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Influence of etherel and gibberellic acid on carbon metabolism, growth, and essential oil accumulation in spearmint (*Mentha spicata*)

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Abstract

Changes in growth parameters and $^{14}CO_2$ and [U-^{14}C]-sucrose incorporation into the primary metabolic pools and essential oil were investigated in leaves and stems of *M. spicata* treated with etherel and gibberellic acid (GA). Compared to the control, GA and etherel treatments induced significant phenotypic changes and a decrease in chlorophyll content, CO_2 exchange rate, and stomatal conductance. Treatment with etherel led to increased total incorporation of $^{14}CO_2$ into the leaves wheras total incorporation from ^{14}C sucrose was decreased. When $^{14}CO_2$ was fed, the incorporation into the ethanol soluble fraction, sugars, organic acids, and essential oil was significantly higher in etherel treated leaves than in the control. However, [U-^{14}C]-sucrose feeding led to decreased label incorporation in the ethanol-soluble fraction, sugars, organic acids, and essential oils compared to the control. When $^{14}CO_2$ was fed to GA treated leaves, label incorporation in ethanol-insoluble fraction, sugars, and oils was significantly higher than in the control. In contrast, when [U-^{14}C]-sucrose was fed the incorporation in the ethanol soluble fraction, sugars, organic acids, and oil was significantly lower than in the control. Hence the hormone treatment induces a differential utilization of precursors for oil biosynthesis and accumulation and differences in partitioning of label between leaf and stem. Etherel and GA influence the partitioning of primary photosynthetic metabolites and thus modify plant growth and essential oil accumulation.

Additional key words: amino acids; chlorophyll; $^{14}CO_2$- and ^{14}C-sucrose incorporation; organic acids; primary photosynthetic metabolites; stem; stomatal conductance; sugars; transpiration rate.

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Abbreviations: Chl = chlorophyll; E = transpiration rate; g_s = stomatal conductance; P_N = net photosynthetic rate.
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Introduction

Mentha spicata (spearmint) is one of the main sources of aroma compounds menthol, carvone, linalool, and linanyl acetate used widely in food, cosmetic, flavour, and pharmaceutical industries. Essential oil biosynthesis in mints including *M. spicata* is strongly influenced by several intrinsic and extrinsic factors (Lawrence 1986, Bernard *et al.* 1990) including temperature (Clark and Menary 1980b), photoperiod (Burbott and Loomis 1967), photosynthetic photon flux density (Clark and Menary 1980a), nutrition (Srivastava and Luthra 1994, Srivastava *et al.* 1997), genotype (Srivastava and Luthra 1991), ontogeny (Srivastava and Luthra 1991b), and osmotic stress (Charles *et al.* 1990). Essential oil, composed mainly of monoterpenes, is synthesized through the mevolanate-isoprenoid pathway in the epidermal oil glands which are carbon-heterotrophic and hence depend on the adjoining mesophyll cells for precursors (McGarvey and Croteau 1995). However, essential oils may not only be accumulated but also biosynthesized in glandular trichomes (Gershenzon *et al.* 1989). Among precursors, CO_2 and sucrose are preferred for essential oil biosynthesis (Gershenzon and Croteau 1991, 1993). Essential oil biogenesis is also linked to the contents of primary metabolites (Srivastava and Luthra 1991a), and a positive but insignificant association has been shown with net photosynthetic rate, P_N (Srivastava *et al.* 1990). Thus the secondary metabolic pathway is closely associated and dependent on the primary metabolic pathway.

Growth hormones play a dominant role in the regulation of growth and development by affecting sink-source relationship (Marschner 1986). El-Keltawi and Croteau (1986a,b) reported the influence of phosphon-D, cycocel, ethephon, and daminozide on the constituents of essential oil of *M. piperita.* Farooqi and Sharma (1988) reported influence of growth retardants on growth and essential oil accumulation in *M. arvensis* whereas Srivastava and Sharma (1991) reported the influence of triacontanol on photosynthetic characteristics and oil accumulation in *M. arvensis.* Most of the growth hormone studies on *M. piperita* or *M. arvensis* attribute the effects to the influence on enzymes of biosynthetic pathways and on plant and growth characters such as herb yield and leaf/stem ratio. However, it is not clear what changes occur in the photosynthetic C-metabolism of the hormone treated plant and translocation of assimilates to the essential oil accumulation. While studying the influence of growth hormones on yield and growth, we observed significant and persistent effect of etherel and GA on plant phenotype.

