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INFLUENCE OF ETHEPHON AND DAMINOZIDE ON GROWTH AND ESSENTIAL OIL CONTENT OF PEPPERMINT AND SAGE*

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Key Word Index—*Mentha piperita*; peppermint; *Salvia officinalis*; sage; Lamiaceae; metabolism; essential oils; monoterpenes; growth regulators; Ethephon; Daminozide.

Abstract—Foliar application of Daminozide at 1000 ppm reduces the growth of *Salvia officinalis* (sage) and decreases essential oil yield, but increases both growth and essential oil yield of *Mentha piperita* at the same concentration. Ethephon at 250 ppm reduces the growth and essential oil yield of peppermint and slightly increases growth and essential oil content of sage. Both growth regulators markedly reduce the level of menthone and menthol in peppermint oil and increase the level of isomenthone and neoisomenthol. Both growth regulators decrease the level of camphor and increase the level of β-pinene in sage oil. Changes in essential oil composition induced by these growth regulators are most readily explained by alterations in the levels or activities of the relevant biosynthetic enzymes.

INTRODUCTION

The effects of Ethephon (2-chloroethylphosphonic acid) and Daminozide (*N*-dimethylaminosuccinamic acid) on plant growth and development are well known [1–3], yet the influence of these growth regulators on essential oil production, and secondary metabolism in general, has received little attention. Foliar application of both Ethephon and Daminozide at a concentration of 0.1 mM was reported to increase plant height, as well as stem, shoot and leaf number in *Mentha piperita* and *Mentha crispa* under field conditions [4], and both compounds increased the yield of essential oil on a fresh weight basis [5]. The application of Daminozide was reported to decrease the menthol content and increase the neomenthol and isomenthone content of the oil of both species, whereas treatment with Ethephon was reported to increase the menthol level in *M. piperita* and reduce the level of this monoterpenol in the oil of *M. crispa* [5]. These findings, although difficult to rationalize in biogenetic terms based on the known origin of the various menthone and menthol isomers [6–9], imply a direct influence of the growth regulators on the metabolism of the monoterpenes which comprise the essential oil of *Mentha*.

The present study was undertaken to examine in greater detail the effects of Ethephon and Daminozide on *M. piperita* (peppermint), and to extend this approach to *Salvia officinalis* (sage), another common essential oil producing species for which the biosynthetic origins of all the major monoterpene components are known [10].

RESULTS

Influence of Daminozide on peppermint

Foliar application of Daminozide (B-Nine, Alar, *N*-dimethylaminosuccinamic acid) at the 500 or 1000 ppm levels resulted in darkening of the leaves and a 10–30% increase in plant weight, leaf length and width, number of leaves per branch and number of internodes. Total leaf and branch weight doubled at the 1000 ppm level and the weight of the main stem was increased by about 50% compared to the untreated controls. Daminozide up to 1000 ppm had no influence on plant height (*ca* 14.5 cm); however, the internode length was consistently reduced by over 10% (the compensation to plant height being provided by the increase in number of internodes). Application at the 2000 ppm level resulted in a general decrease of vegetative characteristics, most notably a reduction (*ca* 35%) in plant height and internode length relative to controls. However, leaf size and weight remained higher for treated plants than for untreated controls, by roughly 30% and 50%, respectively.

Daminozide at the 500 and 1000 ppm levels increased the essential oil content of peppermint by roughly 50% on both a fresh weight and per plant basis compared to controls (Table 1). At 2000 ppm of this growth regulator the yield per plant declined to values only slightly higher than the controls. In terms of oil composition, Daminozide had a pronounced effect in decreasing the level of the ketone (−)-methone and its reduction product (−)-menthol, and in increasing the level of the epimeric ketone (+)-isomenthone and its reduction product (+)-neoisomethol (Table 1). The level of 1,8-cineole in the distilled oil was also significantly reduced by Daminozide treatment compared to the control.

