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## **Review**

# **Secretion mechanisms of volatile organic compounds in specialized cells of aromatic plants**

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### ***Abstract***

The present review focuses on cells secreting volatile odorant compounds. This cell type is found in a wide variety of plants, grouped under the term aromatic plants. Such secreting cells are very diverse in morphology, from highly specialized trichomes to non-specialized cells, including the secretory epidermal cells of petals and osmophores. In these various types of cell, the biosynthetic pathways of three main groups of volatile organic compounds are recognized: isoprenoids, fatty acid derivatives and aromatic compounds. The precise cellular localization of these pathways has not yet been elucidated in all cases, though many of the enzymes involved have already been cloned. These have been found to be frequently located in plastids but also in endoplasmic reticulum or even cytosol. Two alternative mechanisms of secretion termed granulocrine and eccrine have been postulated to exist. Recent studies support the fact that both mechanisms could exist for different compounds and different plants. This review will discuss also the route by which secreted molecules make their way through the cell wall and cuticle.

## ***Introduction***

Aromatic plants are always confused with medicinal plants because they secrete chemicals which sometimes have pharmacological effects. They include all the plants which produce odoriferous secondary metabolites or medicinal active compounds. Some of them are sometimes used as herbs because of their culinary properties. From an horticultural point of view, aromatic plants are often considered as plants which secrete odors by, at least, one vegetative tissue, often leaves but also roots or stems. However, in an ecological perspective, plants which produce scents by flowers can be included in aromatic plants because both produce volatile organic compounds (VOCs). These compounds are generally used as attractants for species-specific pollinators or to protect plants by repulsing herbivores and pathogens.

VOCs are organic molecules that have a high vapor pressure or a high volatility, i.e. they form vapors at normal pressure and temperature. In aromatic and scented plants, they originate from three categories of chemicals (Figure 1): phenolic compounds, fatty acid derivatives and isoprenoids [reviewed in 1,2,3,4,5]. Phenolic compounds, also named aromatic compounds or benzenoids, come from tryptophan, phenylalanine and tyrosine. Examples of these include: methylbenzoate, methylsalicylate, benzylacetate, methyleugenol, benzylbenzoate... The second category of VOCs, fatty acid derivatives, are often associated with the green leaf odor emitted immediately following the breakdown and lipoxygenation of lipid membranes (e.g. linolenic acid) after mechanical damage. However, these green leaf volatiles are sometimes also produced by flowers. The most famous among them is the phytohormone jasmonic acid involved itself in induction of other VOCs after insect or fungi injuries. The third category

of VOCs, isoprenoids, also termed terpenes, comes from the mevalonic acid pathway or from the 2-C-methyl-D-erythritol-4-phosphate pathway. These two pathways synthesize isopentenyl diphosphate, but the first one is located in the cytosol and the second one in plastids. Only carotenoid metabolites, monoterpenes and some other terpene derivatives are volatile e.g. limonene, menthol, linalool, caryophyllene, damascenone, and beta-ionone etc... Such benzenoids, fatty acids derivatives and terpenes can be found in flowers or leaves of different species leading to the hypothesis that the cellular secretion pathway(s) could be the same in both these types of organ. According to evolutionary theories of secondary metabolite biosynthesis [6] and plant-insect interactions [7,8], flower scents and leaf odors could have evolved from the same metabolite pathways by co-adaptation [9].

In plants, the main role of scented VOCs is clearly an ecological interaction with insects or other animals, even if some other roles are known [e.g. 10,11,12]. Examples exist of messages with benefits only for the receiver (kairomones), only for the transmitter (allomones) or for both (synomones). For example, kairomones can be involved in parasitism, allomones in plant defense and synomones in pollination. Insects and animals can associate a particular bouquet of VOCs with the presence or absence of food, with an anti-feeding product, with a toxic molecule, with a source of nectar or with a reproduction site [4,5,7,8,13]. It is not yet known how plants produce and secrete these chemical messages.

