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DEVELOPMENTAL CHANGES IN THE MONOTERPENE COMPOSITION OF *MENTHA* × *PIPERITA* LEAVES FROM INDIVIDUAL PELTATE TRICHOMES

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Key Word Index—*Mentha* × *piperita*; Lamiaceae; peltate trichomes; monoterpenes; biochemical variations.

Abstract—Different steps of monoterpene metabolism—disappearance of limonene, accumulation of 1,8-cineole, reduction of menthone to menthol and acetylation of menthol—have been studied in different parts of *Mentha* × *piperita* leaves of different ages. The analyses of different samples (leaf strips, disks and individual peltate trichomes, translucent or containing crystals from either epidermis) show that all these dynamic changes start at the distal extremity of the leaf and shift progressively towards the base. Except for the peltate trichomes localized within the leaf area, in which a metabolic step is being realized, the trichomes of other parts present a homogeneous monoterpene composition. The measurements of amounts of chlorophyll in two parts, distal and basal, of youngest leaves of terminal buds show that chlorophyll biosynthesis starts also at the distal extremity of the leaf. The regulation of the menthone reduction is discussed. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The oil composition of *Mentha* × *piperita* growing in natural conditions depends on leaf age. Initially, the young apical leaves contain mainly limonene, followed by menthone. Thereafter, in the midstem leaves, menthol becomes the major component, while the older senescent leaves produce only oil rich in menthyl acetate [1–2]. In a previous study on the effects of ageing on monoterpene composition in *Mentha* × *piperita* leaves [3] we have observed that the developmental changes in monoterpene metabolism proceed at different rates when one compares the top to the basal parts of the lamina. However, these results do not correspond to the data of Maffei *et al.* [4] who note that 'a striking monoterpene compositional variability is present among individual trichomes and among different parts of the leaf' and that 'this situation . . . suggests that trichomes at different stages of maturation may be situated very closely on the same leaf'. Because this difference in results might be due to a difference in sampling, new experiments using the desorption–concentration-introduction (DCI.) technique coupled to a gas chromatograph [5] have been carried out on individual peltate trichomes, either translucent or containing crystals, isolated from each epidermis [6], disks or strips of dried leaves, and sampled as a function of different parts of leaves. Moreover, analyses were carried out on individual peltate trichomes sampled from three different parts of a growing leaf as a function of time. We report the results of detailed analyses of monoterpene metabolic steps, i.e. reduction of menthone to menthol, acetylation of menthol, disappearance of limonene and accumulation of 1,8-cineole from different parts of leaves at different maturity stages from growing plants naturally harvested in June. Measurements of chorophyll content, made on two parts of the youngest leaves of the terminal bud of plants, show that the greening of these leaves also starts at the distal extremity.

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RESULTS

Reduction of menthone to menthol

Experiment 1: *oil composition of individual peltate trichomes from lower epidermis.* The DCI technique coupled to a gas chromatograph allows measurements of the monoterpene composition of a single peltate trichome. Thus, individual analyses were carried out on the eight secretory cells surrounded by the extracellular subcuticular space manually separated from the stalk cell (see Experimental). A first set of analyses (Fig. 1) was performed on three consecutive levels of dried leaves (numbered from the base of plant) of plant no. 1 (leaf no. 8, 61 mm long), nos. 9, (50 mm long) and 9′, (52 mm long—opposite leaves) and leaf no. 10 (35.5 mm long). Totals of 59, 79, 64 and 29 individual trichomes were sampled, respectively, from the top to the base of lower epidermis of each leaf and the

