Phytochemistry, Vol. 26, No. 4, pp. 891–895, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

INFLUENCE OF FOLIAR APPLIED CYTOKININS ON GROWTH AND ESSENTIAL OIL CONTENT OF SEVERAL MEMBERS OF THE LAMIACEAE*

NAIEM E. EL-KELTAWI† and RODNEY CROTEAU

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340, U.S.A.

(*Revised received* 15 *June* 1986)

Key Word Index—*Mentha piperita*; *M. spicata*; *M. suaveolens*; *Lavandula vera*; *Salvia officinalis*; Lamiaceae; metabolism; essential oils; monoterpenes; cytokinins.

Abstract—Foliar application of the cytokinins kinetin, diphenylurea, benzylaminopurine and zeatin at the 1–10 ppm level has a general growth promoting effect on *Mentha piperita*, *M. spicata* and *Salvia officinalis*, but not on *M. suaveolens* and *Lavandula vera*, grown under controlled environmental conditions. The essential oil yield of cytokinin-treated plants is also increased up to two-fold on a fr. wt basis relative to untreated controls, with only a minor influence on oil composition in most cases. The increase in oil yield cannot be attributed to alteration in growth or development of the treated plants, or to changes in oil gland populations. *In vitro* assay of the enzymes catalysing the rate limiting steps of camphor biosynthesis in *S. officinalis* and of menthone biosynthesis in *M. piperita* indicated that the increase in oil yield under the influence of cytokinin is a result of increased monoterpene biosynthesis.

INTRODUCTION

The physiological responses of plants to applied cytokinins are numerous and diverse [1, 2]. Most investigations of the biological activity of these compounds have focussed on aspects of plant growth and development, or of primary metabolism; however, the effects of cytokinins on the metabolism of natural products such as alkaloids [3], betacyanins [4] and anthocyanins [5] are also known. Whether the influence(s) of cytokinins on 'secondary metabolism' may be direct or incidental is in no instance certain.

The influence of cytokinins on the biosynthesis and accumulation of terpene components of essential oils appears to have received little experimental attention, in spite of the fact that these natural products are easily analysed and their accumulation is invariably associated with highly segregated structures (e.g. secretory trichomes) [6, 7], a feature which could more readily permit distinction between primary and secondary cytokinin effects. Pretreatment of peppermint rhizomes with kinetin, benzylaminopurine and related cytokinins increased both herbage (by 15–20%) and essential oil content (by 15–50%) without altering oil composition of the resulting plants [8–10]. It was not possible from these studies to distinguish any direct influence of bioregulator on terpene metabolism from those alterations in growth which may have resulted from differences in early development.

The present study was undertaken to examine the influence of four foliar-applied cytokinins (kinetin, zeatin, benzylaminopurine and diphenylurea) on established plants (*Mentha piperita*, *M. spicata*, *M. sauveolens*, *Salvia officinalis* and *Lavandula vera*) grown under controlled conditions. These commercially important oil producing species were chosen for this work because the oil compositions are well known [11–13] and because the biosynthetic origins of most of the relevant monoterpene constituents have already been deciphered [14–16].

RESULTS AND DISCUSSION

Influence of cytokinins on growth

Foliar application of the four cytokinins tested (kinetin, diphenylurea, benzylaminopurine and zeatin) at concentrations of 1–10 ppm produced fairly uniform effects on the growth of *M. piperita*, *M. spicata* and *S. officinalis*. Kinetin resulted in a 30–60% increase in total fr. wt compared to controls, with *ca* 40% increase in leaf weight and 50–60% gain in weight of the mainstem. Leaf length increased (10–20%) as did width (12–18%) and number of leaves per branch (*ca* 10%). The mainstem length was markedly increased (30–50%) as were the number of internodes (20–30%). Internode length was increased by *ca* 15%. Diphenylurea provided a less pronounced increase in fr. wt (15–30%), which was nearly equally distributed between leaves, mainstem and branches. Leaf length and width were again increased (by 25% and 20%, respectively) as was the number of leaves per branch (*ca* 30%). Total stem length was increased by up to 40%, and while the internode length actually decreased (by 20%) compared to controls, the increase in number of internodes (50%) more than compensated. Benzylaminopurine provided an increase in fr. wt of up to

