Acta Physiol Plant
DOI 10.1007/s11738-010-0636-1

ORIGINAL PAPER

The response of terpenoids to exogenous gibberellic acid in *Cannabis sativa* L. at vegetative stage

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Received: 13 July 2010 / Revised: 2 October 2010 / Accepted: 12 October 2010

Abstract In this study the influence of gibberellic acid (GA_3) on plastidic and cytosolic terpenoids and on two key enzymes, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), for terpenoid biosynthesis was compared in vegetative cannabis plants. Treatment with GA_3 resulted in a decrease of DXS activity in comparison with the control plants. The amount of chlorophylls *a*, *b* and total carotenoids declined when plants treated by GA_3 in a concentration dependent manner. The α-tocopherol content of cannabis plants decreased in 50 μM GA_3 treatment and increased in 100 μM GA_3 treatment. Exogenous GA_3 caused an increase in HMGR activity. Concomitant with this result, the amount of squalene and phytosterols increased with GA_3 treatment. The amount of THC and CBD did not change at 50 μM GA_3 treatment, but applying of 100 μM GA_3 increased THC and CBD content in leaf plant in comparison with control plants. GA_3 treatment declined number and percentage of monoterpenes in treated plants. Also the number of sesquiterpenes decreased in response to GA_3 treatment but among the remainder of them, the amount of some sesquiterpenes decreased and some sesquiterpenes increased with GA_3 treatment. Our results showed that GA_3 treatment had opposite effect on primary terpenoid biosynthesis by the plastidic 2C-methyl-D-erythritol 4-phosphate (MEP) and mevalonate (MVA) pathways. But secondary terpenoids showed different response to GA_3 treatment probably due to interference of two biosynthetic pathways in their formation.

Keywords α-Tocopherol · Δ^9-Tetrahydrocannabinol · Gibberellic acid · Phytosterols · Terpenoids

Abbreviations

GA_3	Gibberellic acid
THC	Δ^9-Tetrahydrocannabinol
HMGR	3-Hydroxy-3-methylglutaryl coenzyme A reductase
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
MEP	2-Methyl-D-erythritol-4-phosphate
MVA	Mevalonate

Introduction

The plant *Cannabis sativa* L. is among one of the world's oldest and best known plants/illicit drugs. It has been used for millennia for its fiber and intoxicating properties. *C. sativa* was one of the first non-food industrial plants to be cultivated by man. Cannabinoids are a group of terpenophenolic compounds found in the hemp plant. The highest cannabinoid concentration is found in the resin secreted by the plants' flowering buds. Δ^9-Tetrahydrocannabinol (THC) is the psychoactive component of the hemp plant; other major non-psychoactive constituents include cannabidiol (CBD) and cannabinol (CBN). On the basis of

Communicated by P. Sowinski.

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Published online: 17 November 2010

THC content, *C. sativa* plants are divided into fiber-type and drug-type (Pellegrini et al. 2005).

Terpene biosynthesis in plants goes through both the mevalonate (MVA) pathway in the cytosol and the 1-deoxy-D-xylulose-5-phosphate/2-methyl-D-erythritol-4-phosphate (DOX/MEP) pathway in plastids (Rohmer 1999). Terpenes include many metabolites like pigments such as carotenes (van den Berg et al. 2000), phytohormones such as abscisic acid, gibberellins and brasinosteroides (Crozier et al. 2000), and phytoalexins (Lange and Ghassemian 2003; Kanno et al. 2006). The production and accumulation of cannabinoids in plants of *C. sativa* follow a non-mevalonate pathway (Fellermeier et al. 2001).

Gibberellins form a large family of diterpenoid compounds, some of which are bioactive growth regulators. GA is widely regarded as a growth-promoting compound that positively regulates processes such as seed germination, stem elongation, leaf expansion, pollen-tube growth, trichome, flower and fruit development and floral transition (Olszewski et al. 2002; Razem et al. 2006). Until recently, the isopentenyl diphosphate used for GA biosynthesis was thought to be derived from mevalonic acid. However, it has been established that cytosolic mevalonate is not involved in GA biosynthesis in plants (Est'evez et al. 2001).