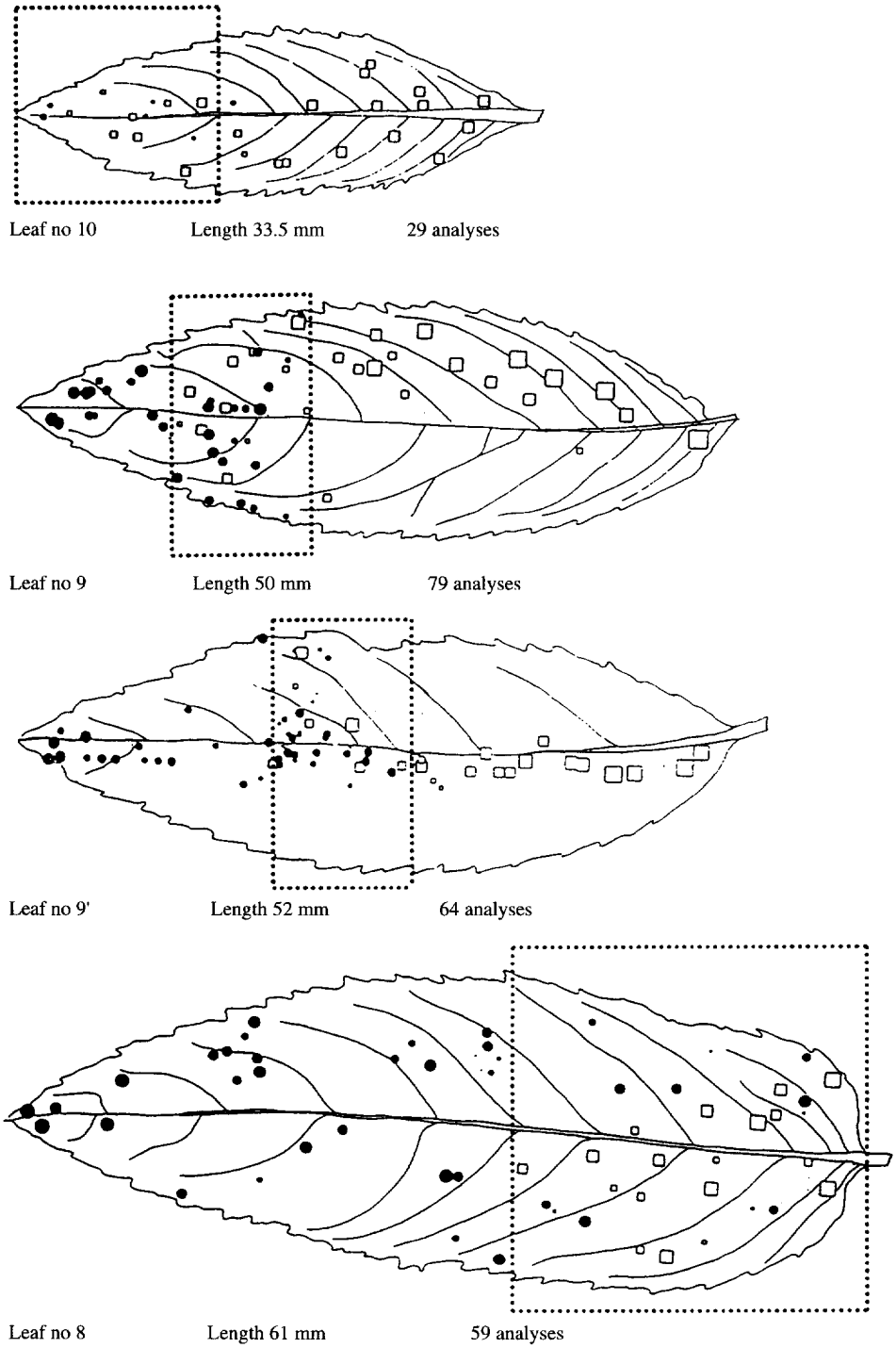

Fig. 1. Differences of percentages of menthol minus percentages of menthone of individual peltate trichomes sampled out from lower epidermis of three consecutive leaf levels (plant no. 1); the filled circles represent oils richer in menthol than in menthone, the squares represent oils richer in menthone than in menthol; the size of each symbol is proportional to the difference (positive or negative). Leaf 10: circle maximum size: +36.5%, minimum size: +12.5%, square maximum: −77.6%, minimum: −7.8%. Leaf 9: circle maximum: +60.7%, minimum: +0.8%, square maximum: −77%, minimum: −2.2%. Leaf 9′: circle maximum: +60.7%, minimum: +11.5%; square maximum: −79%, minimum: −1%. Leaf 8: circle maximum: +63.4%, minimum: +1.1%, square maximum: −44.2%, minimum: −4.1% (see Acknowledgement). Dotted boxes: area in which the monoterpene composition of trichomes is heterogeneous.