In the present study, we report the influence of GA and etherel on the photosynthetic efficiency and oil accumulation studied during the incorporation of $^{14}CO_2$ and [U-^{14}C]-sucrose into primary photosynthetic metabolites, sugars, amino acids, and organic acids, and simultaneously into the essential oil in *M. spicata.* Changes in P_N, chlorophyll (Chl) content, and stomatal conductance (g_s) were also determined.

Materials and methods

Uniform suckers of *M. spicata* cv. MSS-5 obtained from the farm nursery of the Institute were treated with etherel and GA (1 kg m^{-3} each) by dipping in respective solution for 24 h. Later the treated suckers were planted in 10 000 cm^3 earthen pots maintained in a glasshouse at ambient temperature (30-35 °C) and irradiance (800-1000 μmol m^{-2} s^{-1}, measured by a *LiCOR* light meter model *188 B*). Values of growth characters, essential oil, and tracer feeding were recorded 100 d after the treatment.

Chl (*a*+*b*) content was measured on the third leaf. A known mass of leaf tissue was extracted with 80 % acetone and the absorbance was recorded by a *Milton Roy* spectrophotometer *Spectronic 21 D* using the method of Arnon (1949). P_N, initial transpiration rate (*E*), and g_s of the third leaf were measured in a closed system using a portable computerized photosynthesis model *Li-6000* (*LiCOR*, Lincoln, USA) as described in Srivastava and Luthra (1991a). For determining the extraction of essential oil from the control plants (untreated) or after feeding of $^{14}CO_2$ or [U-^{14}C]-sucrose, a known mass of shoot (leaf + stem) material was finely chopped and subjected to steam distillation in a mini-Clevenger apparatus (Clevenger 1928). The volatile oil was recovered by ether extraction. The radioactivity in ether samples was determined in a scintillation counter (*LKB Rack Beta 1215*) using a PPO-POPOP-toluene cocktail (Srivastava and Luthra 1991a).

The tracer studies were carried out with $^{14}CO_2$ and [U-^{14}C]-sucrose that were fed to the freshly excised shoots of treated and control plants and the amounts of label incorporated into essential oil and simultaneously into the pool of primary photosynthetic metabolites (sugars and sugar phosphates, amino acids, and organic acids) were determined. Before the labelling studies, the shoots were cut under water and tested to ensure that they were able to take up water properly. For $^{14}CO_2$ studies, 12 unbranched main shoots (of GA-treated, etherel treated, and control plants) having 6 leaf pairs were placed in vials with the cut ends dipped in half strength Hoagland and Arnon (1938) solution. The vials were then placed in a sealed plexiglass chamber (20 000 cm^3 capacity) around a central vial containing $Na_2{}^{14}CO_3$ solution (3.7 MBq, 2.8 GBq $mmol^{-1}$) obtained from the isotope division of *Bhabha Atomic Research Centre* (BARC), Trombay, India. $^{14}CO_2$ was generated by injecting 4 N H_2SO_4 into carbonate solution through a PVC tube and uniformly distributed with the help of a small electric fan. The leaves were allowed to assimilate $^{14}CO_2$ for 1 h in sunlight (800-1000 μmol m^{-2} s^{-1}). At the end of 1 h, a saturated solution of KOH was run into the central vial and left for 15 min to absorb excess $^{14}CO_2$. The chamber was then opened for the remaining incorporation period of 6 h (Srivastava and Luthra 1991a). For feeding experiments with [U-^{14}C]-sucrose, unbranched shoots having 6 leaf pairs each were placed in vials containing an aqueous solution of 1 μmol [U-^{14}C]-sucrose (185 kBq) obtained from the Isotope Division of BARC, Trombay, India (specific activity 21.5 GBq $mmol^{-1}$). After the uptake of labelled material, the vials were kept filled with half strength Hoagland solution, and the samples were harvested after 6 h.