*This is Scientific Paper No. 7427, Project 0268, College of Agricultural Research Center, Washington State University, Pullman, Washington, 99164-6340, U.S.A. This investigation was supported in part by Department of Energy Contract DE-AM06-76RL02221, Agreement DE-AT06-82ER12027, and by grants from the Washington Mint Commission and Mint Industry Research Council.

†AMIDEAST Peace Fellow from the Department of Horticulture, Assiut University, Assiut, Egypt.

25% and was in most other effects similar to kinetin. Zeatin afforded less than 10% increase in all of the growth parameters examined. Identical treatment of *M. suaveolens* and *L. vera* with all four cytokinins produced no discernible effect on growth.

In an attempt to access the influence of cytokinins on development, the time to flowering (of 50% of the plants) at the highest treatment level was compared to that of untreated controls. No significant differences were observed for *M. piperita*, *M. spicata* or *S. officinalis*; however, both *M. suaveolens* and *L. vera*, for which no growth effects were observed, appeared to flower with somewhat greater profusion from 7–10 days earlier. This time difference was minor (< 10% of the control) and of uncertain significance since too few samples were carried through this length of time to permit adequate statistical analysis.

Leaf oil gland number, size and surface density were microscopically assessed for the highest treatment level at the time of harvest. Although these analyses are difficult to carry out with precision, it was clear that in oil gland size and total number the treated plants could not have exceeded the untreated controls by more than 20%. The treated plants also evidenced a corresponding decrease (by 10–20%) in abaxial and adaxial surface gland density. It does not appear that these differences could account for the differences in essential oil yield observed (see below).

Influence of cytokinins on essential oil yield

Of the four cytokinins tested, kinetin and diphenylurea were most effective in increasing essential oil yield. Foliar application of kinetin at 4 ppm resulted in a 60–80% increase in oil yield on a percentage fr. wt basis (Table 1) in all test species except *L. vera* (*ca* 30%), and nearly doubled oil yield on a mg/plant basis for *M. piperita*, *M. spicata* and *S. officinalis*. Diphenylurea at 10 ppm was even more effective, essentially doubling oil yield on both a percentage fr. wt basis and mg/plant basis in all cases. The influence on peppermint was particularly notable, a 2.7-fold increase in oil yield per plant being observed.

Benzylaminopurine and zeatin were generally less effective, promoting lesser, but still significant, increases in oil yield on both bases (Table 1). *L. vera* was a notable exception in responding quite favourably to low concentrations of benzylaminopurine. A doubling of the highest concentration of each cytokinin indicated in Table 1 resulted in a decline in maximum oil yield by *ca* 15% with all cytokinins for all species tested.

The observed increases in oil yield per plant are attributable, in part, to cytokinin induced growth of *M. piperita*, *M. spicata* and *S. officinalis*. However, in no instance was total fr. wt increased by more than 40%, nor was leaf weight or size increased by more than 50%. As the leaf oil glands represent the major sites of monoterpene biosynthesis [6, 7], the doubling of oil yield observed certainly cannot be fully explained by the increase in leaf size and weight. Additionally, both *M. suaveolens* and *L. vera* exhibited significant increases in oil yield on both percentage fr. wt and per plant bases in response to cytokinins (Table 1), yet neither species evidenced a discernible increase in growth. Thus, the growth-promoting influence of cytokinins could be eliminated as an influence on oil yield in these cases.

