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Drought stress increases the accumulation of monoterpenes in sage (*Salvia officinalis*), an effect that is compensated by elevated carbon dioxide concentration

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Summary

The influence of drought stress on the accumulation of cineole, camphor and α - β -thujone in sage plants (*Salvia officinalis* L.) was determined by comparing the content of monoterpenes of well-watered plants and those restricted in irrigation (70% of the optimal water supply). In order to modulate the impact of drought stress, these plants were cultivated either at ambient or elevated CO₂-concentrations (385 ppm or 700 ppm).

Leaves of sage grown under moderate drought stress reveal significant higher concentrations of monoterpenes (about 33%) than those of plants cultivated under well watered conditions. Under ambient CO₂ also the total content of monoterpenes per plant is higher in the stressed plants than in the well-watered ones. The enrichment in the CO₂-concentration, which is thought to impair the metabolic effects of drought stress, resulted in a marked decrease in the monoterpene concentration, i.e., 17.8% in the case of well watered plants, and 21.8% in the drought trial. From this it is deduced that the increased reduction capacity arising from drought stress, pushed metabolic activity towards the biosynthesis of highly reduced compounds.

Introduction

It is well known that spice-plants are much more aroma intensive when grown in semi-arid regions in comparison to those cultivated in moderate climates. Analogous quality differences are observed with respect to medicinal plants, which in general reveal an enhanced content of relevant secondary plant products, when they grew in barren regions (SELMAR, 1996). Based on reasonable plant physiological considerations this phenomenon could be explained intelligibly: in semi-arid regions – due to limited water supply and much higher light intensities – plants are exposed to a higher level of drought stress than the well watered plants grown e.g. in Central Europe. The corresponding stress conditions may result in enhanced accumulations of secondary plant products (GERSHENZON, 1984; SELMAR, 1996). Because of the stress induced stomata closure, the uptake of CO₂ markedly decreases (CORNIC, 2000). Consequently, the consumption of NADPH + H⁺ for the CO₂-fixation via Calvin cycle declines considerably. In contrast, the input of light energy is enormous, and thus a massive oversupply of NADPH + H⁺ is generated (MAO et al., 2007). Whereas under standard conditions this surplus of reductive power is successfully dissipated either by non-photochemical quenching (MÜLLER et al., 2001) or by an effective re-oxidation of NADPH + H⁺ by the xanthophyll cycle (MÜLLER et al., 2001; LOSCIALE et al., 2008) and photorespiration (WINGLER et al., 2000), under stress conditions the electron pressure in the photosynthetic electron transport chain increases massively (OELZE et al., 2008). As consequence of such an “electron congestion”, electrons overspill to molecular oxygen and superoxide radicals are generated, which have to be detoxified by the typical stress related enzymes, i.e. superoxide dismutase and ascorbate peroxidase, denominated as water-water-cycle (ASADA, 1999). In this context we have to consider that the high reduction power could affect also other metabolic processes and may push corresponding reactions towards

the synthesis of highly reduced compounds, like isoprenoids, phenols or alkaloids (SELMAR, 1996; MAGEL et al., 2006). Unfortunately, these relations have not been considered adequately in plant biology and applied botany, respectively, albeit these considerations reveal a special relevance for any attempts in quality improvement of spices and medicinal plants by increasing the concentration of secondary compounds, e.g. by deliberately applying drought stress (SELMAR, 1996). Accordingly, we attempted to verify these possible relationships by exemplarily analyzing the influence of drought stress on the accumulation of monoterpenes in sage. In order to modulate the extent of “electron pressure” on the plant system, we enhanced the CO₂-concentration, to compensate the reduced CO₂-supply caused by stress induced stomatal closure. Moreover, under well watered conditions, due to the open stomata, the CO₂-concentration in the mesophyll cells increases even more. Consequently, CO₂ assimilation rate is higher than under ambient conditions (AINSWORTH and ROGERS, 2007), which should be accompanied by a decrease of the overall reductive capacity.