After exposure to $^{14}CO_2$ or [U-^{14}C]-sucrose feeding, plants were separated into leaf and stem, finelly chopped, and divided into two parts: (*1*) for determining incorporation of label into essential oil a known mass of shoot material (leaf + stem) was processed as described in the "extraction of essential oil"; (*2*) for determining incorporation of label into primary photosynthetic metabolites a known mass of tracer-fed leaves and stem was extracted immediately in boiling 80 % ethanol. The stem sample did not include the basal portion which had been immersed in labelled sucrose. The ethanol soluble material was separated into neutral (sugars and sugar phosphates), basic (amino acids), and acidic (organic acids) fractions by *Amberlite* ion exchange column chromatography. Ethanol-insoluble material was hydrolyzed by diastase in 0.05 M acetate buffer (pH 5.2) at 50 °C. The radioactivity in hydrolyzed alcohol insoluble material and in eluates after ion exchange separation was measured using Bray's scintillator (Srivastava and Luthra 1994). Total ^{14}C incorporated was calculated as the sum of the total label incorporated in ethanol-soluble and -insoluble fraction and expressed on fresh mass basis.

All measurements were taken in triplicate and the results are given as means ± SE. Values were statistically analysed for significance by paired *t*-test.

Results and discusion

Treatment with etherel and GA resulted in significant changes in plant phenotype which was evident even at 100 d of growth (Fig. 1). Normally the plant metabolizes the externally applied hormones and even if there are some phenotypic differences,

Fig. 1. Changes in plant characters of *Mentha spicata* due to hormone treatment. *Left*: etherel, *middle*: gibberellic acid, *right*: control.

Table 1. Changes in growth and yield characters of *M. spicata* treated with etherel and gibberellic acid (GA). Chl = chlorophyll; P_N = net photosynthetic rate. */** Mean values significant at 5/1 % level of significance by pair *t*-test; NS - nonsignificant.

Characters	Etherel	Control	GA
Chl *a* [g kg^{-1}(FM)]	2.03±0.07*	2.92±0.26	1.09±0.01*
Chl *b* [g kg^{-1}(FM)]	0.84±0.008^{NS}	1.25±0.12	0.45±0.02*
Chl (*a*+*b*) [g kg^{-1}(FM)]	2.87±0.06	4.18±0.37	1.54±0.01*
P_N [μg(CO_2) m^{-2} s^{-1}]	114±7**	229±4	139±12*
Initial transpiration rate [mmol m^{-2} s^{-1}]	436±30*	661±90	426±20*
Stomatal conductance [mmol m^{-2} s^{-1}]	224±10**	440±10	250±20**
Plant height [cm]	3.5±0.21**	6.1±0.10	11.85±0.05**

these are temporary and the plant reverts soon to its normal phenotype, but in the present case the hormone effects were evident much longer. This was accompanied by marked changes in physiological characteristics.

The etherel-treated plants had significantly lower contents of Chl (*a*+*b*), Chl *a*, decreased P_N, g_s, *E*, and plant height as compared to control (Table 1). Thus the overall growth was stunted. There was a difference in utilization pattern of $^{14}CO_2$ and [U-^{14}C]-sucrose. When $^{14}CO_2$ was fed, the total $^{14}CO_2$ fixed in leaves in etherel treatment was significantly higher than in the control. Also the ethanol-insoluble fraction, the sugars, organic acids, and essential oil had a significantly higher ^{14}C-incorporation in etherel treated leaves than in the control (Table 2). Thus the etherel-treated plants allocated more photosynthetic metabolites towards essential oil than the control plants. Partitioning of photosynthetic metabolites between leaf and stem is an important factor in yield determination (Srivastava and Luthra 1991a). In stems, ^{14}C incorporation in ethanol soluble fraction, sugars, and organic acids was significantly higher in the etherel treated plants than in the control. Thus, ethanol soluble compounds remained untranslocated in the stem (Table 2).

