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DIFFERENCES IN PHOTOSYNTHESIS AND TERPENE CONTENT IN LEAVES AND ROOTS IN WILD-TYPE AND TRANSGENIC *Arabidopsis* PLANTS

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Abstract---We investigated the hypotheses that two different varieties of *Arabidopsis thaliana* show differences in physiology and terpene production. The two varieties of *A. thaliana* used in this study were wild-type (WT) and transgenic line (*CoxIV-FaNES I*) genetically modified to emit nerolidol with linalool/nerolidol synthase (COX). Photosynthetic rate, electron transport rate, fluorescence, leaf volatile terpene contents and root volatile terpene contents were analyzed. For both types, we found co-eluting α-pinene+β-ocimene, limonene, and humulene in leaves; and in the roots we found co-eluting α-pinene+β-ocimene, sabinene+β-pinene, β-myrcene, limonene, and humulene. At the end of the growing cycle, COX plants tended to have lower pools of terpene compounds in their leaves, with 78.6% lower photosynthesis rates and 30.8% lower electron transport rates, compared with WT plants at that time. The maximal photochemical efficiency *Fv/Fm* was also significantly lower (25.5%) in COX plants, indicating that these varieties were more stressed than WT plants. However, COX plants had higher (239%) root terpene contents compared to WT plants. COX plants appear to favor root production of volatile terpenes rather than leaf production. Thus we conclude that there were significant differences between COX and WT plants in terms of terpenoid pools, stress status and physiology.

Abbreviations: *A*---CO_2 uptake; COX--- transgenic line (*CoxIV-FaNES I*); ETR---electron transport rate; F_v/F_m---maximum photochemical efficiency of PSII; $\Delta F/F'_m$---actual photochemical efficiency of PSII; g_s---stomatal conductance; TPSs---terpene synthases; VOCs---volatile organic compounds; WT---wild-type.

Keywords: Arabidopsis thaliana, FaNES I, leaf terpene contents, root terpene contents, photosynthesis

INTRODUCTION

Plants produce a variety of volatile organic compounds (VOCs) of which isoprenoids are the most representative and abundant group [1]. Mono- and sesquiterpenes are C10 and C15 isoprenoid compounds that can be produced in the chloroplasts (MEP pathway) and in the cytosol (MVA pathway) [2]. One of the generally accepted physiological functions of these isoprenoids is to avoid damage in cellular membranes when the plants are under physiological stresses, for example, water stress, high temperatures, oxidative stress and high irradiation [3]. Mono- and sesquiterpenes also have ecological functions; they contribute to the defense strategies of the plants against pathogen attack [4], they can act as pollinator attractants [1], and may also play a role in allellopathy [5]. Terpenoids can also have impact on regional air quality reacting with anthropogenic and biogenic nitrogen oxides, contributing to tropospheric ozone and photochemical smog formation [6].

Volatile isoprenoids are mostly produced and emitted by the aerial parts of the plant (leaves and flowers). However, Janson et al. [7] suggested roots as a possible source of monoterpenes in soil and studies also show that there is terpenoid production and emission in roots [8]. This has been supported by measurements of monoterpene emissions in laboratory experiments from pine roots with qualitative and quantitative evidence of the existence of monoterpenes in soils under pine trees [9]. Root emitted terpenes have an ecological role in plant-animal interactions; for example, nematodes are attracted to emission of (E)-β-cayophyllene from western corn maize roots damaged by rootworm [10].

Arabidopsis thaliana flowers produce and emit terpenes [11, 12]. This species is thought to have over 30 putative genes associated with terpene synthases (TPSs), a multigene family [12, 13]. Most of them are almost exclusively expressed in flowers [12, 14, 15].

Other parts of *A. thaliana* are likely to produce and emit terpenes: trace amounts of the monoterpenes limonene and β-myrcene were emitted within its leaves [12], β-ocimene was emitted by rosette leaves [16] and even a release from roots to the rhizosphere (namely, 1,8-cineole) was suggested [17]. Although Chen et al. [12] reported leaf and root emissions, there is generally a lack of information regarding leaf or root production of terpenes in this species.

Recent studies show wide genetic variation among *A. thaliana* from diverse habitats. Different varieties of this species are likely to have different genotypes that might affect both primary and secondary metabolism [18]. We aimed to test the prediction that different *Arabidopsis* varieties will show differences in physiology and terpene content in leaves and roots. The two selected varieties

were a wild-type (WT) and a transgenic line (*CoxIV-FaNES I*) with linalool/nerolidol synthase, targeted specifically to the mitochondria (COX) developed by Kappers [19]. These varieties were selected as contrasting types with high potential to display such differences.

