

***Mentha x piperita* volatiles promote *Brassica oleracea*- A pilot study for sustainable vegetable production**

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ABSTRACT

White cabbage was grown in glasshouses with two *Mentha x piperita* varieties in randomly designed arrangements during spring and autumn 2011/12. The plots were supplemented with one or four *Mentha* plants of the same variety possessing three main shoots of 7 nodes. Compared to the controls, most pronounced differences were found when the leaves were already exposed to *Mentha* volatiles during primordium initiation. When applied at sensitive stages of the *Brassica* seedlings, *Mentha* volatiles enhanced the productivity and increased the quality and quantity of the aboveground biomass. In another experiment, white cabbage seedlings were exposed to menthone, menthol or their 1:1 combination. No promoting effect on leaf development or leaf weight was found, but menthol and the combination of menthol/menthone had effects on the shapes of leaves. The stimulatory effects strongly depended on the developmental stage of leaves. Hence, natural mint volatiles lead to other responses of young *Brassica* plants than single compounds or their combination.

Key words: *Brassica oleracea*, cabbage, mentha, *Mentha x piperita*, menthol, menthone, vegetables, volatiles, young plant promotion

INTRODUCTION

Some plants emit the volatile compounds to interact with their biotic environment. Whereas volatiles from flowers attract pollinators, the release from vegetative organs as a reaction to herbivore damage serves indirect plant defence by attracting natural enemies of the herbivores (6,16,17). Bioorganic volatiles such as monoterpenes have also functions in plant-plant and plant-microorganism communication (5,18,25). There are even hints that plants can discriminate between species via sensing the different bouquets of volatiles emitted by the neighbored plants. Recently, some monoterpenes have been found to act as signal volatiles between plants. These volatiles support systemic resistance induction within and between neighbored plants (4,16,29,32,38).

Depending on the concentration, many terpenoids also have strong phytotoxic properties (3,11,19,21,30,37). Monoterpenes of the essential oil of *Artemisia scoparia* generate reactive oxygen species (ROS) and oxidative damage in receiver plants (12,39). In cucumber roots, menthol increases the of cytosolic calcium ion concentration, which may trigger many cellular responses (27). For example, calcium influx caused a protein

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phosphorylation/dephosphorylation dependent expression of numerous genes, including phenylalanine ammonia-lyase (PAL), farnesyl pyrophosphate synthase (FPS), and pathogenesis-related (PR) encoding genes (4). Due to their herbicidal activity, terpenoids may have a strong impact on the composition of species in plant communities. For instance, 1,8-Cineol and camphor released by *Salvia leucophylla* or fruit material from *Tagetes minuta* inhibited the cohabitant species (22,28). Monoterpenes can selectively inhibit the plant growth by suppressing seed germination, cell proliferation, or respiration. Membranes can be damaged by induced lipid peroxidation and microtubules are affected (9,11,43). Monoterpenes can influence the plant phenotype by altering the leaf size and shape, transpiration, the actin cytoskeleton and gene expression (18,21). Prolonged exposure to certain monoterpenes led to desiccation and death of *Arabidopsis* plants. Inhibition of DNA synthesis was also observed (12). Batish *et al.* (6) described the volatile oil from *Eucalyptus citriodora* as phytotoxic with a differential species-specificity. The use of terpenoids for weed management in agriculture is therefore suggested (40).

In turn, terpenoids are reported to have beneficial effects on plant health and physiological intactness. Isoprene, a simple five-carbon terpene, and other herbivore-induced monoterpenes and sesquiterpenes have been reported to increase tolerance of photosynthesis to high temperatures by stabilizing thylakoid membranes or by quenching the reactive oxygen species (ROS) and thus protect the plant from internal oxidative damage (10,23,24,36). Terpenoids with antifungal properties or the capacity to activate plant defense responses have also been identified. Monoterpenoids can have positive effects on plant growth. In a study we found that low dosages of camphor and menthol resulted in stronger *Arabidopsis* plants (37). Thus, the effects of plant volatiles on other species seem to be very much depended on the dosage, time of exposure and composition, which is related to the density and the species of the emitter plants, the wind direction and most likely in combination with other abiotic factors.