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Table 1. Effect of growth regulators on yield and composition of peppermint oil

Treatment	ppm	Oil yield		Oil composition (%)				
		% fr. wt	mg/plant	menthone	isomenthone	menthol	neoisomenthol	1,8-cineole
Control		0.24	43.2	40.0	16.3	21.6	3.8	4.9
Daminozide	500	0.34	50.5	39.4	20.5	17.3	10.9	3.9
Daminozide	1000	0.32	50.8	32.9	21.8	14.7	13.0	3.6
Daminozide	2000	0.31	44.5	27.5	30.9	13.7	16.7	3.4
LSD (0.05)		0.08	4.9	3.8	4.6	5.9	1.7	0.4
Control		0.26	48.1	41.5	14.6	18.6	1.8	5.8
Ethephon	125	0.30	51.3	39.6	19.4	16.7	4.9	6.1
Ethephon	250	0.22	25.5	27.4	35.2	11.3	11.3	6.7
Ethephon	500	0.15	8.9	3.1	55.1	10.7	12.0	5.8
Ethephon	1000	0.11	1.8	1.8	45.8	8.2	11.0	5.2
LSD (0.05)		0.06	8.4	3.5	11.5	2.4	1.7	0.5

Influence of Daminozide on sage

The response of sage to Daminozide was similar to that observed with peppermint, application at 500 or 1000 ppm resulting in leaf darkening and a 15–30% increase in leaf size and tissue weight compared to untreated controls. However, a notable difference was observed with sage in both plant height and internode length, which were decreased by 32% and 44%, respectively, even at the 500 ppm level. The greater sensitivity of sage to stunting by Daminozide was confirmed by the 2000 ppm treatment which resulted in a decrease of all vegetative characteristics, including a reduction in plant height of nearly 60%.

Oil yield on a fresh weight basis was slightly increased by treatment with 250 ppm Daminozide where stunting was minimal, but decreased slightly at the 500 and 1000 ppm treatment levels. Yield rose again at 2000 ppm where stunting was severe. However, oil yield on a per plant basis was not significantly influenced by Daminozide at the levels tested compared to the untreated control. The effects of Daminozide on the composition of sage oil were less pronounced than those observed with peppermint. Treatment with the growth regulator did increase the proportion of the monoterpene olefin β-pinene, with lesser effect on α-pinene and camphene (Table 2). The proportions of both (+)-3-thujone and (+)-camphor were reduced by treatment with the growth regulator (Table 2), but the levels of other monoterpenes and of the sesquiterpene olefins caryophyllene and humulene were essentially uneffected (data not shown).

Influence of Ethephon on peppermint

Foliar application of Ethephon (Ethrel, Bromeflor, 2-chloroethylphosphonic acid) in the 125–1000 ppm range resulted in a general loss of green coloration, a pronounced increase in anthocyanin pigmentation in both leaves and stems, and variable degrees of chlorosis at the highest application level. Stunting was slight at the 125 ppm level, but with a notable reduction in leaf size (*ca* 50%), whereas stunting was severe at the 1000 ppm concentration. At 500 ppm Ethephon, tissue weight was reduced by 50%, leaf size by 70%, and both plant height and internode length by 75%. At all concentrations of this regulator rosetting of the plants was prominent, and at the 500 ppm level the leaf number was fully doubled.

Treatment of peppermint with Ethephon in excess of 125 ppm resulted in a decrease in oil yield on both a fresh

Table 2. Effect of growth regulators on yield and composition of sage oil

Treatment	ppm	Oil yield		Oil composition (%)					
		% fr. wt	mg/plant	α-pinene + camphene	β-pinene	1,8-cineole	isothujone	thujone	camphor
Control		0.15	40.1	7.9	4.2	8.8	37.7	5.1	23.6
Daminozide	250	0.17	41.7	7.7	5.0	8.9	38.3	5.3	22.4
Daminozide	500	0.14	41.2	7.5	5.4	7.4	42.0	5.1	20.0
Daminozide	1000	0.13	40.2	11.0	6.8	8.2	37.4	4.0	18.3
Daminozide	2000	0.18	42.4	8.0	5.6	9.1	38.8	3.7	20.5
LSD (0.05)		0.01	9.5	1.3	0.9	0.8	NS	0.8	2.0
Control		0.16	39.7	9.7	3.7	9.2	42.9	4.2	18.7
Ethephon	250	0.18	44.7	8.6	8.2	9.2	43.0	5.5	15.0
Ethephon	500	0.13	36.6	8.8	11.0	8.4	39.3	6.1	14.6
Ethephon	1000	0.12	29.7	8.5	13.5	8.4	36.5	7.3	14.3
LSD (0.05)		NS	9.9	NS	2.1	NS	NS	2.2	1.9