MATERIALS AND METHODS

Plant material and plant growth. We used 15 specimens each of *Arabidopsis thaliana* ecotype *Landsberg erecta* (*Ler-0*) (WT) and the transgenic ecotype *Columbia CoxIV-FaNES 1* line (COX) supplied by Iris Kappers (Wageningen UR, Plant Research International), which expresses a linalool/nerolidol synthase gene. The seeds were germinated for 4 days at 4ºC in Petri dishes, and were cultivated in 475 cm^3 plastic pots filled with peat and perlite (2 : 1, v/v) in a controlled environment chamber (14 h photoperiod, 130–150 μmol quanta /(m^2 s), 21ºC air temperature).

The growth medium used was based on that optimized by Gibeaut et al. [20]. The final contents were 1.5 mM $Ca(NO_3)_2$, 1.25 mM KNO_3, 0.75 mM $MgSO_4$, 0.5 mM KH_2PO_4, 70 μM Fe-diethylenetriamine pentaacetate, 50 μM KCl, 50 μM H_3BO_3, 10 μM $MnSO_4$, 2 μM $ZnSO_4$, 1.5 μM $CuSO_4$, and 0.075 μM ammonium molybdate (chemicals were from Fluka, Buchs, Switzerland).

Plant measurements: basal rosette diameter, CO_2 exchange and chlorophyll fluorescence. The diameter of the basal rosette was measured in each of the 30 plants 5 times throughout the experiment at 15, 18, 22, 28 and 31 days after germination respectively. CO_2 uptake (A) and stomatal conductance (g_s) were measured in leaves of the basal rosette only at the end of the growing cycle 31 days after germination of the seeds, using a portable non-dispersive infra-red gas analyzer (IRGA), model ADC-LCi (ADC Inc. Hoddesdon, Hertfordshire, England) connected to an Arabidopsis leaf chamber (ADC Inc. Hoddesdon, Hertfordshire, England). A and g_s values were expressed on a projected leaf area basis, which was measured with Li-Cor 3100 Area Meter (Li-Cor Inc., Nebraska, United States).

The maximum photochemical efficiency of PSII (F_v/F_m) and the apparent photosynthetic electron transport rate (ETR) were also measured at the end of the growing cycle with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). ETR was estimated as:

$$\mathrm{ETR} = \Delta F/F'_m \times PPFD \times 0.84 \times 0.5,$$

where $\Delta F/F'_m$ (actual photochemical efficiency of PSII) was calculated within the software, 0.84 is the coefficient of absorption of the leaves, and 0.5 is the fraction of electron involved in the photoexcitation produced by one quanta [21]. Chlorophyll fluorescence was measured twice: after turning the lights on and after 7 hours of lighting. The maximum PSII photochemical efficiencies (F_v/F_m) were measured after keeping leaves in the dark for at least 25 min.

Laboratory analyses: leaf and root terpene contents at the end of the growth cycle. Samples for terpene extractions were taken from leaves and roots 35 days after germination, from the plants growing in the controlled environmental chamber. Leaf and root material was ground in liquid nitrogen and repeatedly extracted (three times) with pentane, with a non-terpenoid internal standard (0.1 μl dodecane). The pentane-extracted leaves and roots were centrifuged at 10 000 rpm for 10 min. Extracts were then concentrated with a stream of nitrogen, because low concentrations were expected.

Monoterpene separation was conducted using a GC-MS system (Hewlett Packard HP59822B, Palo Alto, California, USA). Extracts (3 μl) were injected into the GC-MS system and passed into a 30 m x 0.25 mm x 0.25 mm film thickness capillary column (Supelco HP-5, Crosslinked 5% pH Me Silicone. A full scan method was used to perform the chromatography. The GC oven was programmed to start at 40ºC, then the temperature was increased at 30ºC/min up to 70ºC, and thereafter at 10ºC/min up to 150ºC, when the temperature was maintained for 5 minutes, and thereafter at 70ºC/min up to 250ºC, which was maintained for another 5 min. Helium flow was 1 ml/min. For both varieties of *A. thaliana*, two blank analyses per day were also conducted.

The identification of terpenes was conducted by comparison with standards from Fluka (Buchs, Switzerland), and with the GCD Chemstation G1074A HP with the Wiley275 library. The internal standard dodecane was used to determine extraction efficiency. Dodecane did not co-elute with any terpene. Calibrations was performed with the common terpenes α-pinene, 3-carene, β-pinene, β-myrcene, p-cymene, limonene, and sabinene standards once every five analyses. The quantification of the terpenes was conducted using the fractionation product with mass 93 [22]. Terpene calibration curves (n=4 different terpene contents) were always significant ($R^2 > 0.99$) in the relationship between signal and terpene contents. The most abundant terpenes had very similar sensitivity (differences were less than 5%). Total terpene contents were calculated as the sum of these main terpenes.