The regulation of the biosynthesis of terpenoids in plant cells is poorly understood. We do not know how the metabolite fluxes between the primary and the secondary metabolisms are orchestrated. Plant growth regulators have essential roles in growth and development of plants and plant responses to environment. In order to understand how they interact with terpenoid biosynthesis, we focused on the role of GA_3 in the regulation of primary and secondary terpenoid production in *C. sativa* at vegetative stage.

Materials and methods

Plant material

The seeds of *C. sativa* L. (with geographical source from Iran) were sown in pots (15 cm, i.d soil-leaf mold-perlit = 2:1:1) and cultivated in a phytotron (25°C 14 h light, 10 h dark). The plants were fertilized weekly with a Hoagland's nutrient.

GA_3 treatment of plants

Cannabis plants were treated when they had seven pairs of leaves. We applied six levels of GA_3 concentration (5, 10, 30, 50, 70, 100 μM). Chlorophyll and carotenoid contents were measured and according to the results we selected two levels of GA_3 concentration (50 and 100 μM). The higher and lower concentrations did not show significant effect.

The plants were subjected to GA_3 (Merck, Germany) treatment by spraying the whole plants with 10 and 100 μM GA_3 solutions and distilled water as a control, until the solution started dripping. The treatment took place with three times spraying at 24 h intervals. The plants were harvested 1 day after the treatment.

Chlorophyll and carotenoid determination

Chlorophyll and carotenoids were extracted from leaves with 95% ethanol and quantified by measuring the absorbance at 664, 648 and 470 nm as described by Lichtenthaler et al. (1997).

Quantitative analysis of squalene and phytosterols by GC

Quantitative analysis of squalene and phytosterols (campesterol, β-sitosterol and stigmasterol) was performed by GC. Freeze-dried leaves (500 mg) were extracted with ethyl acetate at 100 rpm on a gyratory shaker (20 mL, twice, 25°C for 6 h). The acidic compounds were removed with aqueous 5% KOH (10 mL, thrice) followed by the removal of the basic compounds with aqueous 5% HCl (v/v) (10 mL, twice). The organic fraction was washed with water (10 mL, twice) and then dried with anhydrous sodium sulfate. The solvent was evaporated, and the residue was dissolved in hexane (2 mL) and then centrifuged for 10 min at 6,000 rpm to remove the suspended particles.

Chromatography was performed with Agilent Technologies (Wilmington, DE, USA) equipment including a 7,683 automated sample-injection system, a split/split less injector, a 30 m 9 320 lm I. D., 0.25 lm film thickness HP-5 fuse-silica capillary column coated with 5% Phenylmethyl siloxane (J&W Scientific, Folsom, CA, USA) and a flame ionization detection (FID) controlled by the Agilent Chemstation software. The oven temperature was held at 240°C for 10 min, then raised to 260°C at 2°C min^{-1} and then held at that temperature for 30 min; injection port 270°C; detector 300°C; split ratio 15:1, injection volume 1 μL; nitrogen carrier gas 1 mL min^{-1}, hydrogen 30 mL min^{-1} and detector flow of make-up gas (nitrogen) 400 mL min^{-1} in the constant make-up flow mode. Stigmasterol, campesterol, β-sitosterol, and squalene standards were obtained from Sigma (USA).

α-Tocopherol extraction and measurement

Tocopherols were extracted basically as described for cereal seeds by Panfili et al. (2003), by grinding and homogenizing 25 mg of freeze-dried leaf material in 500 μL 100% methanol. After 20 min of incubation at 30°C, the samples were centrifuged at 14,000 rpm for

5 min, the supernatant was transferred to new tubes, and the pellet was re-extracted twice with 250 µL 100% methanol at 30°C for 30 min, pooling all supernatants.

