}. Leaf samples were obtained from 9-week old plants with 9-10 leaves.

Microscopy. For light and transmission electron microscopy, leaf samples (the middle part near the leaf vascular bundle) were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) under vacuum for 1 h and left in fixative for 2 days at 4 °C. Following a 2-h wash in buffer, material was postfixed in 1% osmium tetroxide in phosphate buffer at 4 °C over 24 h. Fixed material was washed, contrasted with 0.5% uranyl acetate, dehydrated and embedded in Araldite resin CY 212 (Agar Scientific Ltd. England). For light microscopy, semithin cross sections (1-1.5 μm thick) were cut on a LKB III ultramicrotome and stained with 0.1% methylene blue in 1% borax. Sections were examined and photographed using a Zeiss Axiovert light microscope (Carl Zeiss GmbH, Göttingen, Germany). For electron microscopy, ultrathin sections (80 nm thick) were double stained with uranyl acetate and lead citrate and examined under a MORGAGNI 268 transmission electron microscope (FEI) operated at 100 kV.

Histochemical characterization. Histochemical analyses were performed on hand-sections of fresh leaves, to detect the presence of mucilage, lipids, phenolics, terpenoids and alkaloids in the actively secreting trichomes. The following histochemical tests were used: Sudan IV and Sudan Black B for total lipids (Jensen 1962); osmium tetroxide for unsaturated lipids (Jensen 1962; Gahan 1984); Nile Blue A for neutral and acidic lipids (Cain 1947); Nadi reagent for terpenes (David & Carde 1964); ferric trichloride for phenolic compounds (Gahan 1984); toluidine blue O for tannins (Baker 1966); periodic acid-Schiff (PAS) reaction to water-insoluble polysaccharides (Jensen 1962); Ruthenium Red for mucilage/pectin (Johansen 1940); Sudan Red 7B/haematoxylin for lipophilic and hydrophilic secretions, simultaneously (Liebman 1942); phloroglucinol-HCl for lignin (Gahan 1984); iodine/potassium iodide (Johansen 1940) and Wagner reagent (Wagner 1993) for alkaloids. For all the histochemical methods used, control tests were carried out following the suggestions of the respective authors. Sections were examined and photographed using a Zeiss Axiovert light microscope (Carl Zeiss GmbH, Göttingen, Germany).

RESULTS AND DISCUSSION

Morphology. The surface of a young, developing tobacco leaf (representative of the surface of over 80 sampled leaves) was covered with GTs, which differed in size and exhibited a variety of structural complexity. The tall GTs had multicellular stalks and uni- (Fig. 1A) or multicellular (Fig. 1C-E) glands. Occasionally branched hairs, having a gland at each tip, were observed (Fig. 1B).

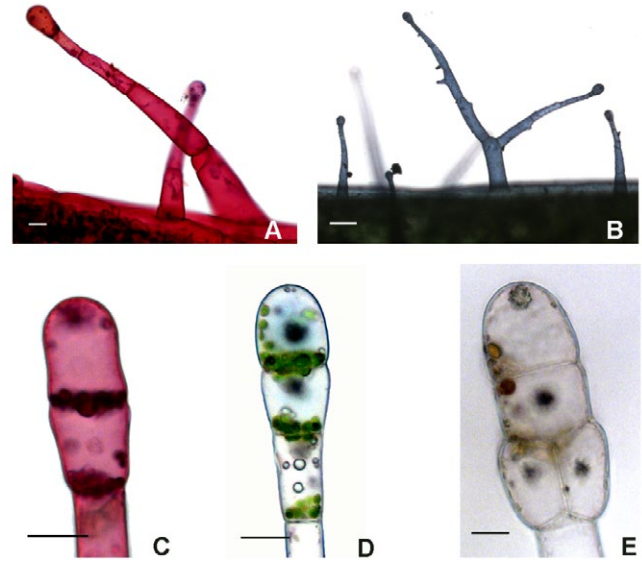

Figure 1. Morphology of tall glandular trichomes on the leaves of *in vitro* grown *Nicotiana tabacum* plants. (**A**) Glandular trichome having four stalk cells and unicellular head. (**B**) Branched glandular trichome with unicellular gland at each tip. Note that cell walls of both stalk and head cells and the secretion are stained dark blue to black with Sudan Black B. (**C**) Tip of the glandular trichome with two-celled head, stained bright pink with PAS. (**D**) Tip of the glandular trichome with three-celled head. The head cells contain bright green chloroplasts and show a faint blue-green color in the vacuolar space after staining with Toluidine Blue O. (**E**) Tip of the glandular trichome with four-celled head. Note crystals of calcium oxalate in the apical cell and orange-to-brown droplets in both apical and subapical gland cells, after staining with Sudan red 7B/haematoxylin. *Scale bars:* 100 μm (B), 10 μm (A, C-E).