Leaf and root dry weights were determined after drying the plant material at 60ºC until constant weight in each of the 30 plants at the end of the growing cycle 31 days after germination of the seeds.

Statistical analyses. Analysis of variance (ANOVA) with Fisher post hoc tests for all the studied dependent variables, and Student's *t*-tests were used to test the significance of differences in response between transformed (COX) and wild type plants (WT), using R 2.7.2 software for Windows (R Foundation for Statistical Computing, Vienna, Austria). Differences were considered significant at a probability level of $P < 0.05$.

RESULTS

Growth: mean diameter of the basal rosette

The growing pattern of WT and COX varieties differed: at the end of the experiment COX plants achieved 34% larger basal rosettes compared with WT plants (table 1). WT plants reached their maximum diameter half way through the experiment, with very low increase during the two last weeks. During this time WT plants increased from 4.16 to 4.72 cm. COX plants had larger basal rosettes diameters that increased continuously during the 4 weeks of the experiment. During the two last weeks of the experiments, COX plants grew from 4.0 to 6.3 cm (table 1).

Plant biomass

The dry weight of the aerial part of the plants was significantly higher in WT (0.082 ± 0.009 mg) compared with COX plants (0.054 ± 0.007 mg) (table 2). The dry weight of the roots was significantly lower in WT (0.056 ± 0.009 mg) compared with COX plants (0.141 ± 0.06 mg) (table 2).

Net photosynthetic rates, stomatal conductance and fluorescence measurements at the end of the growth cycle

Net photosynthetic rates at the end of the experiment (*A*) were 78.6% lower in COX plants than in WT plants ($P < 0.001$; table 2). Stomatal conductance (g_s) tended to be lower in COX plants compared to WT plants (not significant $P = 0.12$, table 2). The apparent photosynthetic electron transport rate (ETR) was 30.8% lower ($P < 0.001$) in COX plants than in WT plants (table 2). The maximum photochemical efficiency of PS II (F_v/F_m) was 25.5% lower ($P < 0.001$) in COX plants than in WT plants (table 2).

Leaf terpene contents

For both varieties, leaves contained α-pinene+β-ocimene (WT 42.67 ± 20.64 μg/g dry wt, COX 10.55 ± 2.45 μg/g dry wt), limonene (WT 24.64 ± 6.73 μg/g dry wt, COX 11.58 ± 2.81 μg/g dry wt) and humulene (WT 10.64 ± 5.76 μg/g dry wt, COX 34.31 ± 7.65 μg/g dry wt) (fig. 1, table 3).

There was no significant difference in leaf terpene contents between the two varieties, but there was a tendency for higher terpene contents in WT plants (fig. 2). Other unidentified compounds were found: “unidentified compound 1” (possibly myrtenal), “unidentified compound 2” (possibly β-ionone) and “unidentified compound 3” (fig. 1, table 3). COX plants tended to

produce lower amounts of terpenes than WT plants (fig. 1). The “unidentified 2” compound was not produced in COX plants (fig. 1).

Root terpene contents

For both varieties, roots contained α-pinene+β-ocimene (WT – 9.5 ± 1.32 μg/g dry wt, COX – 21.58 ± 3.61 μg/g dry wt), sabinene+β-pinene (WT – 32.16 ± 3.66 μg/g dry wt, COX – 100.91 ± 15.34 μg/g dry wt), β-myrcene (WT – 0 ± 0 μg/g dry wt, COX – 23.44 ± 5.95 μg/g dry wt), limonene (WT – 2.35 ± 1.25 μg/g dry wt, COX – 16.75 ± 2.56 μg/g dry wt) and humulene (WT – 5.44 ± 1.45 μg/g dry wt, COX – 2.97 ± 1.34 μg/g dry wt) (fig. 3, table 3).

COX plants showed significantly ($P < 0.001$) higher (239%) contents of terpenes compared WT plants (fig. 4). Other terpenes were found: “unidentified 3” and “unidentified 4” (fig. 3, table 3).

DISCUSSION

Compounds detected in leaf and root extracts

There is clear evidence of terpene production in leaves and roots of both WT and COX varieties of *A. thaliana*. Our results agree with and expand the previous results who found traces of terpenes in leaves of *Arabidopsis thaliana* plants, such as β-caryophyllene, thujopsene, β-farnesene, and β-chamigrene [12, 23].