When [U-^{14}C]-sucrose was fed to etherel treated leaves, the total ^{14}C incorporation was significantly higher than in the control. Incorporation into ethanol soluble fraction was significantly higher than that measured in the insoluble fraction. However, the label in sugars, organic acids, and essential oil fraction was significantly lower than in the control (Table 3). When these fractions were analysed in the stem, the ethanol-insoluble fraction had significantly higher label whereas the ethanol-soluble fraction had significantly lower amounts of labelled sugars, amino acids, and organic acids than the controls (Table 3). Thus the amount of compounds derived from added [U-^{14}C]-sucrose was higher in leaves and significantly lower in stems in etherel treated plants. Hence the capacity to utilize end products of photosynthetically fixed CO_2 and the externally applied sucrose was entirely different. Ontogenic changes exist for distribution of photosynthetically fixed $^{14}CO_2$ in peppermint leaves. The incorporation of $^{14}CO_2$ into sugars was maximum followed by organic acids, amino acids, and essential oil at all stages of leaf development. The incorporation into sugars and amino acids

Table 2. Changes in incorporation pattern of $^{14}CO_2$ into primary photosynthetic metabolic pool and essential oil in leaves and stems of *Mentha spicata* treated with etherel and gibberellic acid (GA). All values in 10^3 dps kg^{-1}(FM). */** Mean values significant at 5/1 % level of significance by pair *t*-test; NS - nonsignificant.

	Fractions	Etherel	Control	GA
Leaves	Ethanol-soluble fraction	225±26^{NS}	262±37	2667±3^{NS}
	Ethanol-insoluble fraction	1723±133**	1057±63	1608±50**
	Total incorporation	1948±157**	1320±97	4275±48*
	Sugar	128±3**	60±3	85±2*
	Amino acids	340±7^{NS}	195±800	703±183^{NS}
	Organic acids	140±5**	83±2	103±2^{NS}
	Oil	1.56±0.04**	0.51±0.03	1.28±0.03**
Stem	Ethanol-soluble fraction	160±8**	89±3	345±53*
	Ethanol-insoluble fraction	1377±717^{NS}	497±17	2753±377**
	Total incorporation	1538±717^{NS}	587±20	3098±358**
	Sugar	158±15*	35±5	177±47^{NS}
	Amino acids	933±283^{NS}	132±43	1300±400^{NS}
	Organic acids	160±8**	47±3	167±13**

declined as the leaf matured whereas the incorporation into essential oil and organic acids increased with leaf expansion and then decreased (Srivastava and Luthra 1991b). In onions, the older was the plant the more of ^{14}C-assimilate left the source leaf (Khan 1981).

The GA-treated plants had significantly lower contents of Chl pigments, P_N, E, and g_s, however the plant height was significantly higher than in the control (Table 1). GA treatment resulted in both higher total fixation of $^{14}CO_2$ and ^{14}C incorporation in ethanol-insoluble fraction and sugars of leaves. Significantly higher amounts of photosynthetic metabolites were translocated towards essential oils because the label was significantly higher in essential oil (Table 2). Amino acid and organic acid contents were not significantly affected over control. Similarly, the stem of GA-treated plants showed significantly higher total incorporation, contents of ethanol-soluble and -insoluble fraction, whereas the contents of organic acids, amino acids, and sugars were not significantly different than in the control (Table 2). Thus overall incorporation of $^{14}CO_2$ into metabolites and their higher subsequent translocation to oil biosynthetic pathway were higher in GA-treated plants.

As concerns the utilization pattern of [U-^{14}C]-sucrose, GA treatment resulted in leaves in significantly higher total incorporation, incorporation in ethanol-soluble fraction, amino acids, and essential oil, whereas ethanol-insoluble fraction and sugar contents were not significantly influenced (Table 3). In contrast, the contents of all these metabolites in stem were significantly not affected (Table 3).

Application of etherel and GA significantly decreases growth and physiological parameters which negatively affects herb yield. Hormone application in general does not bring a simultaneous increase in growth and oil accumulation. In Japanese

Table 3. Changes in incorporation pattern of [U-^{14}C]-sucrose into primary photosynthetic metabolites and in essential oil in *Mentha spicata* treated with etherel and gibberellic acid (GA). All values in 10^3 dps kg^{-1}(FM). */** Mean values significant at 5/1 % level of significance by pair *t*-test; NS - nonsignificant.