Since the beginning of microscopic studies, numerous aromatic and scented plants have been investigated at the cytological level. These studies are too numerous to be described in this review and only some of them are cited as examples here [but see 14,15,16 and references therein]. Surprisingly,

organelles involved in biosynthesis and especially in secretion are poorly known. For example, one question to be answered concerns the possibility for chemicals to be transported directly through the plasma membrane (eccrine secretion) or by specific vesicles of exocytosis (granulocrine secretion). Evidence relating to this question is often contradictory and the existence of both mechanisms could be an attractive hypothesis. The use of molecular biology, confocal microscopy and gas chromatography coupled to mass spectrometry has enabled the clarification of some secretion pathways. This review will focus on recent work on elements of secretion mechanisms of scented VOCs at the ultrastructural level. Here, the term “secretion” is used in its broad sense, though when secondary by-products are non-utilizable or harmful for the organism that secretes them, the exact term is “excretion” [17]. So, when necessary, lessons will be taken from cytological works on excretion of non-volatile secondary metabolites. Indeed, these studies often describe new organelles, vesicles or pathways that could be excellent candidates to also explain secretion mechanisms of VOCs.

### ***Diversity of VOCs secreting cells in aromatic plants***

Secreting cells are classified according to their shape or according to the chemicals they secrete [15,16] e.g. mineral salts and water, polysaccharides, essential oils etc... VOCs are very often lipophilic and consequently are secreted in essential oils, even if some of them, like carotenoid derivatives or phenylethanol for example, are also water soluble. They may be secreted by osmophores, conical-papillate cells, glandular trichomes, ducts, cavities and, occasionally by non-specialized cells.

In some flowers, specialized clusters of cells, named osmophores, are distributed on sepals and petals in a way that seems to attract insect pollinators; this

is the case in the *Orchidaceae* for example [18,19,20,21]. Osmophore cells look like the conical-papillate cells that can be found on the whole epidermis of lot of petals (Figure 2) in more than 200 species [22], and even in the model-plant *Arabidopsis thaliana* (*Brassicaceae*) [23,24]. This conical-papillate shape is known to offer a very high surface of evaporation and to participate in the reflection of light. The *MIXTA* gene, giving rise to the conical shape, has been cloned in *Antirrhinum majus* (*Scrophulariaceae*). Surprisingly, its overexpression in *35S::MIXTA Nicotiana tabacum* (*Solanaceae*) leads to ectopic secreting trichomes on the whole plant suggesting a relationship between conical-papillate cells and the differentiation of secreting trichomes [25].

Secreting trichomes are very numerous and have very different morphologies in the plant kingdom (Figure 3). They occur in a high number of families but most of them can be found, for example, in *Lamiaceae* [26,27,28,29,30,31], *Solanaceae* [32,33], *Cannabaceae* [34] and *Rosaceae*. Indeed, in these families, secreting trichomes may exhibit short or long-stalks, unicellular or pluricellular stalks and heads, branched or non-branched stalks, peltate or capitate heads, and may vary in their capacity for the storage of essential oils between the cell wall and the cuticle. Together with VOCs, secreting trichomes often excrete gum, resin, paste or glue. This is also the case in other types of secreting cells like ducts and cavities. For example, in conifers, diterpenoid resin acids are present in ducts, dissolved in volatile turpentine. Upon injury, the turpentine evaporates and the resin forms a crystalline mass that may trap pathogens [13]. Ducts and cavities are also present in *Apiaceae* [35], *Compositae* [36] or *Rutaceae* [37,38] for example.

In these studies, ducts, cavities, secreting trichomes, conical-papillate cells and other VOCs secretory tissues usually contain small vacuoles, a dense cytoplasm and numerous mitochondria. Leucoplasts,

plastoglobules and unusual figures of reticulum or dictyosomes, like periplastidal reticulum, smooth tubular reticulum, myelin-like lomasoma and osmiophilic vesicles or cisternae for example, are also sometimes observed.