We did not find linalool, nerolidol or DMNT ((E)-4,8-dimethyl-1,3,7-nonatriene) in the foliage and root extracts of the COX plants as it was expected. Based on previous findings differences in the outcome of linalool/nerolidol synthase could be due to allelic variation in encoding functional terpene synthase genes, conversion of the enzyme product into other compounds as found by Aharoni et al. [11], differences in subcellular sites of gene expression, different activities of the terpene synthase together with different substrate pools available for the enzyme might be responsible for the product outcome [11, 14], or silent metabolism [24]. It is possible that no linalool or nerolidol was produced; in fact, Kappers et al. [19] detected no linalool emissions from any of their plants’ foliage, and no nerolidol in 25% of the transformed (COX) plants. It is also possible that linalool might have been produced in leaves but released immediately after production (similar to isoprene). It is also possible that our extraction technique would not have captured such compounds. The most likely possibility is that most of linalool and nerolidol are produced in Arabidopsis flowers, but we did not investigate floral emissions because we removed the flowers to retard the senescence processes in the leaves [25]. Aharoni et al. [11] found small amounts of linalool (from 0.02 to to 13.3 μg/day plant depending on the transgenic line) in the

headspace of transformed Arabidopsis plants, with the *FaNES* 1 gene expressed in the plastids, while Kappers et al. [19] expressed the *FaNES* 1 gene in the mitochondria and also observed nerolidol emissions from the transformed plants' foliage. Kappers et al. [19] did not make clear in which plastids the gene was expressed. If it was only in the chloroplasts, then the compounds detected in the root are likely to have been synthesised in the chloroplasts and differentially translocated down to the roots. However Hedtke et al [26] indicate that target plastids might be both chloroplasts and non-pigmented leucoplasts in the roots. In which case, the root terpene content is likely to be synthesised within the root tissue. Further experimentation is needed to confirm this. But in any case, lack of nerolidol and linalool does not detract in any way from the aims and conclusions of this work.

Effect of variety

There were differences in the morphology of the basal rosette in the two varieties used in our study. The WT had higher numbers of smaller leaves that were shed and replaced when they reached a certain size, while the COX species had fewer leaves whose length increased constantly along the vegetative cycle. Despite the fact that the two varieties (WT and COX) are morphologically different, the experimental plants were comparable in terms of health and phenology to satisfy the aims of the experiment, providing two different varieties of the same species.

There were differences in growth in both varieties of *Arabidopsis*. WT plants reached their maximum diameter before the COX plants (table 1). The COX plants' diameters increased gradually and consistently from the germination until the mature state. Kappers et al. [19] also found that first- and second-generation COX plants showed some growth retardation of the basal rosette. Other differences in growth of *Arabidopsis* varieties have been previously reported: Beemster et al. [27] found that growth of roots varied substantially between varieties.

Different varieties of *A. thaliana* showed evidence of differences in primary and secondary metabolism, indicating that metabolism in the two varieties is affected either by their different genotype, or by different post-translational control of metabolic processes. At the end of the experiment (21 days after germination), COX plants showed lower photosynthetic activity and production in leaves than the WT plants. Comparing COX and WT plants, we found that stomatal conductance (table 2) and calculated electron transport rates (ETR) were lower in COX plants than in WT, and this appeared to result in lower photosynthesis rates (table 2). These results might indicate that the COX plants were more stressed than WT plants. In addition, the mean ratio F_v/F_m was significantly lower in COX plants compared with WT, which also indicates that the COX

plants might have been suffering a degree of stress at the end of the experiment [28], although the plants were grown in identical conditions (see MATERIALS AND METHODS section).

COX plants presented lower leaf weight than WT plants but higher root weight (table 1). These plants, under stress conditions may reallocate carbon from its leaves towards its roots. Reallocation of primary compounds within the plant was shown to reflect a strategy to survive the damaging effects of herbivores [29]. Previous authors have shown that stressed plants could be characterized by high terpene biosynthesis in roots [10]. Rasmann et al. [10] showed that production and emission of root terpenes in maize plants is related to indirect defense by attracting entomopathogenic nematodes.

The carbon reallocation theory is supported by terpene foliar and root productions (figs. 2 and 4): there was a tendency for COX plants to have lower foliar terpene contents than the WT plants, though this difference was not significant, and root terpene contents were much higher in COX plants than in WT plants. Basyuni et al. [30] found that leaf isoprenoid contents generally declined while root contents increased in salt-stressed mango plants. In our study, the same relationship of lower leaf terpene contents and higher root terpene contents in the COX plants also reflects the higher stress status in the COX plants, as indicated by the lower F_v/F_m values.

Concluding remarks

We have shown that a modified Arabidopsis variety (which emits nerolidol from mitochondrial synthesis) directs resources towards root production of terpenes rather than leaf production of these compounds. These plants also have generally lower carbon assimilation rate at the end of the growth cycle compared with wild-type plants.