weight and per plant basis, and the effect was strongly concentration dependent (Table 1). As was previously observed using Daminozide, the major influence of Ethephon on oil composition was a severe reduction in the level of menthone and its derivative menthol, and a corresponding increase in the level of isomenthone and the related alcohol neoisomenthol (Table 1).

Influence of Ethephon on sage

In marked contrast to observations with peppermint, Ethephon treatment of sage resulted in a 20–35% increase in tissue weight and a 20–25% increase in plant height at the concentrations tested (250–1000 ppm). As with peppermint, leaf size of sage was reduced (by about 60% at 1000 ppm) compared to the controls, and leaf number was nearly doubled (at 500 and 1000 ppm) due to increased lateral branching. Leaf coloration was unchanged.

Ethephon treatment of sage resulted in an increase in β-pinene content of the oil, with a corresponding, but smaller, decrease in the content of the other monoterpne olefins (Table 2). The (+)-3-thujone level was also elevated by Ethephon treatment, whereas the content of all other monoterpenes decreased somewhat. The proportion of sesquiterpenes in the oil (*ca* 7%) was uneffected by treatment with this growth regulator.

DISCUSSION

Results of this study indicated that sage is far more sensitive to the dwarfing influence of Daminozide than is peppermint, and, conversely, that peppermint is more sensitive to the influence of Ethephon than sage. Independent studies indicated that the effects of the regulators on growth and essential oil content were cumulative, and that three weekly applications at a given level had roughly the same effect on growth and oil production as did a single application at twice the concentration. In general, oil yield decreased with increased stunting, but at low levels of growth regulator, oil yield increased, most notably in peppermint under the influence of Daminozide. Very similar compositional changes were produced by both growth regulators in the essential oils of sage and peppermint, which could not be attributed to gross differences in development. Thus, control experiments afforded essential oils which were typical in both yield and composition [11–13], and the changes induced by the growth regulators were the opposite of those expected from normal maturation (i.e. an increase in the menthol content of peppermint [14] and an increase in the camphor content of sage [15]). Furthermore, maturation of the treated plants was unaffected by Daminozide and Ethephon as judged by time to flowering. Additionally, the changes brought about by growth regulator treatment could not be attributed to changes in leaf oil gland populations, since microscopic examination of control and treated plants evidenced no observable difference in leaf gland numbers. The alteration in oil yield and composition as a result of growth regulator treatment was, therefore, ascribed to selective changes at the enzyme level.

During the normal course of monoterpene metabolism in peppermint the olefin limonene is converted to the α,β-unsaturated ketone pulegone (5-methyl-2-isopropylidene cyclohexanone) [9]. The bulk of the pulegone undergoes NADPH-dependent reduction of the isopropylidene function by a reductase to afford (−)-menthone [6, 7], and the carbonyl function of this saturated ketone is then stereospecifically reduced by a dehydrogenase to afford (−)-menthol [8]. Lesser amounts of pulegone are also converted, by a reductase of opposite stereospecificity, to (+)-isomenthone, this ketone bearing the (*R*)-configuration at the isopropyl-substituted carbon rather than the (*S*)-configuration of (−)-menthone [7]. Reduction of the carbonyl function of (+)-isomenthone, by the same dehydrogenase involved in (−)-menthol biosynthesis, yields (+)-neoisomenthol [8]. The effects of Daminozide and Ethephon in decreasing the production of menthone and menthol, and increasing the formation of isomenthone and neoisomenthol (Table 1) can thus be explained by an lateration in the relative levels or activities of the pulegone reductases specific for the conversion to menthone (decreases) and to isomenthone (increases), and by the operation of a common dehydrogenase which reduces either available ketone to the corresponding (*R*)-alcohol. Consistent with this scheme is the observation that the levels of total ketone isomers and total alcohol isomers remain essentially unchanged. The previous observation by Bosela and Smik [4] that Daminozide increased production of neomenthol could not be verified in the present study (this isomer was observed in but trace levels), and it now seems likely that the earlier workers had simply misidentified neoisomenthol. The earlier report [4] that Ethephon increased the menthol content of peppermint oil was also not confirmed in the present study in which the opposite effect was observed.