	Fractions	Etherel	Control	GA
Leaves	Ethanol-soluble fraction	6863±37**	2577±933	15695±583*
	Ethanol-insoluble fraction	2655±118^{NS}	3097±350	4425±1200^{NS}
	Total incorporation	9520±148**	5673±1300	20120±1300**
	Sugar	665±77**	2635±200	1775±90^{NS}
	Amino acids	52±15^{NS}	90±3	113±3*
	Organic acids	53±7*	137±3	120±5*
	Oil	1.01±0.07**	1.93±0.09	9555±100**
Stem	Ethanol-soluble fraction	10507±1450*	12308±1267	13637±317^{NS}
	Ethanol-insoluble fraction	3457±125*	1757±417	2715±567^{NS}
	Total incorporation	13963±1567^{NS}	14067±1667	16352±267^{NS}
	Sugar	852±55	1020±75	1188±6^{NS}
	Amino acids	68±3*	82±5	100±3^{NS}
	Organic acids	100±2*	108±3	108±3

mint, chlormequat chloride increased oil content but inhibited growth whereas ethephon decreased growth but had no significant effect on oil content (Farooqi and Sharma 1988). Hormones such as phosphon-D and daminozide influence enzymes and interconversion in essential oil biosynthesis (El-Keltawi and Croteau 1986a,b) and endogenous content of other hormones. However, it is not known how the carbon fixation capacity is affected by hormone application. Despite the decrease in herb yield, both hormone-treated plants contained higher amounts of the CO_2 fixation products. This probably results in greater translocation of photosynthetic metabolites to the oil biosynthetic pathway. The higher contents do not necessarily mean higher CO_2 efficiency; it could also mean that the fixed CO_2 is not utilized by the plant growth process whereas in control plants it is utilized and its content is lower.

The fed sucrose was poorly utilized for oil biosynthesis and simultaneously the content of photosynthetic metabolites was also low. Thus the utilization of CO_2 and sucrose for oil biosynthesis was different. The changes in growth could also be due to differences in partitioning of available assimilates between leaf and stem. The essential oil biosynthesis is an integration of several metabolic pathways which require linking of several steps such as continuous production of precursors, their transport and translocation to the active site of synthesis, and finally the oil accumulation. This sequence of steps depends on normal functioning of associated metabolic pathways. Any disruption in normal metabolic pathways affects the sequence of steps in oil biosynthesis. Thus a plant may alter/adopt its metabolic pathway in response to particular effect, such as nutrient imbalance, hormone application, *etc.* Under etherel and GA treatment there is higher accumulation of photosynthetic metabolites, nevertheless, the decrease in herb yield and growth may

be due to energy deficiency, membrane effects, or other control mechanisms which need to be investigated.

References

Arnon, D.I.: Copper enzymes in isolated chloroplasts: Polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Bernard, V., Nathaile, B., Christine, B.: Effect of day length on monoterpene conversion in leaves of *Mentha piperita*. - Phytochemistry **29**: 749-755, 1990.

Burbott, A.J., Loomis, W.D.: Effects of light and temperature on the monoterpene of peppermint. - Plant Physiol. **42**: 20-28, 1967.

Charles, D.J., Joly, R.J., Simon, J.E.: Effect of osmotic stress on the essential oil content and composition of peppermint. - Phytochemistry **29**: 2837-2840, 1990.

Clark, R.J., Menary, R.C.: Environmental effects on peppermint (*Mentha piperita* L.). I. Effect of day length, photon flux density, night temperature and day temperature on the yield and composition of peppermint oil. - Aust. J. Plant Physiol. 7: 685-692, 1980a.

Clark, R.J., Menary, R.C.: Environmental effects on peppermint (*Mentha piperita* L.). II. Effects of temperature on photosynthesis, photorespiration and dark respiration in peppermint with reference to oil composition. - Aust. J. Plant Physiol. 7: 693-697, 1980b.