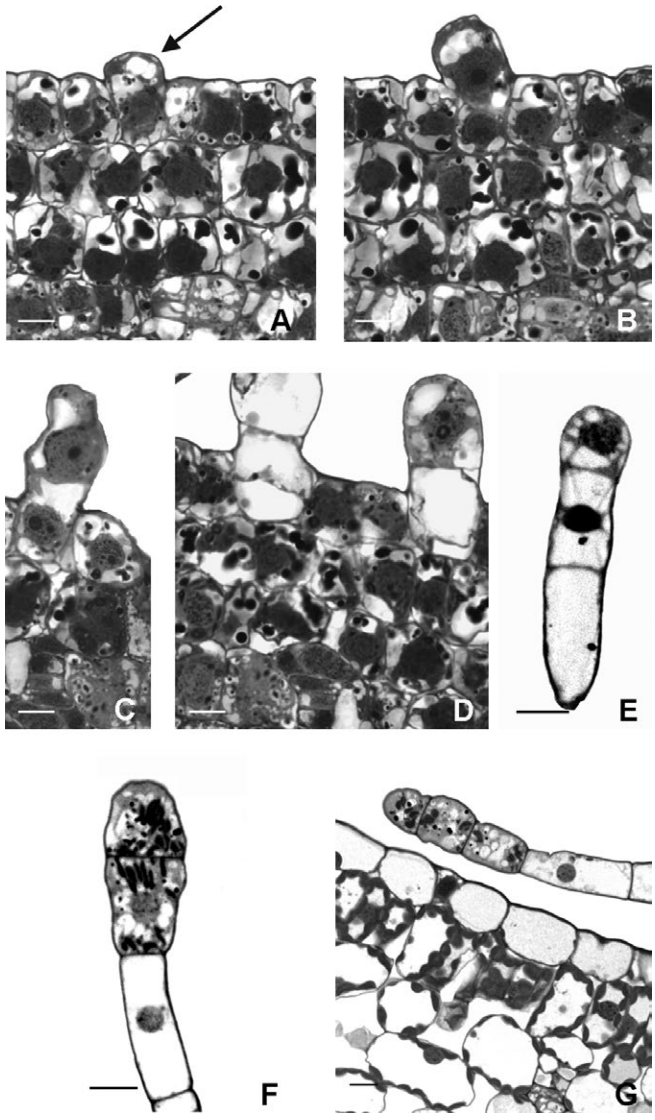

Figure 2. (**A-E**) Early development of tobacco glandular trichome. (**A**) expansion of a single epidermal cell (*arrow*) in a juvenile leaf, (**B**) early two-celled stage after the first periclinal division, (**C, D**) glandular trichome initials consisting of a single vacuolated basal cell and a single cytoplasmically dense apical cell with large nucleus and nucleolus, (**E**) three-celled stage in trichome development (note centrally positioned nucleus in elongated and vacuolated terminal stalk cell). (**F-G**) Tips of glandular trichomes at later developmental stages, with two-celled (**F**) and three-celled (**G**) heads, which are already in the secretory phase. *Scale bars:* 16 μm (A-D), 10 μm (E-G).

Tall GTs developed rapidly on leaf primordia and fully developed forms could be observed already on young, not fully-differentiated leaves (Fig. 2G). Fully-developed tall GTs consisted of a one- to four-celled glandular head, subtended by a stalk of variable length. Adjacent to mature, elongated trichomes with at least six stalk cells, numerous smaller glandular trichomes, with two to four stalk cells could also be seen. The stalk was made up of elongated cells, the length of which gradually decreased from the base upward, with a one- to four-celled head atop a terminal stalk cell (Fig. 1A, B). All stalk cells had centrally positioned nuclei, large vacuoles and a number of plastids at the cell periphery. The basal cell differed from surrounding epidermal cells by the presence of only a few plastids (not shown).