Further work of a similar nature is needed on other plant species, particularly root crop species that have been genetically modified or bred for certain characteristics of pest resistance and productivity. Breeding or genetic modifications resulting in redirection of resources to root production of terpenes might be advantageous for the defence strategies of root crops against soil herbivores and pathogens. However, the associated lower carbon assimilation might adversely affect root crop yield.

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Table 1. Basal rosette diameter (cm) evolution for wild type (WT) and transformed (COX) *A. thaliana* plants after germination

Days after germination	WT	COX	*P*-values
15	4.16 ± 0.32	4.01 ± 0.65	0.83 (ns)
18	4.14 ± 0.25	4.27 ± 0.71	0.85 (ns)
22	4.60 ± 0.32	4.64 ± 0.70	0.86 (ns)
28	4.71 ± 0.36	5.81 ± 0.68	0.015 *
31	4.72 ± 0.35	6.33 ± 0.55	0.0051 **

Statistical significance for the effect of variety is indicated (WT n = 15, COX n = 15); ns – not significant, * $P < 0.05$, ** $P < 0.01$.

Table 2. Leaf and root weights, net photosynthetic rates, stomatal conductance, apparent photosynthetic electron transport rate and photochemical efficiency (F_v/F_m) for wild type (WT) and transgenic (COX) *A. thaliana* plants 35 days after germination

Variables	Units	WT	COX	*P*-values
Leaf weight	mg dry wt	0.082 ± 0.009	0.054 ± 0.007	< 0.05
Root weight	mg dry wt	0.056 ± 0.009	0.141 ± 0.060	< 0.05
Net photosynthetic rates	μmol/(m^2 s)	1.58 ± 0.17	0.96 ± 0.17	< 0.001
Stomatal conductance	mol/(m^2 s)	0.036 ± 0.001	0.030 ± 0.001	ns (0.12)
Electron transport rate	μmol/(m^2 s)	0.55 ± 0.02	0.47 ± 0.02	< 0.001
F_v/F_m		0.70 ± 0.01	0.53 ± 0.03	< 0.001

Statistical significance for the overall effect of variety is indicated (WT n =15, COX n = 15); ns – not significant.

Table 3. Retention time and most abundant ions (m/z) for the main terpenes found in leaves and roots

Compound	Tissue	Retention time, min	Most abundant ions	Type of terpene	
α-Pinene/β-ocimene	leaf, root	7.79	93.00 (28%), 90.95 (9.05%), 92.00 (8.51%), 27.05 (4%)	monoterpene	
Sabinene/β-pinene	root	9.18	94.00 (12.95%), 93.15 (12.39%), 41.05 (9.65%), 28.05 (7.22%), 31.10 (7.21%)	monoterpene	
β-Myrcene	root	9.69	105.00 (10.55%), 93.10 (7.40%), 31.00 (6.72%), 119.95 (5.03%), 55.00 (5.01%)	monoterpene	
Unidentified 4	root	10.26	43.10 (34.34%), 107.95 (14.11%), 150.00 (11.36%), 41.00 (9.89%), 92.90 (1.02%)	monoterpene	
Limonene	leaf	10.47	68.05 (7.07%), 67.05 (5.01%), 93.00 (5.43%), 43.05 (5%), 57.05 (4.14%)	monoterpene	
Unidentified 1 (possibly myrtenal)	leaf	15.00	79.00 (6.53%), 28.00 (5.93%), 107.00 (5.88%), 90.95 (5.17%), 93.10 (3.58%)	monoterpene	
α-Humulene (leaf)	leaf, root	16.22	93.00 (21.39%), 80.00 (7.15%), 121.05 (5.54%), 91.00 (4.44%), 92.10 (4.35%)	sesquiterpene	
Unidentified 2 (possibly β-ionone)	leaf	16.53	177.00 (20.69), 43.00 (12.44%), 122.95 (12.21), 135.00 (3.35%), 93.00 (1.64%)	sesquiterpene	
Unidentified 3	leaf, root	17.40	149.00 (43.49%), 176.95 (9.05%), 150.00 (5.11), 175.95 (3.82%), 93.00 (2.2%)	sesquiterpene	

FIGURE CAPTIONS

Fig. 1. Individual foliar contents of identified and unidentified terpenes for wild type (WT, *1*) and trangenic (COX, *2*) *A. thaliana* plants 35 days after germination.

Vertical bars indicate standard errors of the mean (WT n = 15; COX n = 15). Significant differences among varieties are indicated (+ $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig. 2. Total leaf terpene contents for wild type and transgenic (COX) *A. thaliana* plants 35 days after germination.

"Total" only includes the identified terpenes α-pinene+β-ocimene, limonene, and humulene. Vertical bars indicate standard errors of the mean (WT n = 15; COX n = 15).

Fig. 3. Individual root contents of identified and unidentified terpenes for wild type (WT, *1*) and trangenic (COX, *2*) *A. thaliana* plants 35 days after germination.