Surprisingly, numerous plants emit VOCs by non specialized cells. Indeed, in the *Brassicaceae* for example, there are no specialized secretory tissues. Nevertheless, it was shown that volatile monoterpenoids and sesquiterpenoids are emitted from the green leaves of these plants directly or after injury [39,40,41]. These VOCs could be emitted for the defense of the plant. On the contrary, in *Chamaerops humilis* (*Arecaceae*), it was recently demonstrated that pollinators are attracted by terpenoids and benzenoids emitted by leaves [42,43]. Despite these secretion pathways in non-specialized cells, most of the microscopic studies of essential oil secretion were made on specialized cells [e.g. 14,15,16,17].

### ***Localization of VOCs biosynthesis pathways***

In an attempt to understand how VOCs are secreted, it is important to find where they are synthesized in the cell. In microscopic studies, essential oils production was often attributed to plastids because of the observation of oil droplets or plastoglobules in the stroma [e.g. 28,35,36,37,44,45]. This is in accordance with some cellular fractionation experiments and with the immunolocalization of isopentenyl pyrophosphate isomerase, geranyl diphosphate synthase, limonene cyclase and of the enzymes of the 2-C-methyl-D-erythritol-4-phosphate pathway, all involved in the beginning of the monoterpene biosynthesis [38,46,47,48,49,50]. However, there are conflicting reports in the literature concerning the cellular and intracellular sites of geranyl diphosphate synthase in plant cells. Some data suggest the existence of two forms of this enzyme

depending on the methionine used to initiate translation. The larger one could be targeted to plastids and the truncated one could be cytosolic. It is not known yet how these geranyl diphosphate synthase isoforms could operate during isoprenoids metabolism in plants [41].

The next step of terpene biosynthesis involves terpene cyclases or synthases. All the known monoterpene cyclases and synthases seem to possess plastid-targeting sequences [51]. The final steps of monoterpene biosynthesis are known to be located in the endoplasmic reticulum, as demonstrated by the localization of limonene hydroxylases for example [52,53]. Nevertheless, recent data seem to show that the very last steps of some monoterpene biosynthesis pathways could be cytosolic [30]. Thus, the hypothetical pathway of biosynthesis would begin in plastids or in the cytosol up to the very first cyclic or acyclic monoterpenes. These monoterpenes would then pass into the endoplasmic reticulum by an unknown process. This transfer of terpenes between compartments has been hypothesized from the results of cytological studies showing osmiophilic globules between plastids and periplastidal reticulum, sometimes with a continuous membrane network [e.g. 18,30,36,44,54,55,56]. Finally, in some cases, the last steps of monoterpene biosynthesis could occur in the cytosol.

Recently, in the liverwort *Marchantia polymorpha* (*Bryophyta*), a new terpene biosynthesis pathway was demonstrated in oil bodies [57]. This pathway could be specific of liverworts, but it demonstrates that different compartmentations of the same pathways can exist in nature.

The first steps of sesquiterpene biosynthesis seem to be cytosolic, depending on the mevalonic acid pathway [49,58]. Nevertheless, in *Matricaria recutita* (*Asteraceae*), it was demonstrated that the very first steps, before the biosynthesis of the isopentenyl

diphosphate, occur partially in plastids, from the 2-C-methyl-D-erythritol-4-phosphate pathway [59]. It is important to know whether the isopentenyl diphosphate, synthesized both by the cytosolic and the plastid pathways, passes through the plastid envelope, to be used inside or outside this organelle. The answer could depend on the species, on the tissue and the developmental stage. Irrespective of the answer to this question, the last steps of sesquiterpene biosynthesis seem to occur in the endoplasmic reticulum [60,61]. Sesquiterpenes should then share the same subcellular compartment with some monoterpenes.

Few data are available concerning secretion process of carotenoid derivatives, fatty acid derivatives and benzenoids. Concerning carotenoid derivatives, the enzyme involved in the plastidal synthesis of water-soluble carotenoids derivatives, responsible for the colour and aroma of saffron have recently been cloned in *Crocus sativus* (*Iridaceae*) [62]. Cytological data suggest that there is a transfer of secretory inclusions containing these secondary metabolites from plastids, where they are synthesized, to vacuoles, where they could be sequestered. In *Rosa x hybrida* (*Rosaceae*), several genes encoding carotenoid dioxygenases have been cloned [63]. One of them is targeted to plastids and could be involved in biosynthesis of rose cetones.