Trichome development. Short and tall GTs of tobacco cannot be easily distinguished at their inception, as they are both first discernible as protruding protodermal cells with an asymmetrical cytoplasmic distribution. Young trichomes could be found next to well-developed trichomes, suggesting that trichome development was greatly asynchronous. The earliest stage of GT development was found on the juvenile tobacco leaf, where the expansion of a single protodermal cell was observed (Fig. 2A). The trichome initial had a large, centrally-positioned nucleus with prominent nucleolus, dense cytoplasm and a number of small vacuoles. Trichomes that were slightly older were partitioned by periclinal cell division, separating an apical initial and a basal cell (Fig. 2B). A single, more meristematic, spherical or ellipsoidal apical gland cell that would ultimately give rise to GT head cell(s) was observed atop a single, more vacuolated, cylindrical basal cell (Fig. 2C, D). As a result of periclinal divisions, the stalk could become multicellular (Fig. 2E). The apical cell could remain unicellular, or give rise to a multicellular head as a result of periclinal (Fig. 2F, G) or rarely anticlinal cell divisions (Fig. 1E).

Ultrastructure of the tall GT secretory cells. In the presecretory stage, head cells of the tall GTs had a dense cytoplasm, large nuclei with prominent nucleoli and small vacuoles (Fig. 3A). The plastids contained scarce tubular membrane elements, a few starch grains and large globular electron dense intraplastidial bodies, bounded by a single membrane (Fig. 3B). Densely stained globular inclusions were reported in some of the chloroplasts of tobacco cultivar Tobacco Introduction 1068 (Nielsen *et al.* 1991). Similar inclusions in Xanthi tobacco were suggested to contain lipoidal substances (Akers *et al.* 1978). These inclusions could be thylakoid bodies involved in the formation of the lamellae in the developing chloroplasts (Hurkman & Kennedy 1977), but there is no evidence so far that these inclusions are related to the secretory product.

In the secretory stage, numerous mitochondria, ovoid or ellipsoidal in shape and with many well-developed cristae, were found in the parietal cytoplasm (Fig. 3C). In less electron-dense cytoplasm, a network of tubular smooth endoplasmic reticulum and, in some regions, short cisternae of rough endoplasmic reticulum were present (Fig. 3D). In addition, ER profiles were also observed surrounding plastids and mitochondria (Fig. 3C-E). The dominant component of the secretory cells was an elaborate network of smooth endoplasmic reticulum