Vertical bars indicate standard errors of the mean (WT n = 15; COX n = 15). Significant differences among varieties are indicated (*** $P < 0.001$).

Fig. 4. Total root terpene contents for wild type and trangenic (COX) *A. thaliana* plants 35 days after germination.

"Total" includes α-pinene+β-ocimene, sabinene+β-pinene, β-myrcene, limonene, and humulene. Vertical bars indicate standard errors of the mean (WT n = 15; COX n = 15). Significant differences among varieties are indicated (*** $P < 0.001$).

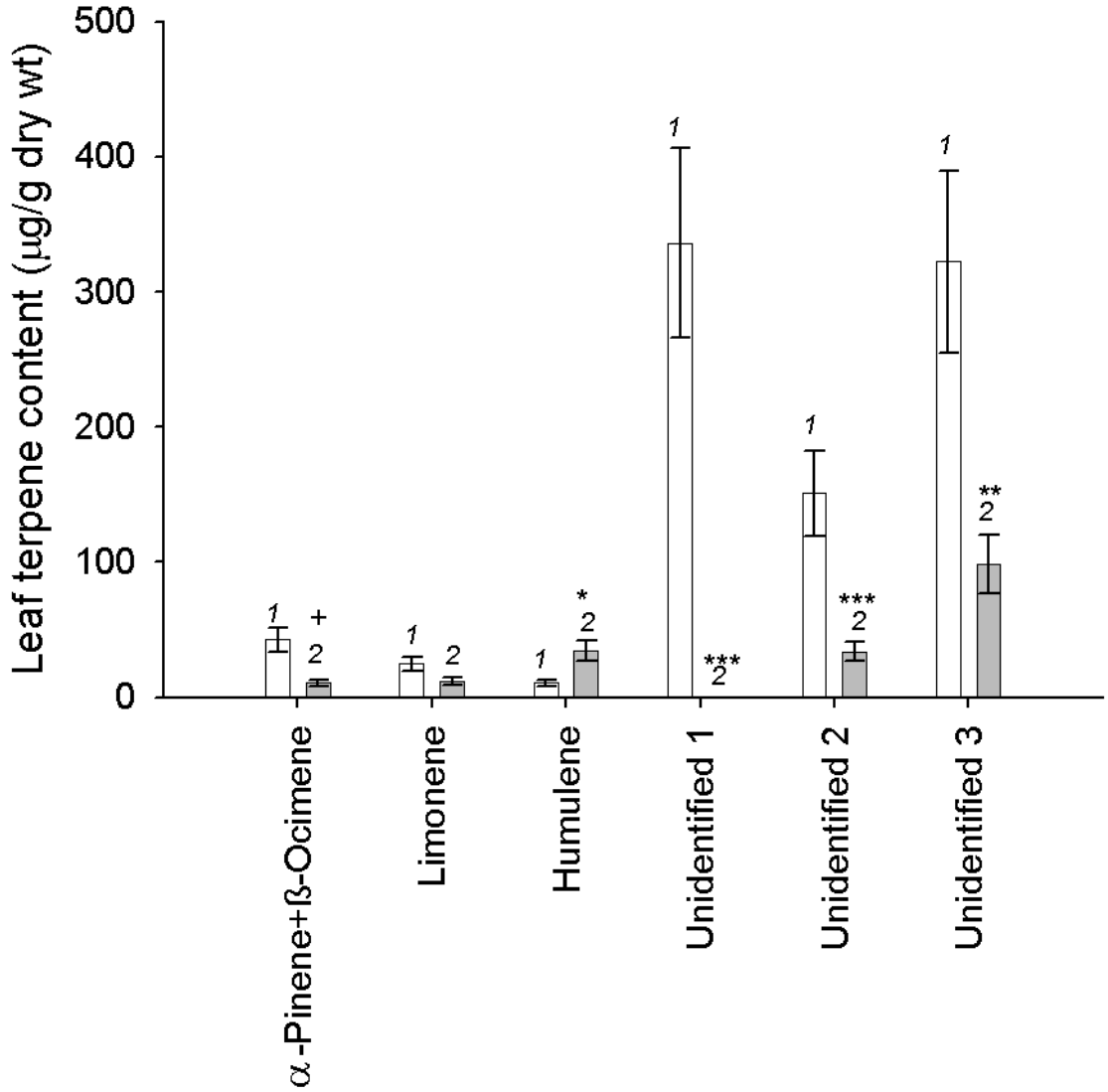
Leaf terpene content (µg/g dry wt)
500
400
300
200
100
0
1
2
+
*
***
**
α-Pinene+ß-Ocimene
Limonene
Humulene
Unidentified 1
Unidentified 2
Unidentified 3

