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BRIEF COMMUNICATION

Effects of abscisic acid on content and biosynthesis of terpenoids in *Cannabis sativa* at vegetative stage

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Abstract

The influence of abscisic acid (ABA) on plastidial and cytosolic terpenoids and on two key enzymes for terpenoid biosynthesis was determined in vegetative stage of *Cannabis sativa* L. Low concentration of ABA (1 μM) increased 1-deoxy-D-xylulose 5-phosphate synthase (DXS) activity in treated plants in comparison to control plants. The amounts of chlorophyll *a* and carotenoids increased in response to ABA treatment but chlorophyll *b* content declined. The accumulation of α-tocopherol was stimulated only by 10 μM ABA. The ABA-treated plants showed a decline in 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity which was followed by a decrease in squalene and phytosterol content. ABA also decreased tetrahydrocannabinol (THC) and cannabidiol (CBD) contents. The essential oil had higher ratios of monoterpenes to sesquiterpenes as ABA-treated plants had less numbers of sesquiterpenes in comparison with control plants. Influence of ABA on the amounts of sesquiterpenes was different, some of them showed decrease of content and others increase of content.

Additional key words: carotenoids, chlorophyll, monoterpenes, phytosterols, sesquiterpenes, α-tocopherol.

Terpenoids are a structurally highly diverse and abundant family of natural compounds. In higher plants, isoprenoids participate in a wide variety of biological functions such as photosynthesis, respiration, growth, cell cycle control, plant defense and adaptation to environmental conditions. Specific examples include photosynthetic pigments (chlorophylls and carotenoids), abscisic acid (ABA), gibberellins (GA), plastoquinone, phytosterols and phytoalexins (Lange and Croteau 1999). In higher plants, the biosynthesis of terpenoids involves the common building block isopentenyl diphosphate and its isomer dimethylallyl diphosphate. These precursors can be synthesized either by mevalonate pathway in the cytoplasm or the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway in plastids (Rodriguez 2002). The cytosolic pathway provides the precursors for sterols and ubiquinone (Laule *et al.* 2003), while the plastidial pathway provides the precursors for monoterpenes, diterpenes, carotenoids, tocopherols, and the prenyl moiety of chlorophyll.

Cannabis is a dioecious plant that has been a source of fiber, food, oil, and medicine. Cannabinoids represent a distinctive class of compounds found only in *Cannabis sativa*. These C_{21} compounds belong to the chemical class of natural terpenophenols and Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most important of these compounds.

ABA regulates many aspects of plant growth and development. In order to understand how ABA interacts with terpenoid biosynthesis, we focused on the role of ABA in the regulation of primary and secondary terpenoid production in *Cannabis sativa* at vegetative stage.

The seeds of *Cannabis sativa* L. were sown in pots (with soil:leaf mold:*Perlite* 2:1:1) and cultivated in a phytotron (25 °C, 14-h photoperiod, irradiance of 70 μmol m^{-2} s^{-1}, air humidity 40 - 50 %). The plants were fertilized weekly with a Hoagland's nutrient solution. When plants had 7 pairs of leaves, they were subjected to ABA (± *cis*/*trans* ABA; *Sigma*, St. Louis, USA) treatment by spraying

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Abbreviations: ABA - abscisic acid; CBD - cannabidiol; DXS - 1-deoxy-D-xylulose 5-phosphate synthase; HMGR - 3-hydroxy-3-methylglutaryl coenzyme A reductase; MEP - 2-methyl-D-erythritol-4-phosphate; MVA - mevalonate; THC - Δ^9-tetrahydrocannabinol.

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the whole plants with 1 and 10 μM ABA solutions and distilled water as a control, until the solution started dripping. The spraying was applied three times spraying at 24 h intervals. The plants were harvested 1 d after the treatment. The third pair of leaves (from one plant) was used for all analyses.

Chlorophyll and carotenoids in leaves were determined as described by Lichtenthaler (1987), and α-tocopherol was extracted according to Panfili *et al*. (2003) and analyzed as described previously (Mansouri *et al*. 2009). Quantitative analysis of squalene and phytosterols (campesterol, β-sitosterol and stigmasterol) followed the procedure of Hartmann and Benveniste (1987). Cannabinoids were extracted and analyzed as described previously (Mansouri *et al*. 2009).

Analyses of essential oil was carried out on a *Shimadzu-QP5050* (Tokyo, Japan) gas chromatograph fitted with an *HP_5-MS* 40 m × 0.18 mm × 0.18 μm column (bonded phase 5 % phenyl siloxane), and interfaced with an *Shimadzu-QP5050* mass selective detector. The injector temperature was set at 280 °C, and the oven temperature program was 60 °C for 5 min, increased at 5 °C min^{-1} to 300 °C, and held for 7 min. A 0.5 mm^3 volume of extract was injected into the chromatograph. Terpenoids and other compounds were identified by mass spectrometry.

Enzymes assayed in this work were extracted from the fresh leaves. The content of protein was determined according to Bradford (1976). The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) was determined by the method of Toroser and Huber (1998). The activity of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) was determined by the fluorometric method according to Querol *et al*. (2001).