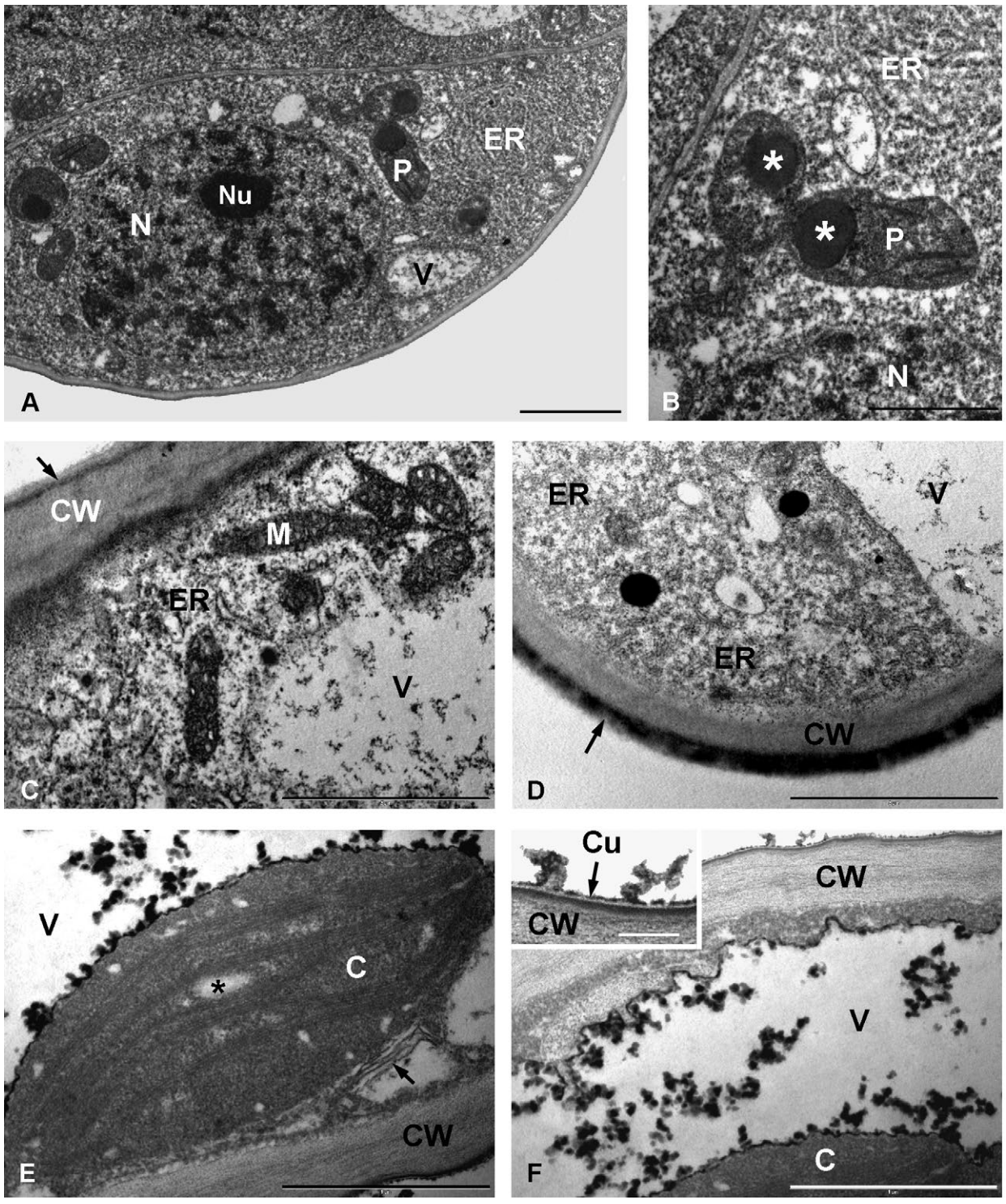

Figure 3. Transmission electron micrographs of tall glandular trichomes of *Nicotiana tabacum* leaves. (**A**) Trichome head cell in the presecretory stage. (**B**) Higher magnification of presecretory-stage head cell showing elaborate network of endoplasmic reticulum (ER), and plastids with poorly-developed lamellar system and large electron-dense globular inclusions (*asterisks*). (**C**) Head cell in the secretory stage, containing numerous mitochondria in the parietal cytoplasm. Note an electron dense layer at the inner side of the cuticle (*arrow*). (**D**) Elaborate network of ER, mitochondria and large globular electron dense inclusions in the cytoplasm. Note remarkably thick electron dense layer (*arrow*) on the outer side of the cell wall. (**E**) Chloroplast with well-developed lamellar system and a few small starch grains (*asterisk*), in close association with ER (*arrow*). (**F**) Large vacuole with granular, extremely osmiophilic content. Inset: electron dense fibrous material released from the outer side of the cuticle. N, nucleus; Nu, nucleolus; V, vacuole; P, plastid; ER, endoplasmic reticulum; M, mitochondrion; CW, cell wall; C, chloroplast; Cu, cuticle. *Scale bars:* 2 μm (A, C, D), 1 μm (B, E, F), 0.1 μm (inset in F).

composed of tubular and cisternal elements that were dispersed throughout the cytoplasm. Generally, high-level exudate accumulators produce di-, tri- and sesquiterpenes as major products. Ultrastructural features that are common in terpene secreting glands are: extended smooth ER; leucoplasts with poorly-defined internal membranes, or normal or sometimes unusually-shaped but otherwise normal-appearing chloroplasts; an association of ER and plastids; and the relative absence of Golgi (Dell & McComb 1978). Most evidence indicates that synthesis of the terpene precursor isopentenyl pyrophosphate occurs in the cytosol. This intermediate then appears to be utilized by plastids or plastid-ER aggregates to synthesize secreted products (Gershenzon & Croteau 1990), and this was confirmed by histochemical analysis (Fig. 4E).