Whereas the herbicidal effects of some volatiles have been investigated in several studies, stimulatory effects on plant growth are rarely reported. In a study of Rentsch *et al.* (31), monoterpenes from peppermint oil and S-(+)-carvone (CAR) were found to interact with the GA-mediated bud dormancy release in potato tubers. Whereas low monoterpene concentrations initiated the bud sprouting, high concentrations led to an inhibition. The accumulation of α -amylase transcripts was also affected by the monoterpenes depending on dosage. The diterpenoid epinodosin significantly promoted growth of *Lactuca sativa* seedlings at low doses, but the higher ones (150-200 μ M) were inhibitory (13). Intriguingly, volatiles emitted from bacteria and fungi were recently found to modify the plant growth (7). For instance, non-pathogenic *Fusarium oxysporum* MSA 35 associated with ectosymbiotic bacteria produces plant growth promoting volatiles, including β -caryophyllene. Without the ectosymbiotic bacteria, another spectrum of volatiles is produced and the fungus develops pathogenicity.

Investigations directed to the question whether a low volatile concentration of emitter plants, such as *Mentha x piperata* L., can have a positive effect on crop growth and development are not yet available. Therefore a pilot study was done to elucidate the effects of *Mentha piperata* volatiles on the growth of cabbage seedlings.

MATERIAL AND METHODS

Plant Material

White cabbage (*Brassica oleracea* convar. capitata var. alba cv LENNOX F1, Bejo Germany) seeds were germinated for 14 days in standard pots commonly used in commercial horticulture (4.5 x 4 x 5 cm pressed peat pots prepared from Potground P, KlasmannDeilmann, Germany). Potground P consists of 90% raised peat H2-5 and H6-H8, pH 5.5, 1.5 g/L KCl with the following nutrients: N= 210 mg/L CaCl₂, P₂O₅ = 240 mg/l (CAL), K₂O= 270 mg/l (CAL), Mg = 100 mg/l, 150 mg/l CaCl₂.

Plant growth was under natural illumination without additional artificial irradiation. Humidity and temperature were electronically controlled (SHT75 Humidity & Temperature Sensors from Sensirion, Switzerland). The sensors were installed 20 cm above the plants. Data were taken every 10 min and transferred to the computerized control systems of the glasshouses. The relative humidity was between 76 % and 81 %. The temperature was 18°C during the day and 14°C overnight. After the germination, the seedlings were cultivated at 14° C (day) and 8° C (night). Watering of the plants was electronically controlled. The watering regime was adjusted to a light integral of 0.6 kW/h to cause flooding of the plants for 9 min. The experiments were started when the seedlings had fully developed cotyledons and a first leaf of 1.5 cm length.

For the co-cultivation the *Mentha x piperita* L. varieties “EnglischeMinze cv Mitcham” and “Multimentha” were used. The plants were obtained from INRES (Institut für Nutzpflanzenwissenschaften und Ressourcenschutz, Bonn). The variety “EnglischeMinze cv Mitcham” has a high content of menthol, a moderate content of menthone and lower menthylacetate, whereas the variety “Multimentha” has a high content of menthone, a lower one of menthol and a moderate content of menthylacetate (Bundessortenamt 2002). Thus, the two varieties differ considerably in the contents of menthol, menthone and menthylacetate. All *Mentha* plants were grown in pots (growing medium Potgrond H, Fa. Klasmann) and used when three main shoots of 7 nodes were achieved. These parameters guaranteed a homogenous plant material with comparable plant size and density of foliage.

Experimental Design of Plant Arrangements

The experimental designs of set 1 and set 2 were the same. The developmental stages at the times of data collections/set were carefully retained. The plant arrangements were in 6 plots, each consisting of 4 boxes with 4x120 plants that were sown in press pots/peat blocks (5 x 4 x 4.5 cm) (one set with four replications). The total number of plants used in the experiment was 2880. Figure 1A demonstrates the experimental design for a box. Four distances to between the *Mentha* plant(s) and the rows of *Brassica* plants were evaluated: 1. row: 5-10 cm; 2. row: 20-25 cm; 3. row: 35-40 cm; 4. row: 50-55 cm. From each box 16 plants were chosen for the measurements of leaf parameters, the plants in between were used to determine the specific leaf weight (Fig. 1). The total number of plants investigated was 384 (N). Data were taken 14 (M1), 17 (M2), 21 (M3), 24 (M4) and 27 (M5) days after sowing.