In *Salvia officinalis* leaves, used as medicinal plant or as spice, the composition of monoterpenes varies strongly and depends on variety and provenance (SALIGŠOVÁ et al. 1997; DOB et al., 2007). However, in most cases the cyclic monoterpenes, cineole, camphor and the mixture of the both stereo-isomers α - β -thujone (Fig. 1) are so much dominant (DOB et al., 2007; CHALCHAT et al., 1998) that their sum represent nearly the entire monoterpene content.

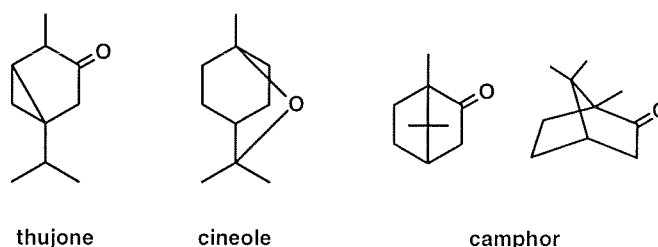


Fig. 1: Formulas of the main monoterpenes from *Salvia officinalis*.

In contrast to cineole and camphor, thujone occurs in sage as mixture of the both stereo-isomers α - and β -thujone.

Materials and methods

Plant material

Sage plants were obtained from a local garden centre. At a time 11 individuals were planted in each of four big plant tubs containing 6 kg of a potting soil / sand mixture (80/20) each. For acclimatisation, all four plant tubs had been cultivated in continuous stirred tank reactors (CSTRs) as described by SCHENK et al. (1995) under conditions as follows: a light / dark period of 16 h (700 $\mu\text{E} \times \text{m}^{-2} \times \text{s}^{-1}$) and 8 h at an air temperature of 26 °C during the day and 16 °C at night and ca. 60% rel. humidity. After the acclimatisation phase of one week, two plant tubs, i.e. one scheduled for the drought stress trial and one control, were grown in a CSTR at ambient CO₂-concentration

(385 ppm), the two others in a CSTR with elevated CO₂-concentration, adjusted to about 700 ppm. The day-night rhythm was changed for all trial to 14 / 10 hrs.

The experiment was initiated by applying moderate drought stress to two of the four plant tubs. Accordingly, in the beginning only about 2/3 of the water amount required for optimal irrigation was applied to the drought stressed trials. Each day, based on gravimetric data on the entire plant tubs, the amount of water lost by evapotranspiration was supplemented. As consequence, the soil water content always was around 8 % in the drought stress approach and around 25% in the well watered control, determined using a TDR-probe (P2Z from IMKO, Ettlingen, Germany). Two tubs, i.e. one well watered control and one drought stress trial were cultivated for 8 weeks at ambient CO₂-concentration, the corresponding two others for 8 weeks at about 700 ppm.

Quantification of monoterpenes

In order to avoid too high variations due to both developmental differences and individual fluctuation, the monoterpene content was determined for pooled leaf samples of all individuals of each trial, partitioned as old, medium and young leaves (Fig. 2). Directly after harvesting, about 10 leaves of each developmental class of each trial were shock-frozen in liquid nitrogen and ground to a fine powder. Each sample was analyzed twice as independent replicates. 300 mg of each frozen sample was suspended with 1 mL hexane in a closed reaction vial for about 3 hours at 30 °C. Exhaustive extraction of monoterpenes was ensured by applying intermittently 3 periods of ultrasonification for 15 min each. After centrifugation (10 min at about 10,000 x g) the hexane phase was filtrated (0.45 µm) and used for gas chromatography on a DB1-like Zebron ZB-AAA capillary column (10 m, 0.25 mm). Injection volume was 1,5 µL, split ratio 1:100, flow rate 450 mL He / min. Temperature program: 2 min for 90 °C, followed by a 20 °C / min-gradient up to 220 °C, remaining for 2 min. Detection was performed using a flame ionization detector; for quantification the area under the peak was compared to that of authentic standards (Carl Roth GmbH, Karlsruhe). Each quantification is based on at least two independent GLC analyses, including independent extraction procedures.