Influence of cytokinins on essential oil composition

The cytokinins tested had no appreciable effect on the oil composition of *M. suaveolens* and *M. spicata*, both species which produce oils containing relatively few components. Thus, the neoiso(iso)pulegol content of *M. suaveolens* oil was essentially invariant (82 ± 2%), while the limonene (12%) and carvone (55%) content of *M. spicata* did not vary significantly regardless of yield. In the oil of *L. vera*, the borneol level increased from 25% in the control to 32–33% at maximal yield with each cytokinin and was accompanied by an increase in 1,8-cineole content (from 7 to 9%); all other components exhibited a slight but uniform decrease. In the case of *M. piperita*, both kinetin and diphenylurea promoted an increase in (−)-menthone content (36–43%) and decrease in (+)-isomenthone content (22–17%) at maximum oil yield, without significantly influencing the levels of the respective derived alcohols (−)-menthol (~ 20%) and (+)-neoisomenthol (~ 2%). Kinetin and diphenylurea are the first of several bioregulators thus far examined [17, 18] to promote an increase in the level of (−)-menthone. Benzylaminopurine and zeatin, which had lesser effects on oil yield, had essentially no effect on menthone content, but did increase the content of isomenthone (from 21 to 26%) and of the derived alcohol (+)-neoisomenthol (2–8%), while decreasing the concentration of (−)-menthol derived from (−)-menthone (from *ca* 21 to 15%). A similar alteration in the composition of *M. piperita* oil was previously observed upon treatment with daminozide [18].

The four cytokinins had a notably uniform influence on the composition of *S. officinalis* essential oil at the treatment levels producing the highest yield. The (−)-3-isothujone concentration (44 ± 1%) was significantly higher than control values (~ 32%), whereas those of both 1,8-cineole and β-pinene decreased, from 10 to 7.5% and from 8 to 5%, respectively. The amount of (+)-camphor (*ca* 20%) was unaffected by cytokinin treatment. Similar alterations of terpene composition in *S. officinalis*, as well as increased oil yield, have been previously observed under the influence of the growth retardant Phosfon D [17], the gross developmental effects of which were quite the opposite of those noted with foliar applied cytokinin.

Although significant differences in the relative percentage of several monoterpenes were observed, most notably in the oxygenated monoterpene fraction, in no instance did cytokinin treatment drastically alter oil composition of the five species tested. It should also be emphasized that although *relative* decreases in the percentage composition of several monoterpene components of the various essential oils were noted, there was a clear increase in the *absolute* levels (as % fr. wt or mg/plant) of all monoterpenes examined in the plants that had been treated with cytokinins in all cases. Thus, the primary influence of cytokinins was to stimulate the overall accumulation of monoterpenes typical of the respective essential oils.

Influence of cytokinins on monoterpene metabolism

Changes in the relative terpene composition of essential oils under the influence of bioregulators [17, 18] have

Table 1. Effect of cytokinins on essential oil yield

Treatment	ppm	*M. piperita* (% fr. wt)	*M. piperita* (mg/plant)	*M. spicata* (% fr. wt)	*M. spicata* (mg/plant)	*M. suaveolens* (% fr. wt)	*M. suaveolens* (mg/plant)	*L. vera* (% fr. wt)	*L. vera* (mg/plant)	*S. officinalis* (% fr. wt)	*S. officinalis* (mg/plant)
Control		0.20	34.5	0.31	41.7	0.17	23.0	0.047	6.4	0.18	33.3
Kinetin	1	0.26	66.5	0.42	76.7	0.21	26.3	0.061	8.0	0.26	37.5
Kinetin	2	0.26	64.8	0.44	77.1	0.23	31.7	0.078	10.3	0.30	50.7
Kinetin	4	0.34	71.7	0.51	83.3	0.27	29.3	0.060	7.8	0.23	64.2
Diphenylurea	2.5	0.38	75.0	0.56	86.2	0.30	33.3	0.082	11.6	0.28	44.4
Diphenylurea	5	0.38	72.5	0.59	88.7	0.33	35.7	0.087	13.2	0.35	61.5
Diphenylurea	10	0.41	93.5	0.61	99.3	0.37	38.7	0.089	13.5	0.39	67.5
Benzylaminopurine	1	0.21	35.8	0.34	43.0	0.21	26.3	0.081	12.2	0.25	37.1
Benzylaminopurine	2	0.22	38.5	0.34	46.3	0.24	30.0	0.078	9.3	0.26	37.8
Benzylaminopurine	4	0.24	44.7	0.36	49.2	0.25	25.7	0.057	8.7	0.28	39.3
Zeatin	1	0.20	48.2	0.32	53.1	0.21	27.2	0.078	9.2	0.27	45.4
Zeatin	2	0.21	50.0	0.33	54.4	0.21	26.3	0.074	10.1	0.27	45.1
Zeatin	4	0.23	47.2	0.35	54.7	0.24	27.8	0.063	9.3	0.28	46.3
LSD (0.05)		0.03	2.9	0.04	3.2	0.04	2.4	0.014	0.7	0.03	3.3