Fig. 1

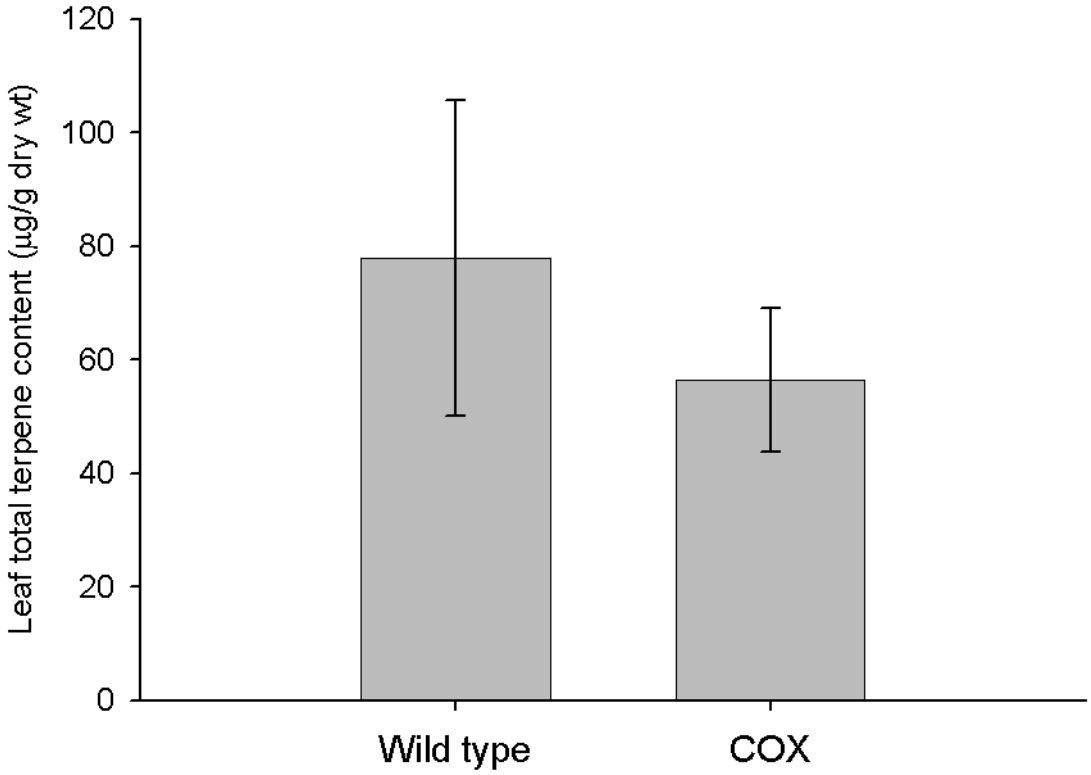
Leaf total terpene content (µg/g dry wt)
120
100
80
60
40
20
0
Wild type
COX