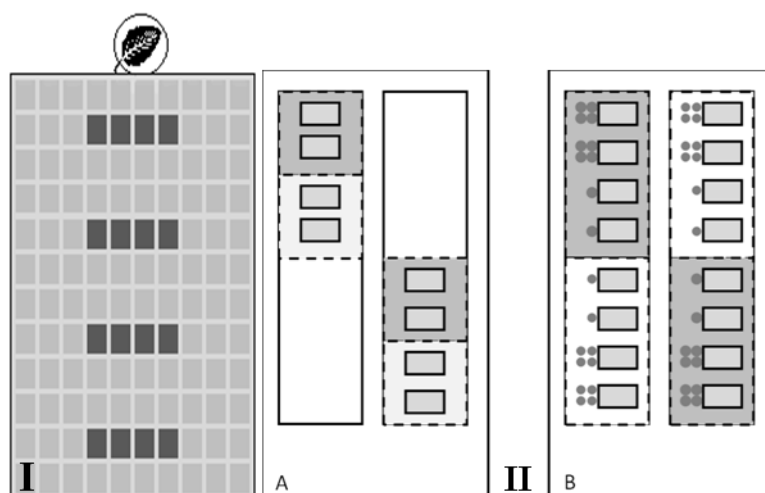


Figure 1. I) Experimental design of a plant box. ■ = Scored plants, the *Mentha piperita* plants are placed outside the box in pot. II) Experimental design of 6-different plots (plots shown by dashed boxes). Control experiments without *Mentha* plants (glasshouse A); experimental design with *Mentha* plants (greenhouse B). (Grey background = *Mentha x piperita* “EnglischeMinze cv Mitcham”, white background = *Mentha x piperita* “Multimentha”, ● = Number of *Mentha* plants). II illustrates the arrangement of plots which was set up in a completely randomized design in two glasshouses (A and B). Differences in grey scale are used to mark the different *Mentha* varieties (white background = *Mentha piperita* L. “EnglischeMinze cv Mitcham”, grey background = *Mentha piperita* “Multimentha”). Each plot consisted of two replications with one or four pots of *Mentha* plants. The distance between the boxes was 50 cm. Control plots without *Mentha* plants were placed in a neighbored greenhouse to avoid the influence of *Mentha* volatiles.

Experiments 1. *Mentha* varieties

The first set of study was conducted on May 2011, the second one on September / October 2012 in glasshouses, Experimental Station, University of Applied Sciences, Osnabrück, Germany. White 1L volume of the phytotron. The temperature during vapourization was 12°C in day and 10°C at night. The plants were grown as above.

Experiments 2: Menthol and Menthone

Another series of experiments was performed in phytotrons (HPP 110, Memmert) with menthol and menthone as single compounds or in 1:1 combination. Containers with 66 seedlings, sized as described in I., in standard pots were subjected to monoterpene vaporization for 48 h. The monoterpenes (1.36 mM menthol or 1.38 mM menthone per container; 0.68 mM menthone and 0.69 mM menthone in combination) were applied on glass Petri- dishes and randomly placed between the seedlings. The temperature during evaporation was 12°C during the day and 10°C at night. After that, plants were cultivated as described in experiments 1.

Measurements of Leaf Parameter: To determine the leaf length, leaf broadness and petiole length only leaves longer than 15 mm were considered. Only the increments of leaf

two and three were calculated because these leaves were already present 14 days after sowing and their exposure to the *Mentha* volatiles started at the earliest stage of development.

Specific Leaf Weight : The data collection (set1/set2) for the specific leaf weight (SLW) was taken once a week, starting with 14 days old seedlings (measurement 1; M1) until the plants were 21 days old (M3). Punching within the intercostal planes was performed with plants between the rows used for measurements of leaf parameters (Fig. 1, dotted lines), thus from plants growing in three different distances from the *Mentha* plants. Every plant was punched only once, whereby areas of 37 mm or 77.5 mm in diameter (10.75 and 47.17 mm², respectively) were punched. The smaller leaf discs of 10.75 mm² were only taken at M1 due to the small leaf size, later on, 77.5 mm² discs were the general size. The leaf discs were taken from the oldest leaf (M1) or from the next leaf above (all other Ms). The samples were dried for 48 h at 105° C. The dry matter was weighed and the specific leaf weight (g/m²) was calculated (SLW = dry weight of leaf disc in g/area of hole-punch in mm² and water content (%) = (leaf fresh weight - leaf dry weight)/leaf fresh weight / 100).