Fatty acid derivatives are generated by the activity of lipoxygenases. The intracellular localization of this pathway depends on the lipoxygenase form involved. They have been located in microsomal fractions, plasma membrane, plastid stroma or envelope and lipid bodies depending on the species, tissues and chemicals studied [reviewed in 64], but the localization of enzymes involved in volatile fatty acids biosynthesis is not well known. Concerning jasmonate, the biosynthesis pathway have been located in plastids and peroxisomes [reviewed in 65].

Benzenoids localization has been less studied than that of fatty acids. In *Antirrhinum majus* petals, *S*-adenosyl-L-methionine:benzoic acid carboxyl methyltransferase, leading to methylbenzoate, has been located in the cytosol of conical-papillate cells [66]. Recently, genes encoding *O*-methyltransferases have been cloned in *Rosa chinensis* (*Rosaceae*) [67]. Fusions of this protein with the reporter Green Fluorescent Protein are targeted to unknown compartments in rose petals [63]. The existence of such different biosynthesis pathways suggest that more than one secreting modality of VOCs could exist.

### ***Granulocrine versus eccrine secretion of VOCs***

In granulocrine mechanisms of secretion, vesicles of reverse pinocytosis directly fuse with the plasma membrane or are surrounded and detached from the cytoplasm by invaginations of the plasma membranes [14]. At the opposite, eccrine mechanism is direct (diffusion or active transport across membranes). The major problem encountered in studies of these mechanisms is to locate each VOC in the cell. Indeed, there is no specific histochemical stains or specific reaction for each category of VOCs; all the known staining methods depend, more or less, on the lipophilic properties of the VOCs e.g. Sudan stains and Fluoral Yellow 088 [68,69]. Nile Blue A, is often used because neutral lipids are red and acidic lipids, including some terpenes, are blue [70]. Nile Red correspond to the purification of the oxazone included in Nile Blue A. It produces a yellow to gold color with neutral lipids and an orange one with acidic lipids [71]. NaDi Reagent is often used giving a purple staining of essential oils [72]. Other stains are also used occasionally, e.g. ferrous thiocyanate for sesquiterpenes [73], and nitrosophenol for monoterpenes phenols [74]. Such

cytochemical studies are thus very helpful to locate oil droplets containing terpenes, but are not sufficient to study the secretion pathway. Furthermore, sizes of the vesicles are very often different when observed with a light microscope or with a transmission electron microscope. It is a common belief that the bigger vesicles, observed in light microscopy, are sometimes lost during specimen processing for electron microscopy [e.g. 75,76], or that the smaller vesicles, observed in electron microscopy, aggregate during the histochemical staining for light microscopy [pers. obs.]. Nevertheless, in numerous electron micrographs, vesicles of essential oils have been precisely located in specialized cells.

In secretory trichomes, mechanisms of oil secretion have led to a range of hypothesis which have not always been clearly qualified as eccrine or granulocrine: eccrine secretion in *Origanum dictamnus* (*Lamiaceae*) [26], active transport process from the smooth endoplasmic reticulum to the outside of the cell in *Mentha x piperita* (*Lamiaceae*) [30], light areas passing through the plasma membrane in *Cannabis sativa* (*Cannabaceae*) [77], plasmic membrane budding in *Nepeta racemosa* (*Lamiaceae*) and *Artemisia annua* (*Apiaceae*) [28,68], or clear granulocrine secretion by fusion of vesicles to the plasma membrane in *Prostanthera ovalifolia* (*Lamiaceae*) [78], for example. On the other hand, in osmophores, ducts and cavities, a granulocrine process is most often suspected [18,19, 35,36,69,79]. In all these studies, oil droplets often originate from plastids, periplastidal reticulum and smooth reticulum but also sometimes from dictyosomes or other organelles.

In ducts of *Pinus halepensis* (*Pinaceae*), two secretion pathways have been detailed [44,54]. In the first pathway, resin droplets are surrounded and detached by plasma membrane invaginations in a granulocrine mechanism. In the second pathway, endoplasmic reticulum and plasma membrane fused to release directly

the resin droplets in the extracellular matrix. These two pathways have been then used by numerous authors to interpret their electron micrographs.