Clevenger, J.F.: Apparatus for determination of essential oils. - J. amer. pharm. Assoc. **17**: 346, 1928.

El-Keltawi, N.E., Croteau, R.: Influence of ethephon and daminozide on growth and essential oil content of peppermint and sage. - Phytochemistry **25**: 285-1288, 1986a.

El-Keltawi, N.E., Croteau, R.: Influence of phosphon-D and cycocel on growth and essential oil content of sage and peppermint. - Phytochemistry **25**: 1603-1606, 1986b.

Farooqi, A.H.A., Sharma, S.: Effect of growth retardants on growth and essential oil content in Japanese mint. - Plant Growth Regul. 9: 65-71, 1988.

Gershenzon, J., Croteau, R.: Regulation of monoterpene biosynthesis in higher plant. - In: Towers, G.H.N., Stafford, H.A. (ed.): Biochemistry of Mevalonic Acid Pathway to Terpenoids. Pp. 99-159. Plenum Press, New York 1991.

Gershenzon, J., Croteau, R.: Terpenoid biosynthesis: The basic pathway and formation of monoterpenes, sesquiterpernes and diterpenes. - In: Moore, T.S., Jr. (ed.): Lipid Metabolism in Plants. Pp. 339-388. CRC Press, London 1993.

Gershenzon, J., Maffei, M., Croteau, R.: Biochemical and histochemical localization of monoterpene biosynthesis in the glandular trichomes of spearmint (*Mentha spicata*). - Plant Physiol. **89**: 1351-1357, 1989.

Hoagland, D.R, Arnon, D.I.: The water culture method for growing plants without soil. - Circ. Calif. agr. exp. Stat. **347**: 1-32, 1938.

Khan, A.A.: Effect of leaf position and plant age on the translocation of ^{14}C-assimilates in onion. - J. agr. Sci. (Cambridge) 96: 451-455, 1981.

Lawrence, B.M.: Essential oil production. A discussion of influencing factors. - In: Parliment, T.H., Croteau, R. (ed.): Biogeneration of Aroma. Pp. 363-369. Amer. chem. Soc., New York 1986.

Marschner, H.: Growth. - In: Marschner, H. (ed.): Mineral Nutrition of Higher Plants. Pp. 269-340. Academic Press, New York 1986.

McGarvey, D., Croteau, R.: Terpenoid metabolism. - Plant Cell 7: 1015-1026, 1995.

Shukla, A., Farooqi, A.H.A., Shukla, Y.N., Sharma, S.: Effect of triacontanol and chloromequat on growth, plant hormones and artemisinin yield in *Artemisia annua* L. - Plant Growth Regul. **11**: 165-171, 1992.

Srivastava, N.K., Luthra, R.: Interspecific variation in mints for photosynthetic efficiency, and ^{14}C primary metabolic pool in relation to essential oil accumulation. - J. Plant Physiol. **138**: 650-654, 1991a.

Srivastava, N.K., Luthra, R.: Distribution of photosynthetically fixed $^{14}CO_2$ into essential oil in relation to primary metabolites in developing peppermint (*Mentha piperita*) leaves. - Plant Sci. **76**: 153-157, 1991b.

Srivastava, N.K., Luthra, R.: Relationships between photosynthetic carbon metabolism and essential oil biogenesis in peppermint under Mn-stress. - J. exp. Bot. **45**:1127-1132, 1994.

Srivastava, N.K., Luthra, R., Naqvi, A.: Relationship of photosynthetic carbon assimilation to essential oil accumulation in developing leaves of Japanese mint. - Photosynthetica **24**: 406-411, 1990.

Srivastava, N.K., Misra, A., Sharma, S.: Effect of Zn deficiency on net photosynthetic rate, ^{14}C partitioning, and oil accumulation in leaves of peppermint. - Photosynthetica **33**: 71-79, 1997.

Srivastava, N.K., Sharma, S.: Effect of tricontanol on photosynthetic characteristics and essential oil accumulation in Japanese mint (*Mentha arvensis* L.). - Photosynthetica **25**: 55-60, 1991.