location of each trichome was precisely defined. For each analysis, the difference of percentage of menthol minus the percentage of menthone was represented by an obscured circle if it was positive and by a square if it was negative with a size proportional to this difference (see Acknowledgement). In the same conditions, consistent results were obtained from 87 analyses of leaf no. 8, 45 mm long, plant no. 2, compared to those of leaf no. 9, plant no. 1 on the one hand; and from 28 analyses of leaf no. 6, 61 mm long, plant no. 3, compared to those of leaf no. 8, plant no. 1 on the other hand.

Experiment 2: *oil composition of individual trichomes from upper and lower epidermis and of foliar disks*. The analyses of six foliar disks stamped out from a dried leaf (37 mm long, plant no. 4) at 3, 10, 16, 21, 27 and 33 mm from the tip and the analyses of three peltate trichomes tanslucent or/and containing crystals (six) from each epidermis and located around each disk are described in Fig. 2.

Consistent results were obtained from other samples examined by the same procedure or by analyses of consecutive strips. However, in these different samples, the intersection point of equal percentage in menthone and menthol may be situated at either the tip, the middle part or the base of the lamina, according to the degree of oil maturation in the studied leaf level, as previously observed in the different leaf levels (nos. 8, 9, 9′ and 10) in Fig. 1.

In Fig. 3, the analyses of seven successive disks and different trichomes sampled at 8, 15, 23, 31, 38, 45 and 52 mm from the leaf top (55 mm long, plant no. 5) show the final stage of metabolic conversion of menthone to menthol in a mature leaf.

Experiment 3: *variation of oil composition of trichomes from the same position on the lower epidermis of growing leaf as a function of time*. The sampling was carried out on a fresh leaf (no. 8, 30 mm long; plant no. 6) which remained on the plant growing naturally, permitting studies of trichomes sampled in the same part of lamina few days later. Translucent trichomes of the lower epidermis were sampled at day 0 (11 June) from four parts of leaf: part A at 27 mm (four analyses), part B at 18 mm (two analyses), part C at 10 mm (three analyses) and part D at 2 mm (three analyses) of the tip (Fig. 4a). Four days later, 10 translucent lower tri-