PHYTO 26:4-B

been rationalized largely in terms of an alteration in proportions of monoterpene reductases, such as those responsible for the conversion of sabinone to thujone and isothujone in *S. officinalis* [19] and for the conversion of pulegone to menthone and isomenthone in *M. piperita* [20, 21]. However, the enzymatic basis for the increase in terpene yield observed upon such treatment has not been explicitly addressed. In the present case, the increase in terpene production under the influence of cytokinins, especially kinetin and diphenylurea, was greater than could be readily accounted for by alteration in growth and development, or in oil gland population and density. Thus, the possibility of a more direct effect on terpene metabolism was suggested. To examine this possibility, two key enzymes were examined in cell-free preparations from the leaves of *S. officinalis* and *M. piperita*, the two species with which we were most familiar and upon which diphenylurea had the greatest effect (Table 1). The first of these enzymes was the geranyl pyrophosphate: (+)-bornyl pyrophosphate cyclase from *S. officinalis*, which catalyses the committed step in (+)-camphor biosynthesis and which is presumed to be the rate limiting step in the pathway since the intermediates bornyl pyrophosphate and borneol do not accumulate to a significant extent [22, 23]. The second enzyme was geranyl pyrophosphate: (−)-limonene cyclase from *M. piperita*, which catalyses the committed step in the formation of (−)-menthone and (+)-isomenthone and which, for similar reasons, is assumed to be rate limiting in this biosynthetic sequence [20]. Extraction and assay procedures for these enzymes have been previously described [20, 22, 24].

Crude soluble enzyme preparations (105 000 *g* supernatants) were identically prepared from matched leaves (fourth leaf pairs of *M. piperita* and third leaf pairs of *S. officinalis*) of untreated controls and plants treated with 10 ppm diphenylurea using two isolation procedures in which the entire leaf was homogenized [20,22] or in which only the epidermis, removed by mechanical abrasion, was homogenized prior to centrifugation [24]. The assay for activity was based on the rate of conversion of [1-^{3}H]geranyl pyrophosphate to the relevant, chromatographically purified, cyclic product [20, 22]. The level of activity of bornyl pyrophosphate cyclase obtained from treated *S. officinalis* leaves was an average of 1.7-fold higher than that from comparable preparations from untreated leaves when compared on the basis of leaf surface area or fr. wt, and *ca* 2.5-fold higher when compared on a per leaf or per mg epidermis protein basis. The difference in activity levels of limonene cyclase was even greater between preparations from treated and control leaves of *M. piperita*. On either a surface area or fr. wt basis, the limonene cyclase levels were *ca* two-fold higher from the diphenylurea-treated plants than from untreated controls, whereas three-fold higher levels were observed in the treated plants on either a per leaf or per mg epidermis protein basis. The increase in the two enzyme activities measured would be sufficient to account for the increase in oil yield observed, if these two activities can be taken as a general indicator of terpene biosynthesis.

Whether the increase in the rate of conversion of geranyl pyrophosphate to cyclic product as determined by *in vitro* assay represents an increase in enzyme level or activity *per se* is not presently known, nor have we yet assessed possible alterations in catabolic processes [25, 26]. In spite of these uncertainties, it is clear that foliar application of cytokinin stimulates the accumulation of essential oil terpenes, at least in part, by direct effect on monoterpene metabolism.