Densely-stained material of variable sizes was evident in numerous vesicles and in the cytoplasm of the secreting glandular cells (Fig. 3D), but there was no evidence to link this material to any secretory product. Akers *et al.* (1978) and Nielsen *et al.* (1991) did not observe secretory product in intercellular regions or external to the plasmalemma in the tall glandular trichomes of tobacco. However, these authors argued that the presence of exudates on tall trichomes and the relative lack of exudate on short trichomes supports the conclusion that the head cells of tall trichomes are involved in the demonstrated biosynthesis of exudate compounds (Keene & Wagner 1985; Kandra & Wagner 1988). Nielsen *et al.* (1991) speculated that secretory products did not accumulate within the head cells at a level sufficient to allow observation using standard microscopy techniques, or that these accumulations, along with the exudate, were removed during tissue processing.

The trichome chloroplasts, which sometimes contained small starch grains, had a well-developed internal membrane system with numerous grana (Fig. 3E). Well-developed chloroplasts with normal granal stacking and stromal development were also found in glandular trichomes of *N. tabacum* cv. Xanthi and Tobacco Introduction 1068 (Akers *et al.* 1978; Nielsen *et al.* 1991). Similarly to what Bentley & Wolf (1945) observed in the Oriental tobacco cultivar Xanthi, these authors reported the lack of starch grains in chloroplasts of the trichome head cells. Akers *et al.* (1978) did not observe starch, but reported electron-dense globular inclusions in the chloroplasts of the head of the tall trichome. Electron-dense inclusions were also observed in the chloroplasts and in small vacuoles of the head cells of the tall trichomes (Meyberg *et al.* 1991; Nielsen *et al.* 1991). However, in our study large electron-dense globules were observed only in the cytoplasm and in the plastids with a poorly-developed lamellar system, whereas fully-developed chloroplasts contained very few small electron-dense globules.

Although head cells of glandular trichomes in most plants lack photosynthetic capability (Dell & McComb 1978; Croteau & Johnson 1984), Keene & Wagner (1985) described the green appearance of the glandular trichomes of the *N. tabacum* genotype that they studied. Bright green chloroplasts in secreting cells of certain *Nicotiana* species that are high level accumulators (~5-30% dry weight of leaves) suggest a possible relationship between their capacity to accumulate exudate and their photosynthetic capacity to fix carbon and/or to produce ATP and NADPH (Keene & Wagner 1985; Wagner 1991).