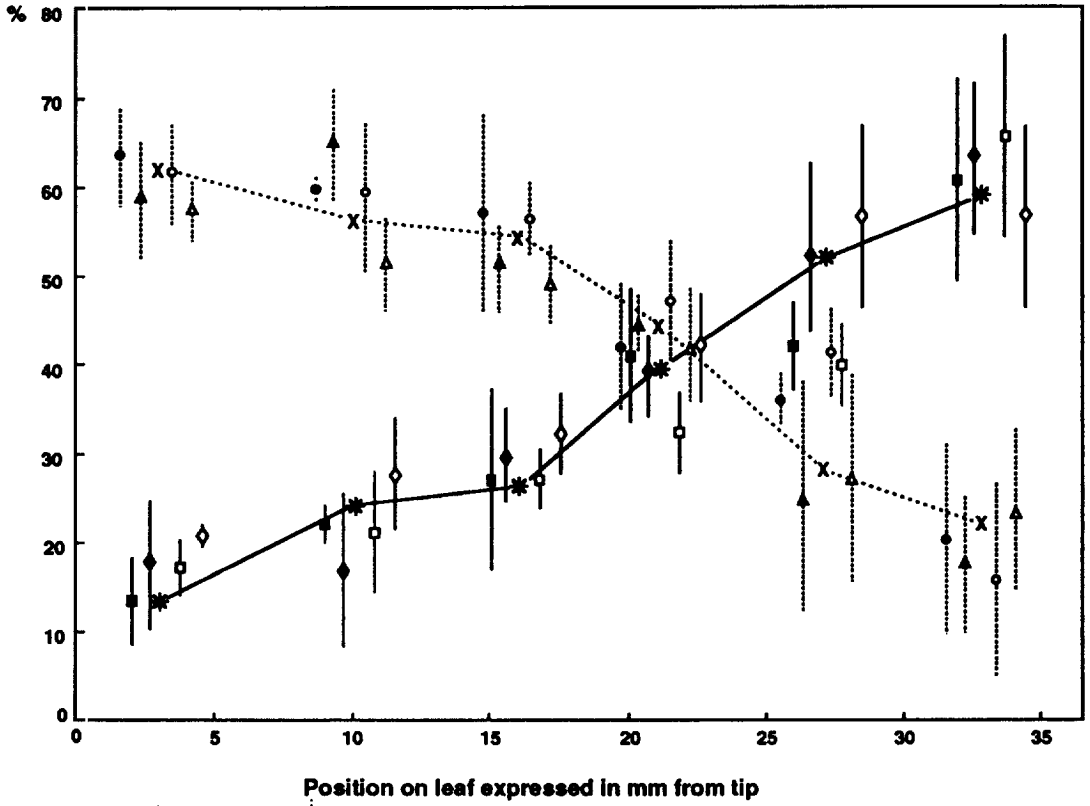

Fig. 2. Menthone (■) and menthol (●) percentages of individual trichomes containing crystals (3 analyses) of upper epidermis; menthone (□) and menthol (○) of individual translucent trichomes (3 analyses) of upper epidermis; menthone (◆) and menthol (▲) percentages of individual trichomes (3 analyses) containing crystals of lower epidermis; menthone (◇) and menthol (△) of individual translucent trichomes (3 analyses) of lower epidermis; menthone (*) and menthol (×) percentages of successive disks of 37 mm leaf.

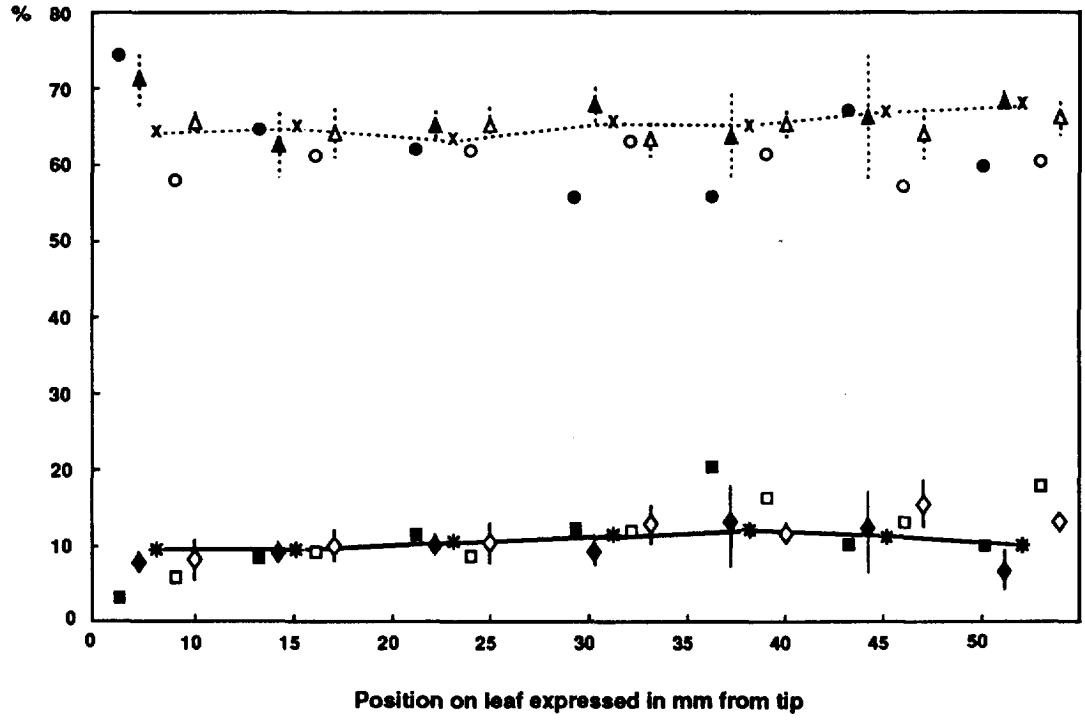

Fig. 3. Menthone (■) and menthol (●) percentages of individual trichomes containing crystals (1 analysis) of upper epidermis; menthone (□) and menthol (○) of individual translucent trichomes (1 analysis) of upper epidermis; menthone (◆) and menthol (▲) percentages of individual trichomes (3 analyses) containing crystals of lower epidermis; menthone (◇) and menthol (△) of individual translucent trichomes (3 analyses) of lower epidermis; menthone (*) and menthol (×) percentages of successive disks of 55 mm leaf.