In osmophores of *Sauromatum guttatum* (*Araceae*), another granulocrine process has been demonstrated [56]. In this species, volatile sesquiterpenes are released from the naked appendix only on one specific day: the day of thermogenic activity. Nevertheless, a few days before emission, pocket-like structures of rough endoplasmic reticulum with an osmiophilic content are observed in the cytoplasm or clearly fused to the plasma membrane. On the day of emission, these pockets are empty. This correlation between electron micrographs and gas chromatography analysis demonstrates that the secretion pathway is granulocrine, at least in this species.

The secretion of scent compounds from petal epidermal cells is poorly understood. Although, this remains to be conclusively demonstrated, essential oils in petal cells are generally supposed to occur in the form of minute cytosolic droplets [16]. A few studies have been made of the ultrastructure of petal epidermis but the majority of these were focused on petal colour [80,81], rather than on scent production. In *Rosa x hybrida*, changes taking place during maturation of rose petal cells have been studied. Like in other secretory cells, plastoglobules are often observed (Figure 4). Furthermore, at the stage of maximal scent emission, tightly whorled structures, reminiscent of lomasomas, supposed to be lipophilic in nature and other vesicular material of unknown function were observed [82]. These vesicles could be associated with the cell wall and putatively concerned with the secretion of petal monoterpenes.

A study of *Dianthus caryophyllus* (*Caryophyllaceae*) petals led to the conclusion that secondary lipid metabolites, including components of fragrance such as aromatic compounds and the so-called green leaf volatiles, could be

formed within membranes of petal tissues. These molecules could subsequently be released from one of the two phospholipid layers of the plasmic membrane into the cytosol by blebbing of lipid-protein particles resembling oil bodies [83,84]. Indeed, the ratio of triacylglycerols and fatty acids of these particles and their protein electrophoretic pattern are close to those of oil bodies, with more free fatty acids. Oil bodies are classical lipid storage organelles of seeds, derived by inflation of only one layer of phospholipids of the reticulum membrane. Nevertheless, they have been observed in other organs such as leaves and anthers [85,86]. Besides, many novel endoplasmic reticulum-derived structures have been recently described [87,88]. These could be new candidates for secretory compartments. For example, in *Rosa x hybrida*, green fluorescent protein fusions with lipid transfer proteins were shown to be targeted to other unknown vacuole-like compartments [63]. Due to their function and abundance in rose petals [89] and mint secretory trichomes [90], lipid transfer proteins could well play a role in an eccrine process.

Recently, the *NpABC1* gene encoding an ATP binding cassette transporter was cloned in *Nicotiana plumbaginifolia* (*Solanaceae*) [91]. This transporter has been located in the plasma membrane by immunolocalization. Its expression is enhanced by sclareol, a toxic diterpene secreted by tobacco trichomes, and it can transport <sup>3</sup>H-labelled sclareolide outside of the cells. This discovery of a terpene transporter throws back the discussion between eccrine versus granulocrine secretion.

### **Structure of the cell wall and the cuticle of VOCs secreting cells**

Once VOCs have passed the plasma membrane, they may be stored under the cuticle in certain cases. In osmophores, in conical-papillate cells and in some secretory trichomes (e.g. most

trichomes of the *Solanaceae* or the *Rosaceae*), VOCs are rapidly volatilized, probably to attract pollinators. At the opposite, in ducts, in cavities and in some secretory trichomes (e.g. most trichomes of the *Lamiaceae*), VOCs are often stored before they can reach the atmosphere, probably to repulse pathogens and herbivores.

Numerous studies have focused on the chemical composition of petal waxes but only one of these has attempted to correlate this with VOC emission during flower development [92 and references therein]. In *Antirrhinum majus* petals, the cuticle seems to be permeable because the internal pool of methylbenzoate is highly correlated to its emission. The cuticle thickness and ultrastructure is not involved in this permeability but chemical composition is involved. Because branched alkanes and hydroxy esters create steric hinderances preventing packing of waxes, they have been associated with this permeability.