EXPERIMENTAL

Plant material. S. officinalis L. and *L. vera* D.C. were grown from seed, and *M. piperita* L., *M. spicata* L. and *M. suaveolens* Ehrh. (*M. rotundifolia*) were propagated from single-node cuttings of etiolated rhizomes [27]. The plants were grown in peat moss in a growth chamber with a 14 hr photoperiod (900 ± 100 fc, fluorescent/incandescent), 29° day/25° night temp. cycle and r.h. of 62 ± 12%. Plants were watered as needed and fertilized weekly with a complete fertilizer (N:P:K, 1:1:1, with microelements and Fe chelate). Plants were thinned and allowed to grow for 5 weeks before treatment with kinetin, diphenylurea, benzylaminopurine and zeatin.

Treatments. Each expt was conducted in randomized complete block design with 3 replications totalling 60 plants per treatment. Cytokinins at the indicated concns were prepd in H_2O containing 0.1% Tween 20 and sprayed to the point of run-off with a hand sprayer. Three additional applications were made at weekly intervals and the plants were harvested 1 week after the last treatment. Controls sprayed without growth regulator were included in each expt. Data on growth characteristics (fr. wt plant, leaves, stem and total branches; length, width and number of leaves; length of stem; number of internodes and internode length) were collected and subjected to analysis of variance with comparison by least significant differences at $P = 5\%$ (LSD 0.5) [28].

Oil analysis. A minimum of 3 representative 10 g samples of fr. tissue were steam distilled using a simultaneous steam distillation–extraction apparatus (J & W Scientific), employing as int. stds (+)-isomenthone for *S. officinalis* and *L. vera*, and (+)-fenchone for the *Mentha* species. On completion of distillation (1 hr) the essential oil collected (in pentane) was dried (Na_2SO_4) and kept under N_2 in a sealed glass tube in the dark at −20° until analysis. Oil analysis (1 μl samples) was performed by capillary GC (FID at 230°, 100:1 injection split at 220°) on a 25 m Carbowax 20 M WCOT column operated at 4 ml/min H_2 and programmed from 45° (5 min hold) to 180° at 10°/min. FID output was electronically integrated and fr. wt yield and per plant yield were calculated based on the int. stds. Yield and relative percentage of major oil constituents (> 3%) were also determined. Data were statistically analysed as before [28], and the LSD (0.05) of the means are reported. Identifications of oil components based on RR_t were confirmed by GC/MS comparison of R_t and MS to authentic standards.

In vitro studies. Extracts of *S. officinalis* and *M. piperita* leaves were prepd as described elsewhere [20, 22] and the resulting homogenates were centrifuged at 105 000 *g* to obtain the sol. supernatant used as the enzyme source. The prepns were concd as necessary by ultrafiltration (Amicon PM 30) and dialysed to the appropriate assay conditions for the Mg^+-dependent conversion of [1-^{3}H]geranyl pyrophosphate to the relevant cyclic monoterpenes. The specific procedures for the assay of geranyl pyrophosphate: (+)-bornyl pyrophosphate cyclase (*S. officinalis*) and geranyl pyrophosphate: (−)-limonene cyclase (*M. piperita*), and for the chromatographic isolation and determination of the radiolabeled products, are described elsewhere [20, 22]. The technique for the selective isolation of leaf epidermis and the prepn of cell-free extracts from this tissue, have also been described [24]. Fr. wt and surface area of mid-stem leaves used in these expts were determined immediately prior to enzyme prepn and protein concns in the resulting extracts were estimated by the dye-binding method [29].