The results presented are the mean of three replicates. Means were analyzed by one-way analysis of variance (*ANOVA*; *SPSS 15.0*). Statistical significance of difference between means was calculated using Duncan test at $P < 0.05$ level.

DXS is the first enzyme for plastidial terpenoid biosynthesis in plants. The treatment of *Cannabis* plants with 1 μM ABA caused a slight increase in DXS activity (Table 1) while 10 μM ABA did not have any significant effect on DXS activity. Estevez *et al*. (2001) reported that transgenic *Arabidopsis* plants showing up-regulation or down-regulation of DXS activity concomitantly showed changes in the contents of MEP-derived isoprenoid end-products. The phytyl C20 conjugates of chlorophylls and tocopherols, and C40 conjugates of carotenoids are produced by the MEP pathway. According to our results, the plants treated with 1 μM ABA showed an increase in the contents of chlorophyll *a* and total chlorophyll, whereas, chlorophyll *b* content decreased (Table 1). Chlorophyll *a* content increased also in plants treated with 10 μM ABA. Positive effects of ABA in increasing of chlorophyll content have been reported by Agarwal *et al*. (2005). At previous work we found that at flowering stage ABA decreased the chlorophyll content in both male and female *Cannabis* plants (Mansouri *et al.* 2009). This result indicates that plant response to ABA is different at various stages of development.

Treatment with ABA induced carotenoid accumulation in *Cannabis* leaves (Table 1). Similar to our results, there are several reports showing carotenoid accumulation stimulated by ABA treatment in wheat and *Stylosanthes guianensis* (Agarwal *et al*. 2005, Zhou *et al*. 2005). The amount of α-tocopherol increased only in 10 μM ABA treatment (Table 1). A positive correlation between ABA and α-tocopherol content was observed in *Cistus creticus*, maize seedlings and female *Cannabis* plants (Jiang and Zhang 2001, Mansouri *et al*. 2009, Munne-Bosch *et al*. 2009).

HMGR activity declined in *Cannabis* plants in response to ABA treatment (Table 1). Consistently with

Table 1. Effect of ABA on DXS activity, contents of chlorophylls, carotenoids and α-tocopherol, HMGR activity, contents of squalene, phytosterols, THC and CBD in *Cannabis sativa* plants. Means of three replications ± SD. Means followed by different letters in a column, are significantly different ($P < 0.05$) according to Duncan test.

Parameters	Control	1 μM ABA	10 μM ABA
DXS [rel.U]	4.92 ± 0.21b	5.65 ± 0.29a	4.82 ± 0.34b
Chlorophyll *a* [mg g^{-1}(d.m.)]	8.38 ± 0.39c	9.45 ± 0.26a	8.97 ± 0.34b
Chlorophyll *b* [mg g^{-1}(d.m.)]	4.64 ± 0.15a	4.69 ± 0.20a	4.11 ±0.10b
Carotenoids [mg g^{-1}(d.m.)]	2.11 ± 0.01c	2.33 ± 0.03a	2.20 ± 0.02b
α-Tocopherol [mg g^{-1}(d.m.)]	0.83 ± 0.10b	0.81 ± 0.11b	1.08 ± 0.07a
HMGR [U mg^{-1}(protein)]	0.10 ± 0.004a	0.04 ± 0.002b	0.03 ± 0.002c
Squalene [μg g^{-1}(f.m.)]	23.47 ± 0.57a	14.53 ± 0.68b	13.11 ± 0.26c
Campesterol [μg g^{-1}(f.m.)]	0.64 ± 0.008a	0.43 ± 0.014b	0.38 ± 0.050b
Stigmasterol [μg g^{-1}(f.m.)]	0.74 ± 0.012a	0.51 ± 0.055b	0.16 ±0.015c
β-Sitosterol [μg g^{-1}(f.m.)]	1.96 ± 0.019a	1.54 ± 0.044b	1.42 ± 0.012c
THC [mg g^{-1}(d.m.)]	3.72 ± 0.39a	3.11 ± 0.36a	2.61 ± 0.23c
CBD [mg g^{-1}(d.m.)]	1.17 ± 0.07a	1.23 ± 0.06a	0.50 ± 0.04b

Table 2. Effect of ABA on contents of terpenoids [%] in the essential oil of *Cannabis sativa* plants. Means of three replications ± SD. Means followed by different letters in a line, are significantly different ($P < 0.05$) according to Duncan test.