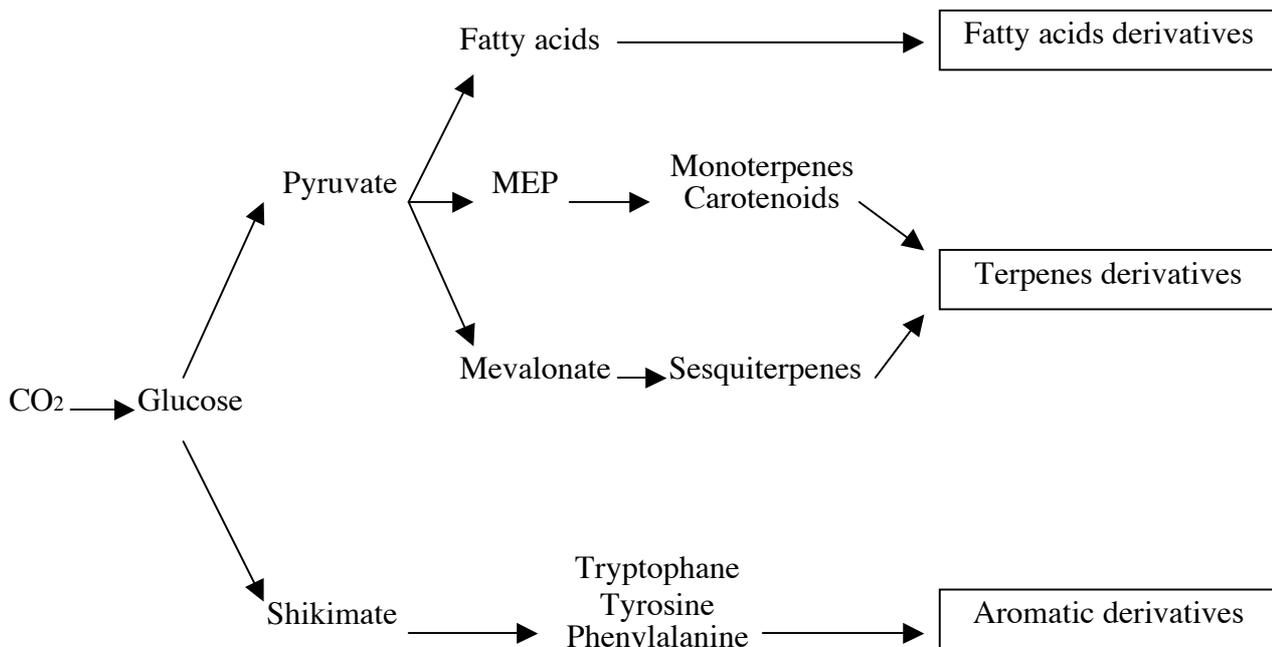
In the osmophores of members of the *Orchidaceae* such as in certain species of *Scaphosepalum*, the permeability of the cuticle could regulate the emission of VOCs [19]. However, pores have been observed in *Scaphosepalum microdactylum*, *Restrepiella sp.* and some *Restrepia* species [18,19]. Pores in the cuticle have been also sometimes observed in trichomes. This is the case of conoidal trichomes of *Plectranthus ornatus* (*Lamiaceae*) [29].

Very often, essential oils are stocked in a sub-cuticular space that expands during secretion and are then released by rupture of the cuticle [30,45]. In *Mentha x piperita*, rates of monoterpene volatilization through the cuticle have been estimated to be < 5% over a 6-month growing period [93]. One can interpret these results in relation to the putative defense role of mint trichomes; in the case of herbivory, repulsive monoterpenes are then released immediately.