REFERENCES

1. Fox, J. E. (1969) in *Physiology of Plant Growth and Development* (Wilkins, M. B., ed.) p. 85. McGraw-Hill, London.
2. Schneider, E. A. and Wightman, F. (1978) in *Phytohormones and Related Compounds—A Comprehensive Treatise* (Letham, D. S., Goodwin, P. B. and Higgins, T. J. V., eds) Vol. 1, p. 29. Elsevier/North Holland, Amsterdam.
3. Stearns, E. M. Jr., and Morton, W. T. (1975) *Phytochemistry* **14**, 619.
4. De Nicola, M. G., Amico, V. and Piattelli, M. (1975) *Phytochemistry* **14**, 989.
5. Pecket, R. C. and Bassim, T. A. H. (1974) *Phytochemistry* **13**, 1395.
6. Croteau, R. and Johnson, M. A. (1984) in *Biology and Chemistry of Plant Trichomes* (Rodriguez, E., Healey, P. L. and Mehta, I., eds) p. 133. Plenum Press, New York.
7. Fahn, A. (1979) *Secretory Tissues in Plants*, pp. 158–222. Academic Press, New York.
8. Zlatev, S., Zlateva, M. and Iliev, L. (1977) *Perfum. Flavor* **2**, 56.
9. Zlatev, S., Iliev, L., Zlateva, M. and Vasilev, G. (1978) *Rast. Nauk.* **15**, 51.
10. Zlatev, S., Iliev, L., Vasilev, G. and Zlateva, M. (1980) *C. R. Acad. Bulg. Sci.* **33**, 555.
11. Lawrence, B. M., Hogg, J. W. and Terhune, S. J. (1971) *Parf. Cosm. Sav. Fr.* **1**, 256.
12. Embong, M. B., Haziyev, D. and Molnar, S. (1977) *Can. Inst. Food Sci. Technol. J.* **10**, 201.
13. Lawrence, B. M. (1981) in *Essential Oils* (Mookherjee, B. D. and Mussinan, C. J., eds) p. 1. Allured, Wheaton.
14. Croteau, R. (1981) in *Biosynthesis of Isoprenoid Compounds* (Porter, J. W. and Spurgeon, S. L., eds) Vol. 1, p. 225. Wiley, New York.
15. Croteau, R. (1984) in *Isopentenoids in Plants; Biochemistry and Function* (New, W. D., Fuller, G. and Tsai, L.-S., eds) p. 31. Dekker, New York.
16. Croteau, R. (1986) in *Herbs, Spices, and Medicinal Plants: Recent Advances in Botany, Horticulture, and Pharmacology* (Craker, L. E. and Simon, J. E., eds) Vol. 1, p. 81, Oryx Press, Phoenix.
17. El-Keltawi, N. E. and Croteau, R. (1986) *Phytochemistry* **25**, 1603.
18. El-Keltawi, N. E. and Croteau, R. (1986) *Phytochemistry* **25**, 1285.
19. Karp, F. and Croteau, R. (1982) *Arch. Biochem. Biophys.* **216**, 616.
20. Kjonaas, R. and Croteau, R. (1983) *Arch. Biochem. Biophys.* **220**, 79.
21. Kjonaas, R., Martinkus-Taylor, C. and Croteau, R. (1982) *Plant Physiol.* **69**, 1013.
22. Croteau, R. and Karp, F. (1979) *Arch. Biochem. Biophys.* **198**, 512.
23. Croteau, R., Felton, M., Karp, F. and Kjonaas, R. (1981) *Plant Physiol.* **67**, 820.
24. Croteau, R. and Cane, D. E. (1985) *Methods Enzymol.* **110A**, 383.
25. Croteau, R., El-Bialy, H. and El-Hindawi, S. (1984) *Arch. Biochem. Biophys.* **228**, 667.
26. Croteau, R. and Sood, V. K. (1985) *Plant Physiol.* **77**, 801.
27. El-Keltawi, N. E. and Croteau, R. (1986) *Sci. Hortic.* **29**, 101.
28. Snedecor, G. W. and Cochran, W. G. (1973) *Statistical Methods*, 6th edn. Iowa State University Press, Ames.
29. Anonymous (1977) Technical Bulletin 1051, Bio-Rad Laboratories, Richmond, California.