	Substances	Control	1 μM ABA	10 μM ABA
Monoterpenoids	α-pinene	5.91 ± 0.22	0	0
	β-pinene	2.78 ± 0.15	0	0
	myrcene	2.03 ± 0.09 a	0.78 ± 0.18 b	0
	limonene	2.23 ± 0.18	0	0
	α-campholenal	1.00 ± 0.25 a	0.52 ± 0.02 b	0
	verbenol	10.79 ± 1.25a	6.54 ± 0.74 b	4.23 ± 0.70 c
	pinocarvone	1.17 ± 0.08	0	0
	α-phellanderene	1.00 ± 0.11	0	0
	myrtenal	3.32 ± 0.35 a	1.01 ± 0.25 b	1.13 ± 0.15 b
	verbenone	2.22 ± 0.16 a	1.85 ± 0.14 b	1.16 ± 0.09 c
	citronellol	2.86 ± 0.76 a	2.54 ± 0.54 a	1.98 ± 0.64 a
	carvone	0.76 ± 0.25 a	0.47 ± 0.09 a	0.35 ± 0.02 b
Sesquiterpenoids	caryophyllene	3.70 ± 0.19 a	2.84 ± 0.45 b	1.02 ± 0.27 c
	β-farnesene	2.18 ± 0.64 a	0.63 ± 0.05 b	0
	α-humulene	1.23 ± 0.07	0	0
	germacrene d	1.72 ± 0.24 a	1.00 ± 0.34 b	0.54 ± 0.07 c
	β-bisabolene	1.08 ± 0.23	0	0
	Δ-amorphene	1.32 ± 0.39	0	0
	spathulenol	2.46 ± 0.42	0	0
	caryophyllene oxide	2.76 ± 0.05 b	2.56 ± 0.21 b	3.05 ± 0.15 a
	γ- eudesmol	0.79 ± 0.06 b	0.95 ± 0.22 b	1.35 ± 0.15 a
	valerianol	0.81 ± 0.09 b	1.28 ± 0.25 a	1.33 ± 0.22 a
	β-eudesmol	0.86 ± 0.09 c	1.54 ± 0.29 b	2.06 ± 0.54 a
	bulnesol	0	0.89 ± 0.11 b	1.67 ± 0.34 a
	α- bisabolol	0.53 ± 0.08 c	0.85 ± 0.09 b	1.59 ± 0.25 a
	cryptomerione	0	0	0.48 ± 0.11
Cannabinoids	cannabichrome	2.50 ± 0.40 c	4.67 ± 0.50b	14.22 ± 3.57a
	cannabinol	1.05 ± 0.35 c	1.99 ± 0.31b	2.87 ± 0.23 a

our results, HMGR specific activity was inversely correlated with endogenous ABA level in maize endosperm during seed development and in avocado fruit growth (Moore and Oishi 1994, Cowan *et al*. 1997). The concentration of squalene in plants treated with 1 and 10 μM ABA was reduced in comparison with control plants (Table 1). The accumulation of phytosterols declined in response to ABA treatment (Table 1) and the reduction in stigmasterol and β-sitosterol content was significant at both levels of ABA. The ABA treatment decreased squalene and phytosterol contents in a pattern similar to the changes in the HMGR activity. Since HMGR is an important control point for the MVA pathway in plants (Kato-Emori *et al*. 2001), the decrease in HMGR activity should result in decreasing the supply of phytosterols.

The Δ^9-tetrahydrocannabinol (THC) content was 3-fold higher than cannabidiol (CBD) content in *Cannabis* leaves at vegetative stage (Table 1). Therefore, according to our results this strain belongs to drug-type. The content of THC decreased at 1 and 10 μM ABA treatment. The amount of CBD declined with 10 μM ABA treatment. These results were opposite of those in productive *Cannabis* plants (Mansouri *et al*. 2009). Cannabinoids are synthesized from MEP pathway (Fellermeier *et al*. 2001). In spite of increasing DXS activity, we did not observe an increase in cannabinoid contents. It shows that MEP pathway is regulated by every end product *via* a special mechanism.

To identify the terpenoid composition of the essential oil of *Cannabis* at vegetative stage and effect of ABA on quality and quantity of these compounds, extracts of leaves were analyzed by gas chromatography-mass spectrometry. In plant extracts, monoterpenes, sesquiterpens, cannabinoids and other compounds such as dodecane, pentadecanol and octadecane were identified (Table 2). Verbenol and caryophyllene showed the highest amount among monoterpenes and sesquiterpenes, respectively, and THC was the most abundant cannabinoid in plant extracts (Table 2). THC and CBD were analyzed separately by ultra-performance liquid chromatography (UPLC; Table 1). ABA treatment declined the number of monoterpenes and sesquiterpenes and percentage of monoterpenes in comparison with control plants (Table 2). Some of sesquiterpenes that were detected in control plants were not observed in ABA-

treated plants (*e.g.*, humulene, bisabolene, amorphene and spathulenol). The percentage of some sesquiterpenes (such as germacrene and caryophyllene) declined and some of them (such as valerianol and β-eudesmol) increased by ABA treatment. The percentage of cannabichromene and cannabinol increased when plants were treated with ABA. The most of these components had been reported in previous investigations (Rodriguez *et al*. 1996, Rothschild *et al*. 2005), except of verbenol which is a monoterpen. It may be because of different roles of these terpenoids in plants.

Recent research tried to shed light on regulatory effects of ABA on terpenoids biosynthesis by MEP and MVA pathways. Our results showed that ABA treatment has reducing effect on the amount of monoterpenes and phytosterols. On the other hand, primary metabolites from MEP pathway increased by ABA treatment. Further studies combining genetical and biochemical approaches may eventually allow a deeper understanding of regulatory mechanisms of terpenoids biosynthesis and the roles of phytohormones in this process.

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