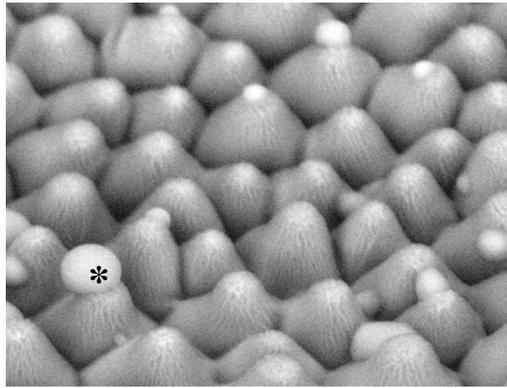
## Conclusion

Many questions remain to be answered concerning the secretory pathway of VOCs. The eccrine and granulocrine mechanisms seem both to exist in aromatic and scented plants. Evidence for the eccrine mechanism is available for study using an ATP binding cassette transporter and evidence for the granulocrine mechanism is provided by numerous histological studies. The discovery of new kinds of vesicles suggests new hypotheses for the cellular channelling involved in secretory pathways. Oil bodies could be a possibility, but not the only one. As evidence of this, the different terpenes, benzenoids and fatty acids derivatives are not synthesized in the same organelles and cell compartments. Finally, if they do not affect the same secretory pathway, many different processes of secretion must exist including: lipid carriers, transfer proteins,

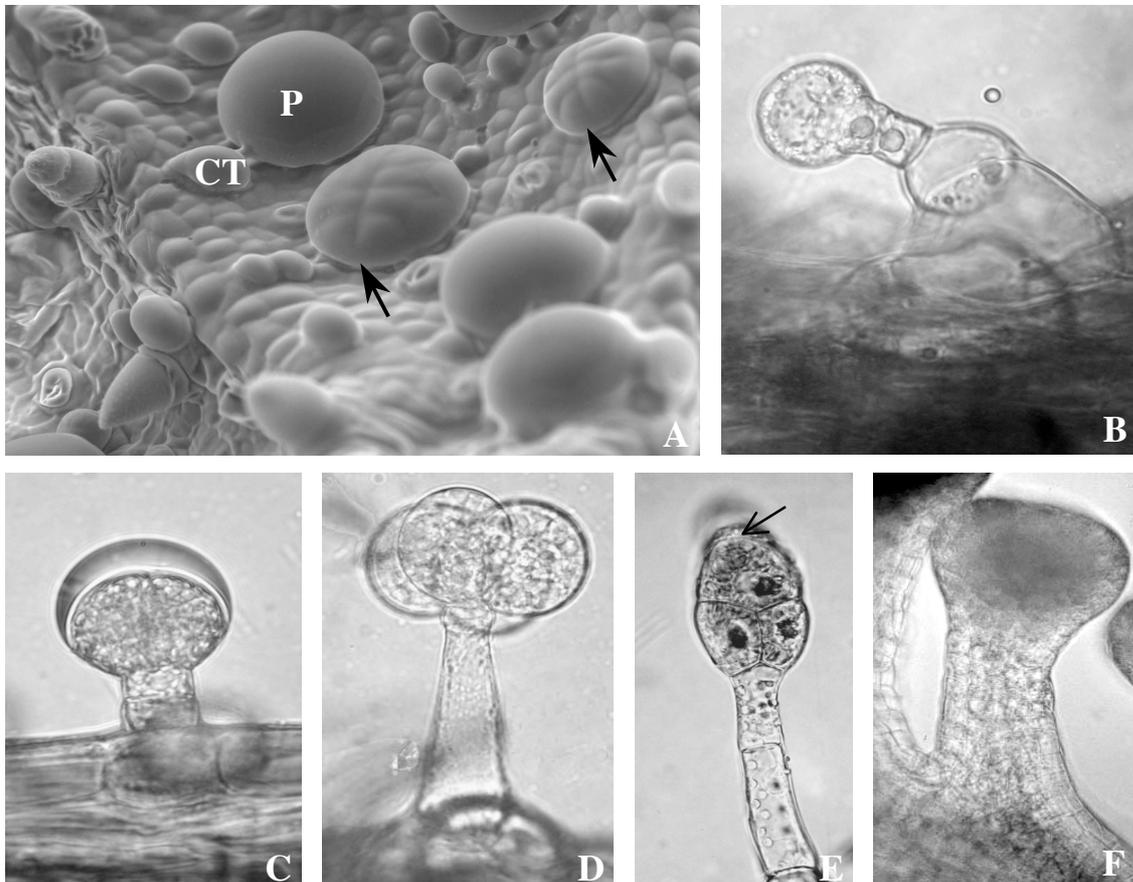
oleosomes and reticulum cisternae. Despite valuable cytological studies, the elucidation of secretion pathways is rendered very difficult by the absence of stains specific for the different secreted compounds. A way to circumvent this problem is to locate at the cellular and subcellular levels the enzymes involved in the biosynthesis pathways. As these pathways are more and more investigated, this alternative approach will certainly lead to fruitful discoveries. Nevertheless, the absence of an aromatic model-plant is another brake on the study of these cellular pathways, even though recent data indicate that *Arabidopsis thaliana* also emits terpenes in small quantities. If an aromatic model emerges, such as tobacco for example, together with mutants, transgenic possibilities and methods for genetic analysis, the field of aromatic secretory mechanisms will undoubtedly be revolutionized.