Fresh and Dry Matter and Leaf Development: At the end of the experiment (set 1/set 2) the rate of the leaf development was analyzed. From 16 plants /box the leaf number of all leaves larger than 15 mm was established. Then, all 384 plants which were used for the earlier data collections were cut above ground. The 4 plants of each row were used to determine the fresh and dry weight. After weighing, the 96 four-plants-packages were transferred into aluminum foil cups and placed in an oven for drying at 105° C for 48 h. The dry mass was weighed and the dry matter (%) was determined.

Statistical analysis:: For statistical analysis, SAS (version 8.2, SAS North Carolina, USA) was used. Analysis of variance was conducted using the GLM procedure to test for the effects of *Mentha* varieties, density and distance between *Mentha* and *Brassica* plants at each sampling date. The comparison of means was done by using the Tukey test at the significance level $p=0.05$. The LS Means procedure was used to test the interactions between the main effects computing the p 's ($p=0.05$) for pairwise differences with the Bonferroni method.

RESULTS AND DISCUSSION

Specific Leaf Weight

The set 1 (spring) data showed that the specific leaf weight was significantly increased, when the *Brassica* seedlings were exposed to four plants of *Mentha x piperita* L. varieties “Englische Minze cv Mitcham” and “Multimentha” plants (14.5 and 19.7% more than control, respectively) (Fig. 2A). The highest increase (24.8%) was found with one Multimentha plant (Fig. 2). Thus, Multimentha had stronger influence than English mint. The repetition of the experiment in autumn (set 2) also revealed a significant increase in specific leaf weight in arrangements with one and four English mint plants (~ +30%) and one Multimentha plant (Fig. 2). Effects were stronger when *Brassica* plants were exposed to one *Mentha* plant (English mint: ~ +50%; Multimentha: ~ +100%) than in spring, which could be an effect of seasonally caused changed air concentrations of monoterpenes, together with changed composition of monoterpenes in the *Mentha* bouquet. For instance, the quantity of limonene, menthone and menthyl acetate is known

to change during the season and with leaf age (20, 33). Those changes may also explain why four Multimentha or English mint plants had fewer effects in autumn. Due to higher monoterpene concentration in the air and a changed composition, positive effects could be lessened. Thus, the differences in the *Brassica* response are explainable by the different seasons as plant growth was under natural illumination. The light quality and quantity should influence both species, *Brassica* and *Mentha*, whereby light quality is an important factor in modulating the essential oil composition of *Mentha* volatiles (14,26). In addition, the emission is influenced by temperature, which results also in diurnal variations in the monoterpene concentration in the air (20). Microorganisms may be crucial in triggering the biosynthesis of monoterpenes in *Mentha piperita* (35). The activity of microorganisms is higher in spring than in autumn. Such influences have to be considered, when volatiles are used to induce growth effects on receiver plants. Nevertheless, both sets of experiments led to similar results. In brief, different menthone and menthol contents of two mint varieties were less important than the composition of entire bouquets.

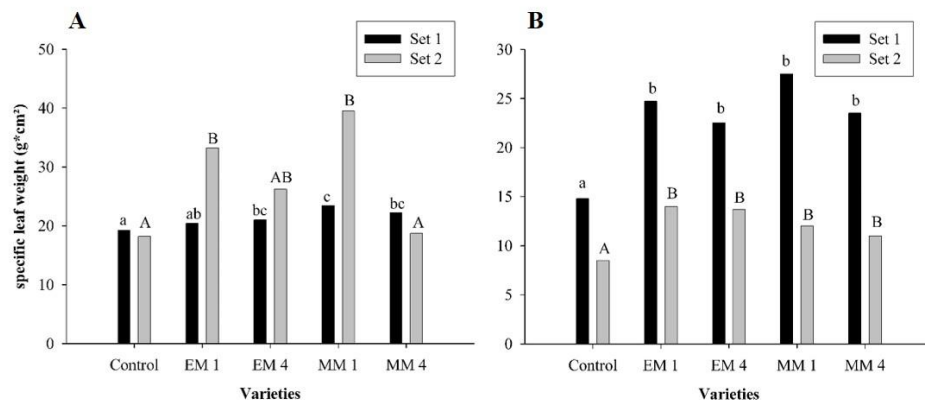


Figure 2. A. Specific leaf weight 18 days after sowing (%). Difference between control and *Brassica* plants exposed to volatiles of the *Mentha* varieties is significant ($p \leq 0.05$), Bonferroni test. B: Rate of leaf development /primordia. Analyzed 27 days after sowing (end of experiment). Difference between the control and *Brassica* plants exposed to volatiles of the *Mentha* varieties is significant ($p \leq 0.05$). Bonferroni test.