chomes of the part A (Fig. 4b) were analysed and nine days later, three translucent trichomes of the part A (Fig. 4b) and six of part C (Fig. 4c) were studied.

Acetylation of menthol

This metabolic step was studied both by analyses of lower epidermis translucent trichomes and of foliar disks. The 17 analyses of translucent trichomes sampled on the senescent leaf no 3 (58 mm long; plant no. 1) are summarized in Fig. 5. The size of obscured circle is proportional to the percentage of menthyl acetate (maximum size: 24.9%; minimum size 1.2%). The results of 13 analyses of disks stamped from the leaf no. 4 (30 mm long) of another sample (plant no. 7) are summarized in Fig. 6.

Decline of limonene and increase in 1,8-*cineole*

Sixteen analyses of consecutive foliar disks stamped out from a young dried leaf (30 mm; plant no. 7) are reported in Fig. 7. Consistent results were obtained from 8 other experiments realized with the same procedure on fresh or dried leaves.

DISCUSSION

Regardless of the sampling technique employed all the results obtained (leaf strips, leaf disks or individual peltate trichomes from each epidermis) show that the different metabolic steps start and shift progressively from the top to the basal part of lamina so that the apical part is metabolically most mature. This trend holds for the disappearance of limonene and accumulation of 1,8-cineole (Fig. 7), the reduction of menthone to menthol (Figs 1–4) and the acetylation of menthol (Figs 5 and 6). Such results confirm our previous data obtained from a smaller number of analyses and a less discriminant sampling [3].

The analyses of numerous individual peltate trichomes relative to the reduction of menthone to menthol show not only that this transformation occurs progressively from the top to the base of leaf as a function of leaf level (Figs 1 and 2), but also that this reduction is realized in a synchronous way as a function of time in the different parts of the same leaf (Fig. 4a-c). Therefore, these detailed results show that the 'striking monoterpene compositional variability (which) is present among individual trichomes and among different parts of leaf' indicated by Maffei *et al.* [4] is observed only within a restricted area of the leaf (Fig. 1, see dotted boxes). Outside this area, all the trichomes possess a major compound—either menthone or menthol (i.e. components reflecting opposite oxidation-reduction levels according to their maturity degree).

When the leaf reaches the mature stage, all tri-

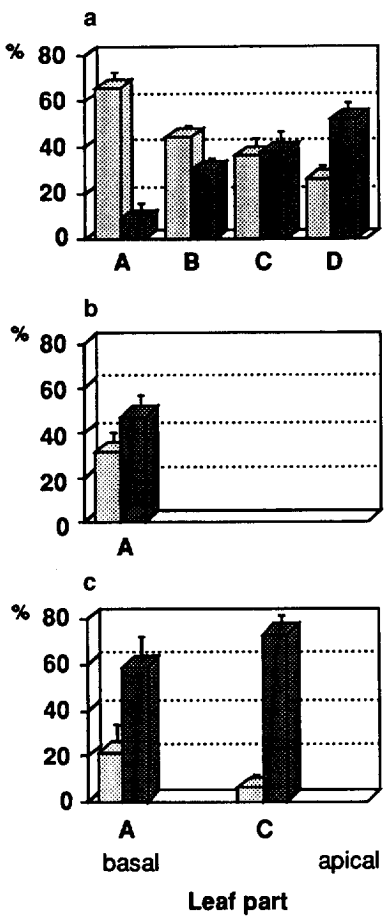

Fig. 4. Menthone (▨) and menthol (■) percentages (mean and standard deviation) (I) of translucent individual trichomes located at four parts A, B, C and D of 30 mm leaf at day 0 (a) at day +4, (b) and at day +9 (c).