Chromatographic conditions

Ultra-performance liquid chromatography (UPLC) analysis was carried out with a Waters Acquity ultra performance liquid chromatograph equipped with a PDA detector. An Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 µm particles) was used for separation. Methanol (100%) was used as mobile phase; the flow rate was 0.48 mL min^{-1}. PDA detector was accomplished at 290 nm for α-tocopherol. Injection volume was 10 µL.

Sample collection for THC, CBD and terpenoid measurement

The third leaves pair were collected and used for cannabinoid measurement. Samples were dried at room temperature in darkness. Sample material (50 mg) was placed in a test tube with 1 mL chloroform. Sonication was applied for 15 min. After filtration, the solvent was evaporated to dryness and the residue was dissolved in 0.5 mL methanol.

Chromatographic conditions

The apparatus and column conditions were the same as those of α-tocopherol analysis. The mobile phase was an acetonitrile–water gradient from 70:30 to 100:0 in 5 min, stay 100:0 in 1 min, return to 30:70 in 1 min (flow 0.4 ml min^{-1}). Buffer (0.05% TFA resulting in a pH of 3) was added to both solvents to eliminate the tailing of phenolic compounds. PDA detector was accomplished at 230 nm for THC and CBD. Injection volume was 7 µl. Cannabinoid peaks were identified by cannabinoids standards (THC and CBD) that were a generous gift from Pr. Jun Szopa Wroclaw University, Wroclaw, Poland.

GC–MS conditions

Analyses were carried out on a Shimadzu-QP5050 gas chromatograph fitted with an HP5-MS 40 m × 0.18 mm × 0.18 µM (Bonded phase 5% Phenyl siloxane) column, 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 30°C, and held for 7 min. A 0.5 µL volume of extract was injected into the chromatograph. Terpenoids and other compounds in plant extracts were identified by mass spectra, and Kovats retention indices calculated from the retention times of hydrocarbon standards.

Enzyme assays

Enzymes assayed in this work were extracted from the fresh leaves with a 50 mM Tris–HCl extraction buffer containing, 10 mM β-mercaptoethanol, 1% (w/v) polyvinylpyrrolidone (PVP), and pH 7.5. The leaves were ground in the extraction buffer (1 gfw mL^{-1}) for 5 min with a pestle and mortar on ice and by liquid nitrogen, followed by centrifugation at 14,000 rpm and 4°C for 30 min to obtain a solid-free extract. The content of protein was determined according to Bradford (1976) method using bovine serum albumin as a standard.

The activity of HMGR was determined by the method of Toroser and Huber (1998). The activity of 1-deoxy-D-xylulose 5-phosphate synthase was determined by the fluorometric method of Querol et al. (2001) which is based on the reaction of 1-deoxy-D-xylulose 5-phosphate with 3, 5-diaminobenzoic acid in an acidic medium to form a highly fluorescing quinaldine derivative. In this method, DXS activity express as relative activity.

Data analysis

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

Results and discussion

Figure 1 shows influence of exogenous GA_3 on DXS activity. Treatment with GA_3 resulted in a decrease of DXS activity in comparison with the control plants. GA_3 treatment had a stronger effect on decreasing of DXS activity in high concentration. There is no report about the effect of

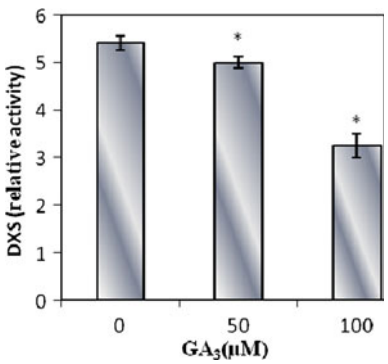

Fig. 1 Effects of gibberellic acid (GA_3) on 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

GA_3 on DXS activity. Many reports support a regulatory role of DXS for the production of MEP-derived isoprenoids in plants (Rodriguez-Concepcion 2006). Est'evez et al. (2001) reported that transgene-mediated upregulation or downregulation of DXS levels in Arabidopsis were correlated with concomitant changes in the levels of MEP-derived isoprenoid end products.