In sage, the monoterpene olefin (+)-sabinene is converted to the α,β-unsaturated ketone sabinone, the double band of which is then stereospecifically reduced to yield either (+)-3-thujone or (−)-3-isothujone [16]. Differences in the proportions of the saturated ketones observed under the influence of growth regulators could therefore result from alteration in the proportion of the corresponding stereospecific reductases. The precursors of 3-thujone and 3-isothujone were present at only minor levels (< 3%) under all circumstances. 1,8-Cineole and camphor are derived via distinct cyclizations of the common precursor geranyl pyrophosphate [17, 18], and the observed decrease in the levels of these monoterpenes under the influence of Daminozide and Ethephon most likely results from diminution of the levels or activities of the relevant cyclases. The influence of growth regulators on the production of monoterpene olefins in sage is somewhat more complex. Sage produces only the (−)-isomer of β-pinene and this compound is formed by the cyclization of geranyl pyrophosphate by an enzyme which also produces (−)-α-pinene and (−)-camphene as lesser co-products [19, 20]. (+)-α-Pinene and (+)-camphene are synthesized from geranyl pyrophosphate by a separate and distinct cyclase [20]. Since Ethephon markedly increases the level of β-pinene, the growth regulator must act to increase the relative rate of (−)-β-pinene formation, and necessarily the rate of formation of (−)-α-pinene and (−)-camphene. At the same time, the relative rate of production of (+)-α-pinene and (+)-camphene must decrease in order that the *overall* levels of these olefins decrease as observed (Table 2).

The results described here provide strong suggestive evidence that growth regulators such as Daminozide and Ethephon can influence essential oil formation by a direct

effect on monoterpene metabolism. The alterations in oil composition observed are most readily explained by changes in the levels or activities of the corresponding biosynthetic enzymes. However, modification of the rates of catabolism of compounds such as menthone [21] and camphor [22] cannot yet be excluded. Studies are now underway to correlate growth regulator-induced changes in oil composition with measurement of enzyme levels in cell-free systems.

EXPERIMENTAL

Plant material. Sage (*Salvia officinalis* L.) was grown from seed and peppermint (*Mentha piperita* L.) was propagated from single-node cuttings of etiolated rhizomes. The plants were grown in peat moss in a growth chamber with 14-hr photoperiod (900 ± 100 fc, fluorescent/incandescent), 29° day/25° night temp. cycle and relative humidity of 62 ± 12%. Plants were watered as needed and fertilized weekly with a complete fertilizer (N:P:K, 20:20:20, with microelements and iron chelate). Plants were thinned and allowed to grow for 5 weeks before treatment with Daminozide (B-Nine, Alar *N*-dimethylaminosuccinamic acid) or Ethephon (Ethrel, Bromeflor, 2-chloroethylphosphonic acid).

Treatment. Each expt was conducted in randomized complete block design with 3 replications totalling 60 plants per treatment. Growth regulators at the indicated concns were prepared in distilled H_2O containing 0.1% Tween 20 and sprayed to the point of run-off with a hand sprayer. Two additional applications were made at weekly intervals, and the plants were harvested one week after the last treatment. Data on growth characteristics (fr. wt of plant, leaves, stem and total branches, length, width and number of leaves, length of stem, number of internodes and internode length) were collected. Controls sprayed without growth regulator were included in each experiment.

Oil analysis. A minimum of 3 representative 10 g samples of fresh tissue were steam distilled using a simultaneous steam distillation–extraction apparatus (J & W Scientific), employing as internal standards (+)-isomenthone for sage and (+)-fenchone for peppermint. On completion of distillation (1 hr) the essential oil collected (in pentane) was dried over anhydrous 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 200°) 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 internal standards. Yield and relative percent of major oil constituents were also determined. Data were statistically analysed [23], 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 retention times and mass spectra to authentic standards.

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