chomes, irrespective of which part of the leaf they are situated in, possess an oil rich in menthol (Fig. 3). Other factors do not affect monoterpene composition. For example, analyses of the oil composition of trichomes carried out on each side of the median vein indicate no significant variations along this transversal axis (Fig. 1); the oil composition of translucent trichomes or trichomes containing crystals on each epidermis also does not differ. The oil composition of trichomes sampled out on young leaf corresponds to that described by McCaskill *et al.* [7] for the oil obtained from glands of the whole leaf. We have never observed as high a percentage of neomenthol in the abaxial epidermis translucent trichomes as Maffei *et al.* [4].

In growing mint plants, the small leaves of the apical tuft are overlapped and the oldest entirely cover the youngest; then, progressively, the leaves become first orthotropic, then plagiotropic. Thus, in growing buds, the first part of these young leaves to be exposed to light is the apical part. Light is one of most important environmental factors regulating monoterpene oil composition [5, 8–11]. Careful observation of these small leaves shows that only their distal extremities are green, while their bases remain yellow. Separated measurements of the amounts of chlorophyll in the apical and basal part of young leaves (see Experimental) in which the (−)-menthone to (−)-menthol reduction occurs, show that chorophyll biosynthesis also starts at the distal extremity of these leaves (Fig. 8). The photosynthetic activity in the tissues of the apical extremity could therefore explain why the activity of NADPH-dependent 3-keto-reductase, responsible for the reduction of (−)-menthone to (−)-menthol [12], is high in this part of the leaf and shifts progressively towards the base, and correlates with the greening of the leaf. Such a suggestion agrees with the model of Burbott and Loomis [9]; according to these authors, 'the oxidation-reduction level of monoterpenes reflects the general oxidation-reduction state of the respiratory coenzymes of the terpene-producing cells' and the changes in monoterpene composition are of photosynthetic origin rather than a response to photoperiodic treatment. Nevertheless, in order to determine the delayed effect and to define a possible correlation between the initiation of chlorophyll content and that of the menthone reduction, new measurements of these metabolic changes in the different parts of the smallest leaves have to be made. However, even in these cases, the

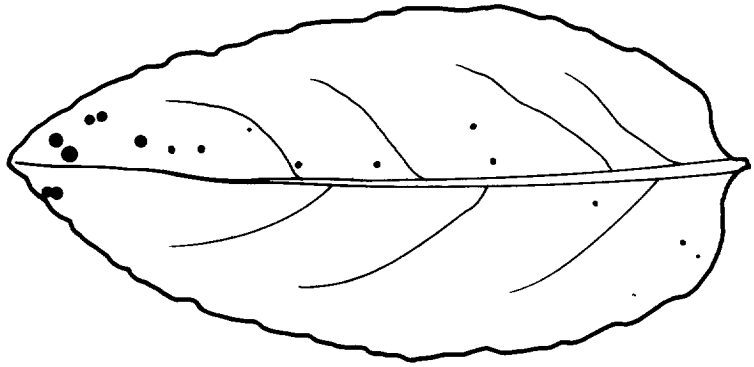

Fig. 5. Menthyl acetate percentages (●) of translucent individual peltate trichomes of leaf level no. 3 (plant no. 1): maximum size of circle, 24.9%; minimum size, 1.2%.