The effects of the concentrations of GA_3 on the chlorophyll contents of cannabis leaf are presented in Fig. 2. The amount of chlorophylls *a*, *b* and total decline when plants treated by GA_3 in a concentration dependent manner. The phytyl (C20) conjugates chlorophylls and tocopherols, and carotenoids (C40) are produced by the MEP pathway. Reduction in chlorophyll content after GA_3 application has been reported in wheat (Misra and Biswal 1980), peach trees (Monge et al. 1994), rice seedlings (Yim et al. 1997) and pea (Bora and Sarma 2006).

Treatment of cannabis plants with 50 and 100 μM GA_3 significantly decreased carotenoid contents in a dose-dependent pattern (Fig. 3). The variations in the level of the carotenoid were similar to those observed for the chlorophylls. Apparently carotenoids and chlorophyll accumulation are controlled through a similar mechanism, because both of them are reduced by GA_3. The changes in chlorophyll and carotenoid contents were parallel with changes in DXS activity. It can show the limiting role of DXS activity in chlorophyll and carotenoid synthesis.

The α-tocopherol content of cannabis plants decreased in 50 μM GA_3 treatment and increased in 100 μM GA_3 treatment (Fig. 4). Tocopherols (α-, *β*-, *γ*-, and *δ*-tocopherol) are lipophilic antioxidants that collectively constitute vitamin E (Crowell et al. 2008). Tocopherols are synthesized by photosynthetic organisms, occurring mainly in leaves and seeds. Literature sources indicate that the major tocopherol form in leaf tissues is α-tocopherol (Szymanska and Kruk 2008). Abdul Jaleel et al. (2007) reported that GA_3 treatment stimulated the α-tocopherol accumulation in *Catharanthus roseus*.

Exogenous GA_3 caused an increase in HMGR activity (Fig. 5). Since HMGR is an important control point for the

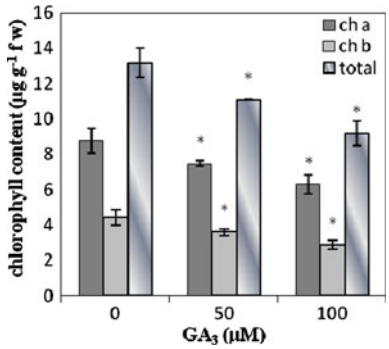

Fig. 2 Effects of gibberellic acid (GA_3) on chlorophylls *a*, *b* and total in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

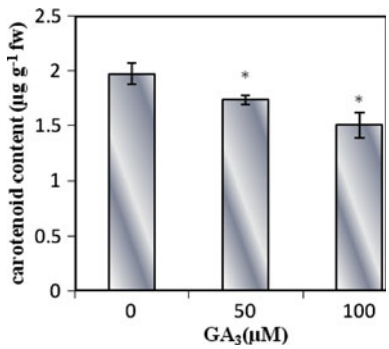

Fig. 3 Effects of gibberellic acid (GA_3) on carotenoids in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

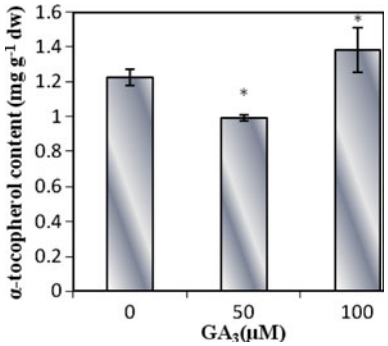

Fig. 4 Effects of gibberellic acid (GA_3) on α-tocopherol content in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