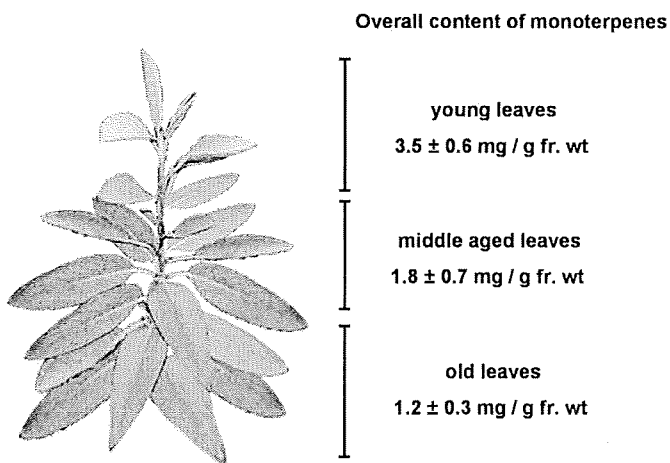


Fig. 2: Partitioning and classification of leaves sampled for monoterpene analyses.

Due to high developmental differences in monoterpene content, a reliable quantification required an age-dependent differentiation of leaves. The average ratio (fresh weight) between young, middle aged and old leaves was about 20:30:50.

Results and discussion

Composition of monoterpenes

The GLC-analyses revealed that the sage plants used for our experiments belong to the major group of *Salvia officinalis* plants, in which the three cyclic monoterpenes, cineole, camphor and α - β -thujone are predominant. Cineole accounts for about 11% - 15% of the overall monoterpene concentration, camphor for 32% - 40%, and the α - β -thujone stereo-isomer mixture for 51% - 57% (data not shown). Since in the leaves of the plants used for this study – apart from these three monoterpenes – only traces of other volatile terpenes are present, the overall concentration of monoterpenes mentioned in this paper is given as sum of that for cineole, camphor and α - β -thujone.

In general, the concentration of monoterpenes in leaves of individual plants varies markedly, depending on the leaf age (e.g. VORIN and BAYET, 1996; DUDAI et al., 2001). Accordingly, we analyzed the monoterpene contents in leaves of different developmental stages. As shown in Fig. 2, the overall concentration in young leaves was about twice that of middle aged leaves and even triple that of old leaves. Consequently, for all further analyses, we determined the monoterpene concentration of leaves partitioned in three classes: young, middle aged and old leaves.

Impact of drought stress and CO₂ on the monoterpene concentration

Leaves of sage grown under moderate drought stress (8% soil water content) revealed significantly higher concentrations of monoterpenes than those from plants cultivated under well watered conditions (25% soil water content), independently of their developmental stage (Fig. 3). This applied for both trials, i.e., the plants grown under ambient CO₂-concentration as well as for those exposed to elevated CO₂-concentrations (Fig. 3). A detailed evaluation of the data presented in Fig. 3 reveals that the average drought stress related enhancement of monoterpene concentration under ambient CO₂, was about 33% (38.8% for old leaves, 29.8% for middle-aged leaves and about 30.3% for young leaves). Under elevated CO₂, the corresponding average enhancement was about 22% (17.8% for old, 22.6% for middle-aged and 25.1% for young leaves).

The comparison of the monoterpene concentrations of the plants grown under ambient CO₂ with those cultivated under enhanced CO₂ (Fig. 3), clearly points out, that – apart from old leaves – an enhancement of the CO₂-concentration yields in a significant decrease in the

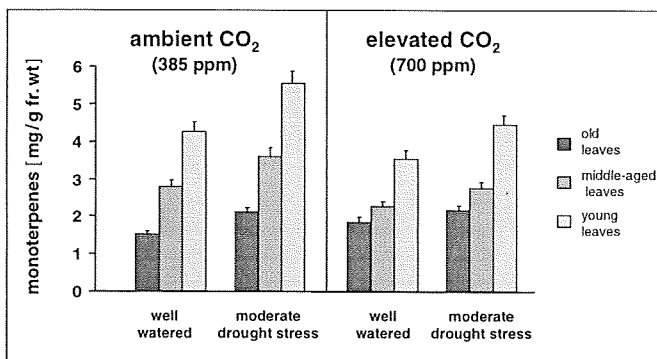


Fig. 3: Monoterpene concentrations in leaves of differentially stressed sage plants.