Leaf Development

In the spring experiment (set 1), *Mentha* volatiles had strong influences on the leaf development. In comparison to control, the number of plants which developed leaf no. 3 after 21 days was doubled to 3-folds higher (Fig 2B). Between the *Mentha* variants, the difference was not significant. In autumn (set 2), the influence of the *Mentha* plants was strongly reduced, compared to set 1 results attributable to the season.

In spring, the influence of mint plants on the leaf shape was ambiguous and no clear tendency was found. The growth of petioles was not affected at any stage and by any treatment. There was, if at all, only a tendency for larger total leaves (petiol + leaf lamina) at node three and four. At M5, both *Mentha* varieties led to less ellipsoid leaves. In set 2, due to seasonal growth retardation, spring stage L3/M3 and L4/M4 matched now stage L3/M4 and L4/M5. Likewise set 1, there were no significant differences in the leaf length or shape.

Leaf development is characterized by the stages of primordium initiation, primary and secondary morphogenesis (15, 42), whereby primordium initiation depends on high auxin and low cytokinin concentrations. During the primary morphogenesis, cell division is high, whereas during secondary morphogenesis the cell size grows (2). The more or less abrupt entering from primary to the secondary morphogenesis stage, (which seemed to be triggered by signaling from the chloroplast), is shown by dramatic transcriptomic changes. *Mentha* volatiles seem to interact with these events, perhaps by hormone interference. Rentzsch *et al.* (31) assume target specific interactions of defined monoterpenes with hormone signaling pathways.

Dry Matter

At the end of data collection, the dry matter (%) was determined. In both *Mentha* varieties, the dry matter (%) was increased in spring, 11 % with one *Mentha* plant and 7-9 % with four *Mentha* plants (Fig. 3). In autumn (set 2), there was no significant increase in dry matter (%) (data not shown). These results are again in line with the assumption of higher concentrations and altered compositions of *Mentha* monoterpenes in spring and autumn. Exposure to one *Mentha* plant in spring resulted in the largest positive effects, whereas with four *Mentha* plants the optimal concentration to induce positive effects was already exceeded. In autumn the emitted bouquet of *Mentha* compounds elicits no effect.

An increase in specific leaf weight is a known, multifaceted response to many factors such as elevated UV B radiation (8,14) and drought tolerance (36). A differential inhibition of the leaf area expansion as well as the accumulation of substances that affect leaf cell density has been considered. An increase in specific leaf weight (SLW) was found associated with the leaf thickness and changes in the rate of cell division or cell elongation (2). The higher specific leaf weight found in *Brassica* leaves fumigated with *Mentha* volatiles could be due to the interaction of menthol and other monoterpenes with the cytoskeleton and altered transpiration rates.

Exposure of *Brassica oleracea* seedlings to the volatiles of both *Mentha* varieties during spring enhanced the productivity and increased the quality and quantity biomass. In autumn, effects on the specific leaf weight were stronger. Leaf development at the early stages was influenced (but less than observed in spring). In the second set, dry matter (%) was not affected. Thus, in both sets we found effects of *Mentha* volatiles on the specific leaf weight and on defined stages of leaf development, whereas effects on dry matter (%) were different. The study, done for application in legume cultivation, raises questions regarding the molecular background of growth stimulation by *Mentha* volatiles in combination with defined developmental stages of the leaves of the receiver plant.

Influence of Menthol and Menthon

In contrast to the mint plants, menthol and menthon or a 1:1 combination of the compounds had no effect on the rate of leaf development, specific leaf weight or dry matter percentage. However, the shape of defined leaves was affected. Leaf 2 was significantly broader at stage M4 and M5 (M4=24, M5=27 days after sowing), when the *Brassica* plantlets were treated with menthol, whereas menthon and the combination of menthol and menthon had no influence (Fig. 4A). The length of leaf 1 was significantly increased during the same growth period, using a combination of menthol and menthon (Fig.4B). The results indicate again, that only defined stages of leaf development are

responsive to growth promoting concentrations of the monoterpenes used in this study. Older leaves showed no response.