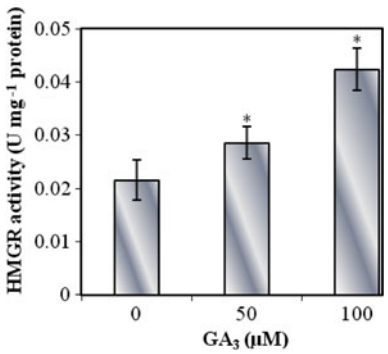

Fig. 5 Effects of gibberellic acid (GA_3) on 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activities in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

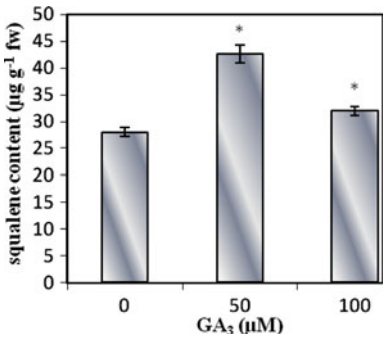

Fig. 6 Effects of gibberellic acid (GA_3) on squalene content in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

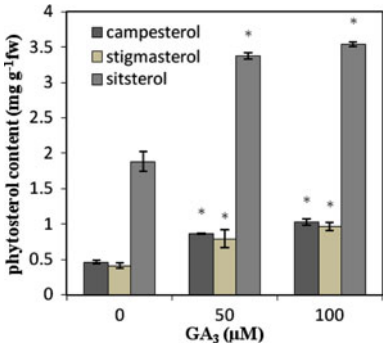

Fig. 7 Effects of gibberellic acid (GA_3) on phytosterol content in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

MVA pathway in plants (Kato-Emori et al. 2001), the increase in HMGR activity should result in increasing the supply of phytosterols. Squalene and phytosterol contents showed similar changes and increased concomitant with HMGR activity (Figs. 6, 7). Consistent with our results pea seedlings treated with GA_3 showed an increase in the HMGR activity (Russell and Davidson 1982).

Control plants had higher amount of THC than CBD. The amount of THC and CBD did not change at 50 µM GA_3 treatment, but applying of 100 µM GA_3 increased THC and CBD content in comparison with control plants (Fig. 8). The increasing of THC content was higher than those of CBD content. Probably, the increase observed in the THC and CBD content at high level of GA_3 is not a direct effect of GA_3 treatment and could reflect the GA_3 interaction with other plant hormones. As it has been shown that exogenous application of GA_3 caused a clear increase in ACC content, ACC oxidase activity and ethylene biosynthesis occur during the breaking of dormancy and onset of germination in *Fagus sylvatica* L. seeds (Calvo et al. 2004). Furthermore, it is possible that ethylene caused the increase observed in THC and CBD content.

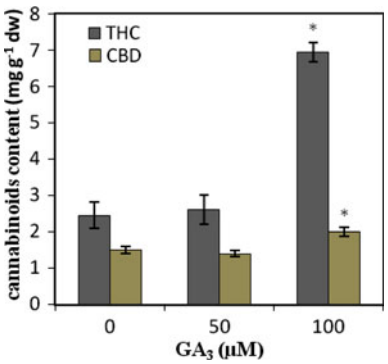

Fig. 8 Effects of gibberellic acid (GA_3) on THC and CBD content in the leaves of cannabis plants. Values are means of three replications ± standard deviation. *Asterisks* indicate the significant difference at $P < 0.05$ level by Duncan test when compared with controls