Fig. 2

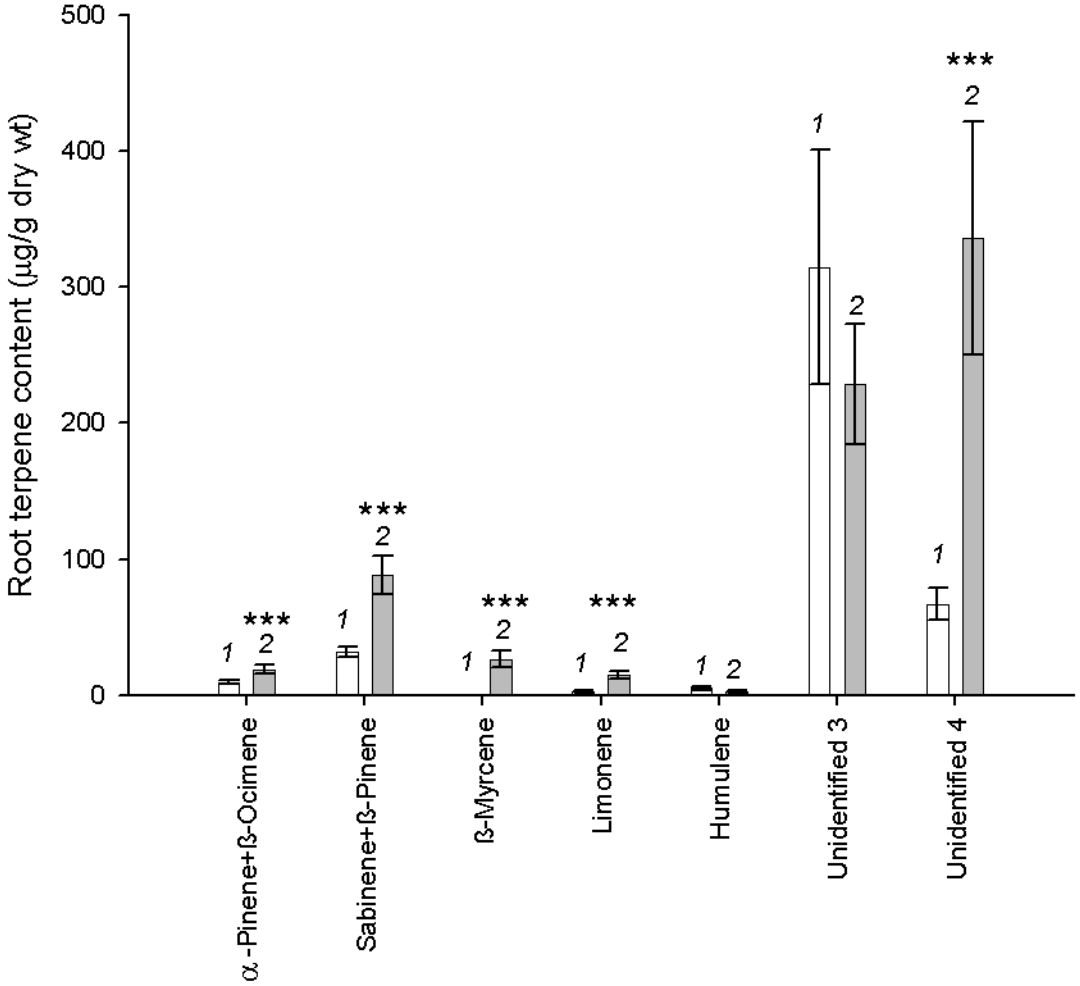
Root terpene content (µg/g dry wt)
500
400
300
200
100
0
***
1
2
α-Pinene+ß-Ocimene
Sabinene+ß-Pinene
ß-Myrcene
Limonene
Humulene
Unidentified 3
Unidentified 4

Fig. 3

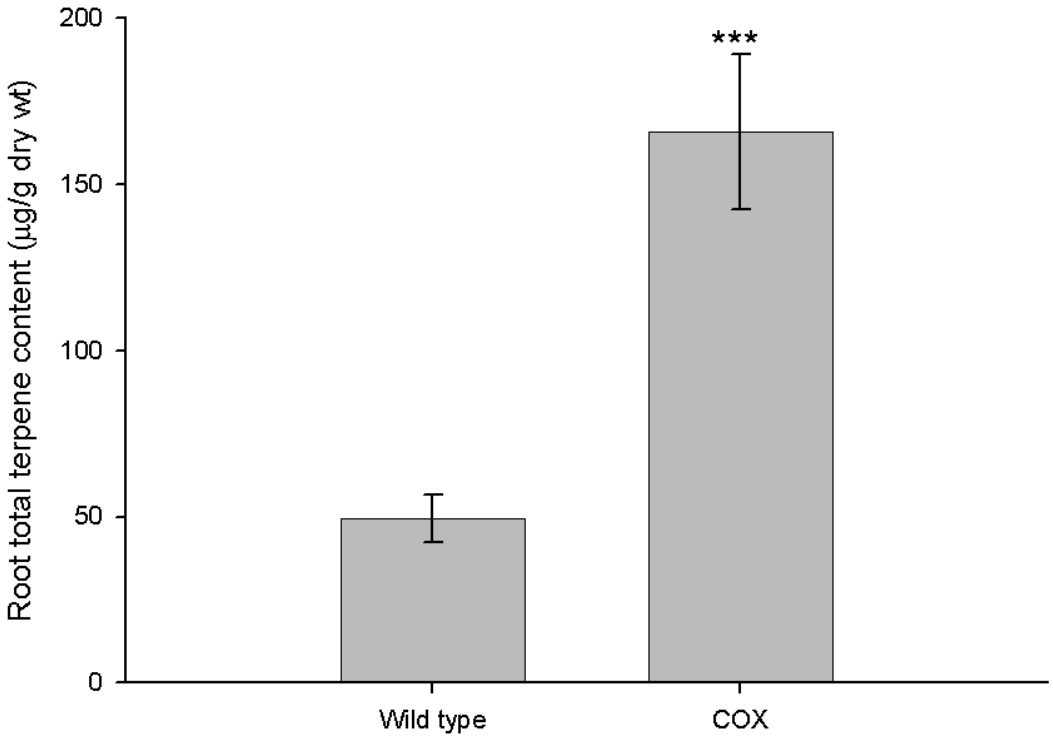
200
150
100
50
0
Root total terpene content (µg/g dry wt)
***
Wild type
COX

Fig. 4