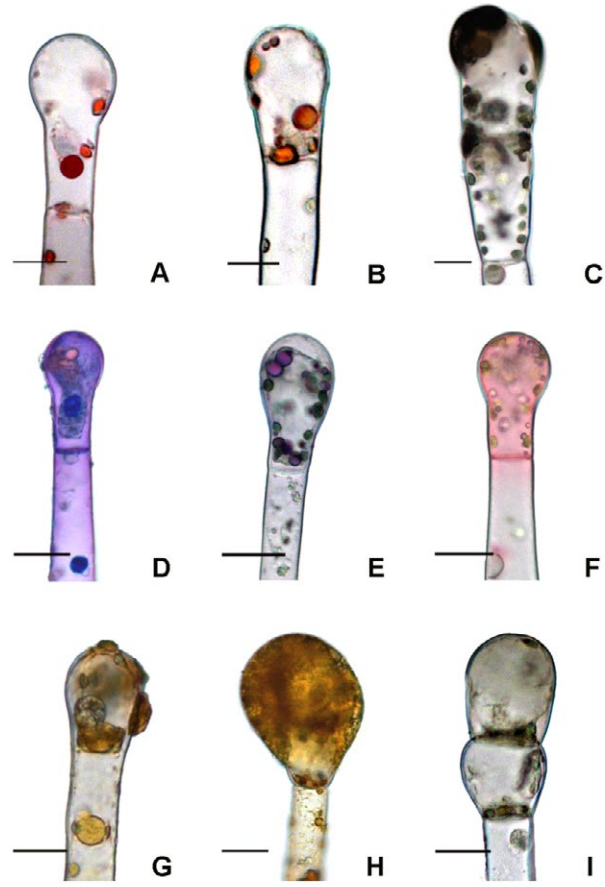

Figure 4. Histochemical characterization of the *Nicotiana tabacum* tall glandular trichome secretion. (**A**) Red-to-brownish staining of lipophilic secretory droplets and plastids in the head cell (Sudan red 7B/haematoxylin). (**B**) Lipophilic droplets and plastids stained bright orange with Sudan IV. (**C**) OsO_4 test showing brown-to-black staining of unsaturated lipids in plastids and droplets within the head cell, and in secreted exudate adhering to the outer surface of the head cell. (**D**) Neutral lipids (nucleus surface and material dispersed within the head cell) stained blue, and acidic lipids (secretory droplets) stained red with Nile Blue A. (**E**) Purple droplets of various sizes observed after staining with Nadi reagent. (**F**) Head cell positive for carbohydrates other than cellulose (Ruthenium red). Note particularly intense staining of the cross cell wall between the head cell and terminal stalk cell. (**G, H**) Tall glandular trichome positive for alkaloids (brown-yellow staining), using Wagner reagent (**G**) and iodine solution (**H**). (**I**) Phenolic compounds in the vacuole stained greenish with $FeCl_3$. *Scale bars:* 10 μm.

At this stage, the vacuoles increased in size and contained phenolic material occurring as net-like deposits and globular masses (Fig. 3F). Dark, granular, extremely osmiophilic content found in the vacuoles of *Teucrium polium* leaf cells (Christodoulakis *et al.* 2010) are condensed tannins that are commonly found in xerophytes. Nielsen *et al.* (1991) observed the large void in the vacuole of tobacco trichome head cells, which was probably the site of a calcium oxalate crystal that was removed during tissue preparation. The inner side of the cuticle was more electron-dense than the outer part (Fig. 3C-D). Krüger *et al.* (1996) found that *N. tabacum* leaf trichomes exhibit a typical bilayered cuticular membrane, composed of a thin outer cuticle proper and a thicker cuticular layer, where the cuticle proper consisted of no more than three to four lamellae. Their results contradicted the classification by Akers *et al.* (1978) of tobacco trichome cuticular membranes as being reticulate in all regions. From the outer side of the cuticle, electron dense fibrous material was released (Fig. 3F). The secreted material, passing through the wall, was possibly released via cuticular micropores

Histochemistry. Glandular heads of the tall GTs were covered with a sticky exudate or secretion (Fig. 4C, H). As short GTs do not appear to accumulate exudate on the surface of the gland, the tall GTs were suggested to be the ones that are involved in biosynthesis of the leaf surface compounds, duvatrienediols and sucrose esters, which affect the host plant resistance to disease and have an impact on organoleptic properties (Keene & Wagner 1985; Kandra & Wagner 1988; Nielsen *et al.* 1991). Nielsen *et al.* (1991) observed no exudate globules on the stalk cells, concluding that a sticky exudate or secretion was apparently restricted to the glandular head of tall GTs. However, they speculated that although not visible, the exudate may be contained by the cuticle as proposed by Bentley & Wolf (1945), or possibly by a layer of oxidized secretion products.

Results of the histochemical tests carried out to characterize the main classes of compounds secreted by the tall GTs of *Nicotiana tabacum* grown *in vitro* are shown in Figure 4. Tall GTs stained positively for lipophilic compounds, as revealed by Sudan Black B (Fig. 1B), Sudan Red 7B/haematoxylin, Sudan IV and OsO_4 staining (Fig. 4A-C). The positive reactions in non-specific tests using Sudan Black B and OsO_4 can be attributed to long chain lipids, aliphatic hydrocarbons with ester or ketonic functions, triglycerides, steroids and free fatty acids (Pearse 1968). As evidenced by Nile Blue A staining, both neutral and acidic lipids were present within the head cells, while the lipid droplets were also found on the outer surface of the cuticle (Fig. 4D). Purple droplets observed within head cells after Nadi reaction demonstrated the presence of both essential oils and resins, which were also present in the halo around chloroplasts; trichome exudate on the lateral gland cell walls also stained positive (Fig. 4E). The leaf surface gum in *N. tabacum* has been shown to contain cembratrienediol (Heemann *et al.* 1983). It is suggested that this diterpene is produced and secreted by the tall trichomes, which secrete a clear resinous material, as short trichomes do not exhibit resinous material on their surfaces (Meyberg *et al.* 1991). Tall GTs stained positively for water-insoluble polysaccharides, as revealed by the PAS reaction (Fig. 1C). Staining with Ruthenium Red indicated that the head cells of tall trichomes secrete copious amounts of mucilaginous polysaccharides (Fig. 4F), but no lignification was observed after staining with phloroglucinol-HCl (not shown). The histochemical test for alkaloids gave positive results using Wagner reagent and I/KI staining (Fig. 4G, H). Alkaloids were abundant in trichome head cells as shown by the intense yellow-brown color of both the head cell and its exudate. Vacuoles of the trichome head cells gave a positive reaction for phenolic compounds (Fig. 4I). In addition, trichome head cell vacuoles stained positively for tannins (polyphenols), as evidenced by Toluidine Blue O staining (Fig. 1D).