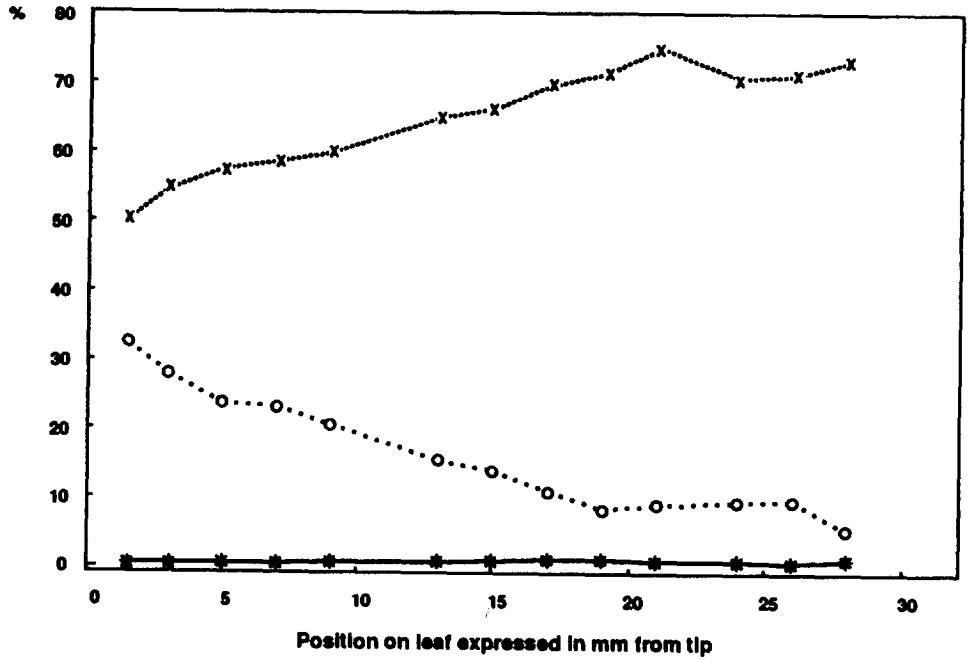

Fig. 6. Menthone (*), menthol (×) and menthyl acetate (○) percentages of 13 successive disks of 30 mm leaf.

participation of phytochrome in photoregulation of monoterpene production, demonstrated by Tanaka *et al.* [11] in thyme seedlings, cannot be entirely discarded because this photoreceptor controls 'en amont', in angiosperm leaves, the expression of the gene encoding the protochlorophyllide reductase that catalyses the reduction of protochlorophyllide to chlorophyll [13]. Moreover, in addition to the indirect control, it should be interesting to determine if phytochrome directly regulates the biosynthesis of 3-keto-reductase itself or

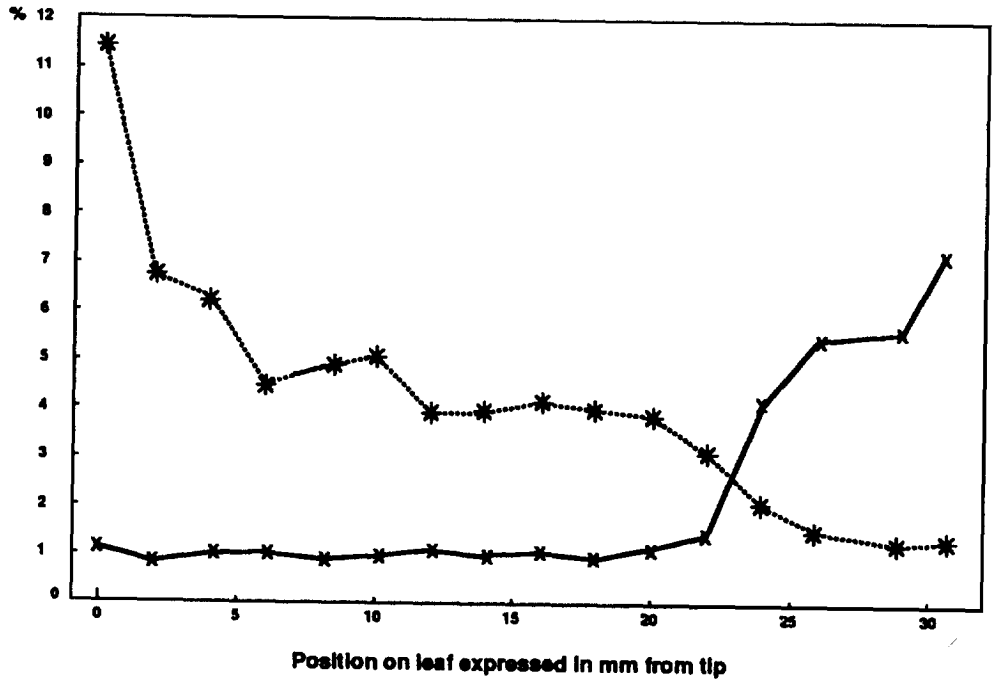

Fig. 7. Limonene (×) and 1,8-cineole (*) percentages of 16 successive disks of 31 mm leaf.