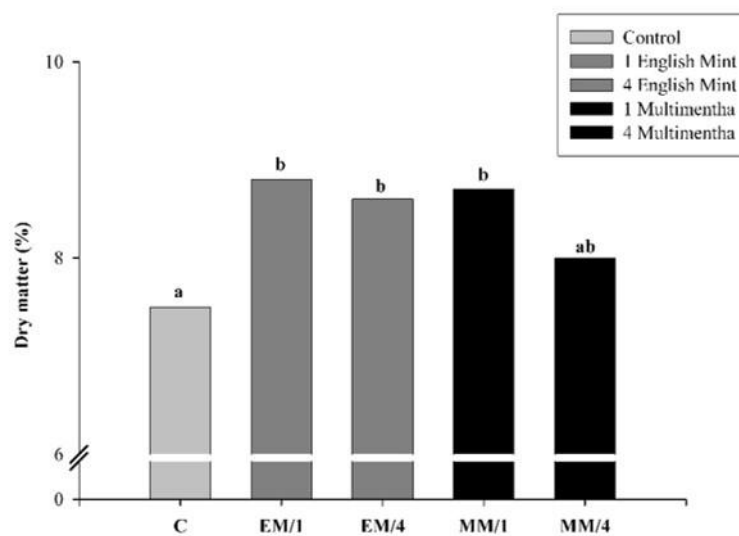


Figure 3. Dry matter (%) at the end of experiment. Difference between control and *Brassica* plants exposed to volatiles of the *Mentha* varieties is significant ($p \leq 0.05$), Bonferroni test.

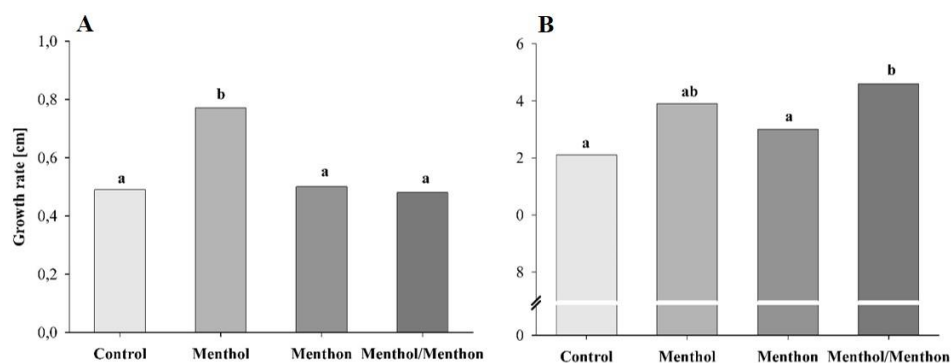


Figure 4. A: Influence of the monoterpenes on the growth rate (average values) of leaf width (leaf 2) between M4 and M5. (a-b = significance, $p \leq 0.05$, Tukey). B: Influence of the monoterpenes and their combination on the length of leaf 1 Mean values and standard deviation ($p \leq 0.05$ Tukey).

The *Mentha* monoterpenoids have been identified many times and published in numerous papers. The major compounds are menthol, menthone and several other-products. The allelopathic effects of highly concentrated monoterpenes are well known. For these reasons, those GC-MS analyses were not done in our study. Rather our intention

was to present a culture regime, which is also practicable for users. The content of monoterpenes in the essential oil fraction and in the volatiles depends on an extremely complex regulation triggered by endogenous and exogenous factors. Transcript abundances, properties of enzymes and most important, epigenetics and DNA methylation patterns are of fundamental importance for biosynthesis and the content of monoterpenoids. (1).

Our study demonstrated that the presence of same aged *Mentha* plants with differently aged leaves, even when cultured under defined conditions (soil, nutrients, watering regime) is useful for *Brassica* vegetables.

CONCLUSIONS

The application of single monoterpenes or their combination did not result in comparable growth stimulation as observed with *Mentha* plants. It has to be elucidated whether intercropping of emitter and receiver plants is also suitable for the field or whether such arrangements can only be used in glasshouses (41). More work is necessary to elucidate, whether other artificial compositions of monoterpenes as used here, could replace the *Mentha* plants. The results strongly indicate that the composition of the *Mentha* bouquet with major and minor constituents including compounds which are only present in traces function in concert in triggering leaf development of cabbage. Future dissecting of the molecular events is therefore of great importance to optimize the use of volatile applications in horticulture.

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