The generally weak staining that we observed in different histochemical tests could be accounted for by lower production of secondary metabolites under *in vitro* conditions (Gurel *et al.* 2011; Rosa & Dornelas 2012). The plants were grown on a culture medium containing lower levels of total nitrogen, which in some cases was shown to increase secondary metabolite production (Coste *et al.* 2011). However, the molar ratio of NH_4^+/NO_3^- in half-strength MS medium did not favor an increase in secondary metabolite production. Moreover, *in vitro* conditions with higher relative humidity, lower light levels and aseptic environments may account for the synthesis of different amounts of monoterpenes (Juliani *et al.* 1999). Nevertheless, plants grown *in vitro* are continuously exposed to a defined microenvironment, providing nearly optimal conditions for plant proliferation, in contrast to marked variations of the growth conditions of field-grown plants.

CONCLUSION

The tall glandular trichomes of *in vitro* grown aromatic tobacco *Nicotiana tabacum* were analyzed using light and transmission electron microscopy. The reaction of numerous hydrophobic droplets to various histochemical stains indicated that they could be a mixture of lipids, phenolics and alkaloids. Granular, extremely osmiophilic material, dispersed within the vacuole, appeared to be condensed tannins. During the secretory phase, the ultrastructure of the head cells was characterized by highly-developed endoplasmic reticulum, numerous mitochondria and well developed chloroplasts, cellular compartments involved in the synthesis and transport of terpenoid secretion. Ultrastructural studies did not reveal the formation of a subcuticular space by cuticle detachment. In the absence of cuticular rupture, the release of secretion

probably occurred through cuticular micropores. Studies on the secretory pathway of different secondary metabolites, including terpenoids, histochemical and cytological analyses of leaf glandular trichomes of plants grown under controlled *in vitro* conditions can provide valuable information on compartmentalization of the secretion process.

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REZIME

In vitro morfogeneza i sekrecija sekundarnih matabolita iz visokih žlezdanih trihoma duvana

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Duge žlezdane trihome mladih listova biljaka *Nicotiana tabacum* gajenih *in vitro* analizirane su na nivou svetlosne i transmisione elektronske mikroskopije. U prethodnim istraživanjima pokazano je da duge žlezdane trihome produkuju različita jedinjenja uključujući i diterpene. U cilju karakterizacije sekretorne aktivnosti dugih žlezdanih trihoma *in vitro*, a posebno struktura uključenih u sintezu, transport i sekreciju diterpena, praćeno je razviće dugih trihoma kao i histohemijske i ultrastrukturne karakteristike njihovih sekretornih ćelija. Na nivou elektronske mikroskopije pokazano je da sekretorne ćelije dugih trihoma karakterišu gusta citoplazma, brojne mitohondrije i male vakuole. U citoplazmi sekretornih ćelija uočena je razgranata mreža cisterni endoplazmatičnog retikuluma, kao i tamne globularne strukture, koje su takođe zapažene i u hloroplastima i vakuolama. Visoka zastupljenost glatkog endoplazmatičnog retikuluma ukazuje da ova organela ima ulogu u biosintezi i/ili transportu diterpena. Histohemijskim testovima potvrđeno je prisustvo lipofilnih i hidrofilnih supstanci, uključujući terpenoide (etarska ulja i smole), kisele/neutralne lipide, kao i fenolna jedinjenja (tanine), alkaloide, mucilageni materijal/pektine i polisaharide.

Ključne reči: duvan, histohemija, morfologija, ultrastruktura, žlezdane trihome