The monoterpene concentrations were quantified after 8 weeks of cultivation under the conditions mentioned. The overall values correspond to the sum of the concentration for cineole, camphor and the mixture of the both stereo-isomers of α - β -thujone.

monoterpene concentration. In the well watered trials the average decrease for middle-aged and young leaves was 17.8%; in case of drought stressed plants, the corresponding CO₂-related average decrease was about 21.8%.

It was assumed that the diminished uptake of CO₂ due to the drought stress induced stomata closure would lead to a lesser consumption of NADPH + H⁺ for the CO₂-fixation, yielding in an increased oversupply of reduction equivalents (SELMAR, 1996). This, in turn, might favour an enhanced synthesis of highly reduced compounds like monoterpenes. The results on the stress induced increase of monoterpene concentration confirm this relationship. Moreover, the CO₂-related decrease in the monoterpene concentration supports this hypothesis even more.

Intriguingly, the CO₂-shortage caused by drought stress induced stomata closure can fully be compensated by enhancing the exogenous CO₂-concentration, expressed by the nearly the same monoterpene concentrations in corresponding leaves of all developmental stages (Fig. 3, outer left and outer right bars). This compensation of the drought stress induced increase of monoterpene content by enhancing the CO₂-supply fully confirms the assumption of a close interference between reduction capacity and the share of synthesis of highly reduced compounds. In particular, this issue becomes obvious, when regarding the extremes: the average monoterpene concentration in drought stressed plants grown under ambient CO₂-concentrations, and thus exhibiting a massive surplus of reduction capacity, is about 60% higher than in well watered plants growing in an enhanced CO₂-atmosphere and therefore revealing a markedly less oversupply of energy. In this context it should be noted that in forthcoming approaches the determination of the actual *in vivo* reducing capacity is required to confirm the theory mentioned here; a putative strategy might be the quantification of reduced cofactors, the comparison of reduced versus oxidized glutathione, or of ascorbate versus dehydroascorbate, respectively.

At this point it should be emphasized that the extra consumption of reduction equivalents needed for the enhanced monoterpene synthesis under stress conditions is far lower than the extent of energy dissipation via non-photochemical quenching or the xanthophyll cycle. Thus, the surplus of monoterpene synthesis does not represent inevitably an effective mechanism for energy dissipation; however, it manifests the high reductive power in stressed leaves and therefore represents a symptom of its physiological state. In contrast, various authors postulate that the energy required for the isoprene biosynthesis that is emitted by leaves of numerous plants indeed could contribute significantly to the dissipation of the excess of photosynthetic energy (e.g. FALL 1999; SHARKEY and YEH, 2001). Indeed, an assessment of the energy used for photosynthesis on the one side and that consumed for isoprene synthesis on the other side revealed that under standard conditions the energy consumption accounts for less than 1% (MAGEL et al., 2006). However, at higher temperature, the amount of energy dissipated by isoprene emission might rise up to 25% of the energy supply for net photosynthesis (MAGEL et al., 2006). Although it has to be taken into account that – due to rising the rates of photorespiration at higher temperatures – the energy entering photosynthetic CO₂-fixation is much higher than that calculated by the authors only from the rate of net photosynthesis, the percental share of energy used for isoprene emission indeed is markedly. On the first sight it seems much likely that the amount of energy or of reduction equivalents, respectively, required for the biosynthesis of monoterpenes additionally accumulated in sage, is several orders of magnitude lower than that necessary for photosynthesis or that for the synthesis of isoprene emitted. However, when calculating the required energy according to MAGEL et al. (2006), with 140 photons per monoterpene (= 70 photons per isoprene unit, i.e. 14 NADPH + H⁺

per isoprene unit, 5 photons per NADPH + H⁺) and an increase in monoterpenes of about 3.2 nmol x m⁻² x s⁻¹ (~ 1 mg / g f.w x 50 d, see Fig. 3), about 0.45 μmol photons are dissipated by the supplementary monoterpene synthesis under stress condition. As this value is about one third of that calculated for the isoprene synthesis by MAGEL et al. (2006), also in the case of the sage monoterpenes a corresponding positive effect of the energy dissipation by their synthesis cannot be excluded. However – apart from any putative potential function – it should be emphasized that the surplus of monoterpene synthesis induced by drought stress just manifests the high reductive power in stressed leaves pushing the biosynthesis towards highly reduced compounds, and therefore represents just a symptom of this physiological state.