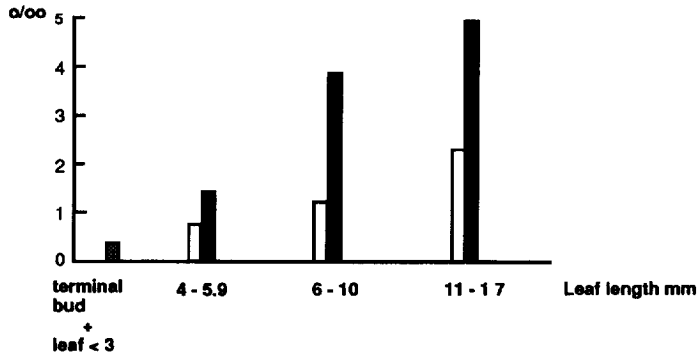

Fig. 8. Amounts (μg chlorophyll mg^{-1} dried leaf) of terminal buds + leaves smaller than 3 mm (▨) and of apical (■) and basal (□) parts of three successive leaf levels (for details see text and Experimental).

that of a protein regulating its activity [14]. This would explain the response to photoperiodic treatments observed by Grahle and Höltzel [8], Clark and Menary [10] and Voirin *et al.* [5]. Further molecular genetic studies, such as isolation and sequencing of the cDNA clone that encodes 3-keto-reductase, determination of the actual products of gene R [15] (a regulatory protein or biosynthetic enzyme), are needed both to determine the effects of light in the control of essential oil composition and to examine the developmental regulation of monoterpene biosynthesis in *Mentha* × *piperita* leaves.

EXPERIMENTAL

Plant material. Peppermint plants were the Black Mitcham variety of *Mentha* × *piperita vulgaris*, propagated vegetatively from the same clone (no. 19, INRA Antibes, France). The eight secretory cells surrounded by the extracellular subcuticular space were manually isolated from the stalk cell under a Zeiss Stemi SV8 stereomicroscope with the aid of a microfeather (Father Safety Razor Co., Ltd, Medical Division, Japan). The precise location of each peltate trichome on the epidermis was defined by measurements with a micrometric scale under the Stereomicroscope. The GLC analysis was performed immediately after isolation. All the samples were harvested in June. Disks 1 mm diameter were stamped out from dried leaves at different positions on leaf (see Figs 2, 3, 6, and 7) or leaves were divided into transverse strips from the tip to the base of the leaf; each disk or strip was successively analysed.

Methods. The direct chromatographical analyses of essential oil of all the samples by coupling a DCI technique with GC have been described in ref. [5]. In the results, only 10 compounds, except for comparisons with the results of McCaskill *et al.* [7], have been taken into consideration; the study of the variability of their percentages was studied from five successive analyses of the same oil extracted from *Mentha* × *piperita*; α = 0.05: limonene 1.08±07; 1,8-cineole 4.09±0.7; pulegone 0±0; menthofuran 1.66±0.66; menthone 35.73±2.8; isomenthone 0.43±0.16; menthol 44.98±3.38; neomenthol 4.17±0.56; isomenthol 2.14±0.54; menthyl acetate 3.04±0.34.

Chorophyll amounts (μg chlorophyll mg^{-1} dried leaf) were measured by the classical method of Bruinsma ref. [16] (λ_{max} 652 nm; Me_2CO–H_2O 8-2; $E^{1\%}$ 1 cm = 360) from 33 leaf samplings. The leaves of each plant were collected node by node; four levels were defined as a function of the successive position and length of leaves: level 1, meristem and youngest leaves smaller than 3 mm; level 2, leaves between 4 and 5.9 mm; level 3, leaves between 6 and 10 mm; level 4, leaves between 11 and 17 mm. Each leaf of levels 2, 3 and 4 was divided in two equal parts, apical and basal.

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