**Fig. 1: Biosynthesis pathways of major volatile organic compounds.** MEP, 2-C-methyl-D-erythritol-4-phosphate.

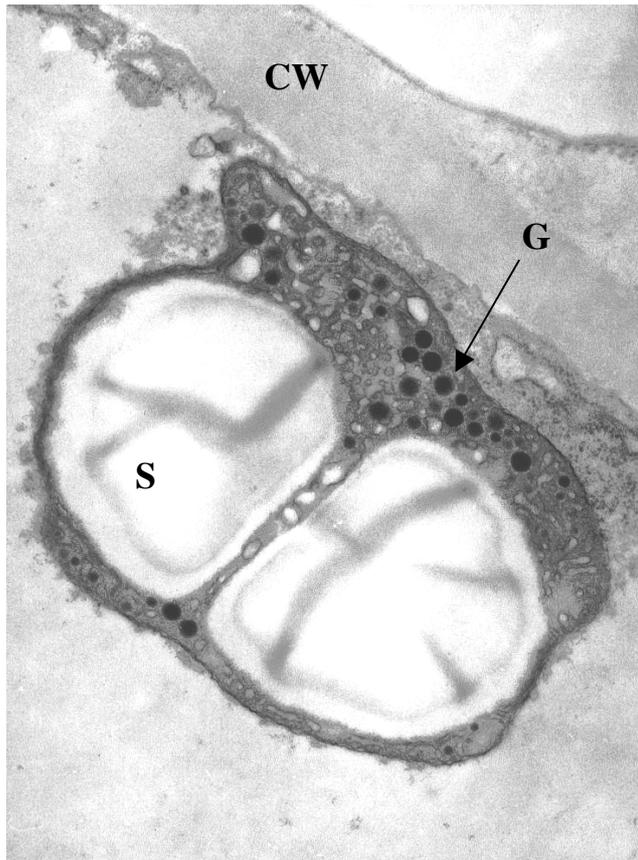


**Fig. 2: Environmental electron micrograph of the petal epidermal cells of *Rosa x hybrida* (Gr. x 600).** Low pressure of environmental microscopy allows to work on fresh material without any fixation or chemical processing. VOCs seem to gather together (star) due to environmental conditions in the microscope chamber.



**Fig. 3: Diversity of secretory trichomes among the Lamiaceae (A, B, C) , Solanaceae (D, E) and Rosaceae (F).**

Environmental electron micrograph of peltate (PT) and capitate (CT) glandular trichomes of *Mentha x piperita* (A, Gr. x 400). Note the shadow (arrows) of the 8 head-cells before the secretory phase of peltate glands. Light micrographs of secretory trichomes of *Ajuga reptans* (B, Gr. x 400) with its thick stalk, *Lamium maculatum* (C, Gr. x 400) with the subcuticular oil droplet, *Lycopersicon esculentum* (D, Gr. x 400) with the 4 head-cells, *Nicotiana tabacum* (E, Gr. x 400) with the sticky secretion (thin arrow) and *Rosa x hybrida* (F, Gr. x 100) with the numerous cells.



**Fig. 4: Transmission electron micrograph of typical plastids of *Rosa x hybrida* petals during secretion (Gr. x 15000). S, starch granules; G, plastoglobules; CW, cell wall.**

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