Impact of drought stress on biomass and monoterpene content

In principle, the monoterpene concentration in stressed leaves could also be enhanced when the rate of synthesis remains unaffected, but the entire biomass production of the plant declines due to the stress conditions applied. This aspect especially has to be considered when the plant material represents an important commodity, i.e., for spices or pharmaceutical preparations. As numerous leaves had to be detached for their analyses in the course of the entire experiment, which consequently influences the further development of the corresponding plants, it is very knotty to calculate the exact gain of biomass during the complex trial. However, based on the segmentation for young, middle aged and old leaves and the average total biomass at the end of the experiment, the total monoterpene content for one plant that theoretically should be achieved without harvesting any sample leaves for the four trials performed, could be estimated (Tab. 1). As expected, as well for plants cultivated under ambient CO₂-conditions as for those grown in a CO₂-enhanced atmosphere, the gain of biomass is markedly reduced in the plants that suffered moderate drought stress. On the other hand, an increase of the CO₂-concentration yields in the well-known effect of a pronounced increase in biomass (KÖRNER, 2000).

Although the plants exposed to the moderate drought stress under ambient CO₂ have gained only 90% of biomass of the well watered controls – due to their much higher concentration of monoterpenes – their overall content is markedly higher and accounts for a drought stress induced increase in the total monoterpene content of about 20%. However, due to the much higher growth rate of the well watered plants under enhanced CO₂-conditions, this effect does not

Tab. 1: Total amount of monoterpenes in differentially stressed sage plants.

The total content of monoterpenes had been calculated on the basis of a segmentation for young, middle aged and old leaves of 20:30:50 and the average total biomass determined for plants that were not influenced by sampling. The overall values correspond to the sum of the concentration for cineole, camphor and the mixture of the both stereo-isomers of α - β -thujone.

trial	biomass (mean f. w. / plant) [g]	monoterpene content (estimated amount / plant) [mg]
well watered, 385 ppm CO ₂	24.5	59.9
drought stressed, 385 ppm CO ₂	21.8	73.9
well watered, 700 ppm CO ₂	34.1	74.3
drought stressed, 700 ppm CO ₂	23.0	65.6

occur when drought stress is applied under elevated CO₂ (700 ppm). In this case the growth enhancement overcompensates the higher concentration of monoterpenes in the stressed plants. In this context it has to be emphasized that the overall monoterpene content of the well watered plants grown in the enhanced CO₂-atmosphere is about the same as that of the drought stressed plants grown at ambient CO₂-concentrations, although their biomasses differs up to 50%.

The data presented in this paper approve the hypothesis that the content of secondary plant products can be enhanced by deliberately applying moderate drought stress. Yet, with respect to quality improvement by increasing the concentrations of the active ingredients, e.g., for medicinal usage or as spices, it could become relevant to differentiate between the concentration of the corresponding compounds and their overall content, i.e., their total yield. Accordingly, when a phytopharmakon exclusively based on leaf material, requires a high concentration of an active agent, the deliberate application of moderate drought stress might be a suitable possibility for increasing drug quality by enhancing the concentration of the required secondary plant products. On the other hand, when the drug should be extracted, it is not the concentration but the overall amount of the substance which becomes relevant. To obtain an optimal yield, also the total biomass produced is determining the output. Therefore, a thorough and comprising assessment of the putative impact of drought stress is required, i.e., the positive influence on the increase of secondary plant products on the one hand, and its negative effect on biomass production on the other hand. Furthermore it should be considered that the application of certain growth conditions, which cause a raised biomass production also, might be accompanied by reduced concentrations of relevant compounds.

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