To identify the terpenoid composition of the essential oil of cannabis at vegetative stage and effect of GA_3 on quality and quantity of these compounds, extracts of leaves were analyzed by gas chromatography–Mass spectrometry. In plant extracts, 12 monoterpenes, 14 sesqueterpens, 4 cannabinoids and other compounds such as dodecane, pentadecanol and octadecane were identified (Table 1). Verbenol with 10.79% and caryophyllene with 3.7% showed the higher amount among monoterpenes and sesquiterpenes, respectively. Because THC and CBD were analyzed separately by UPLC, the results of their analysis have not been shown in Table 1. GA_3 treatment declined number and percentage of monoterpenes in treated plants in comparison with control plants. From 12 monoterpenes that were identified in control plants only 3 and 1 monoterpenes were detected in plants treated with 50 and 100 µM GA_3, respectively. Except of THC and CBD, two other cannabinoids, CBN and cannabichrome, were detected in plant extracts. GA_3 treatment at 100 µM level increased the amount of these cannabinoids. Plants treated with 50 and 100 µM GA_3 had seven and four sesquiterpenes, respectively. Exogenous GA_3 increased the percentage of some sesquiterpenes (Caryophyllene, Spathulenol, β-Eudesmol, α-Bisabolol) in 50 and 100 µM level and decreased the percentage of tree sesquiterpenes (β-Farnesene, α-Humulene, Germacrene D). Other sesquiterpenes were not detected on plant essential oil. Different effect of GA_3 on sesquiterpene content could be explained by different pathways of sesquiterpene biosynthesis (Laule et al. 2003), because both pathways (MVA and MEP) contribute in sesquiterpene biosynthesis. Furthermore, there is this expectance that biosynthesized sesquiterpenes from MVA pathway increase and those of from MEP pathway decrease with GA_3 treatment.

These results showed that GA_3 treatment had opposite effect on primary terpenoid biosynthesis by MVA and

Table 1 Effect of GA_3 on terpenoids ratios in the essential oil of cannabis plants

	Substance	Control	GA_3 (50 μM)	GA_3 (100 μM)
1.	α-Pinene	5.91 ± 0.22	0	0
2.	β-Pinene	2.78 ± 0.15	0	0
3.	Myrcene	2.03 ± 0.09	0	0
4.	Limonene	2.23 ± 0.18[a]	0.43 ± 0.05[b]	0
5.	α-Campholenal	1.00 ± 0.25	0	0
6.	Verbenol	10.79 ± 1.25[a]	9.71 ± 1.08[a]	8.61 ± 0.79[b]
7.	Pinocarvone	1.17 ± 0.08	0	0
8.	α-Phellanderene	1.00 ± 0.11	0	0
9.	Dodecane	1.03 ± 0.15	0	0
10.	Myrtenal	3.32 ± 0.35	0	0
11.	Verbenone	2.22 ± 0.16[a]	0.49 ± 0.02[b]	0
12.	Citronellol	2.86 ± 0.76	0	0
13.	Carvone	0.76 ± 0.25	0	0
14.	Isobornyl acetate	1.16 ± 0.25[a]	0.65 ± 0.09[b]	0
15.	Caryophyllene	3.70 ± 0.19[c]	4.83 ± 0.53[b]	6.94 ± 0.68[a]
16.	β-Farnesene	2.18 ± 0.64[a]	0.91 ± 0.09[b]	0
17.	α-Humulene	1.23 ± 0.07[a]	0.48 ± 0.04[b]	0
18.	Germacrene D	1.72 ± 0.24[a]	0.62 ± 0.07[b]	0
19.	β-Bisabolene	1.08 ± 0.23	0	0
20.	Δ-Amorphene	1.32 ± 0.39	0	0
21.	Spathulenol	2.46 ± 0.42[c]	5.36 ± 1.10[b]	8.53 ± 0.85[a]
22.	Caryophyllene oxide	2.76 ± 0.05	0	0
23.	γ-Eudesmol	0.79 ± 0.06	0	0
24.	Valerianol	0.81 ± 0.09	0	0
25.	β-Eudesmol	0.86 ± 0.09[c]	2.75 ± 0.54[b]	4.13 ± 0.53[a]
26.	α-Bisabolol	0.53 ± 0.08[c]	0.99 ± 0.09[b]	1.77 ± 0.25[a]
27.	Pentadecanol	0.35 ± 0.07	0	0
28.	Octadecane	0.63 ± 0.05[c]	5.87 ± 0.79[b]	12.71 ± 1.5[a]
29.	Cannabichrome	2.5 ± 0.4[b]	1.97 ± 0.35[b]	5.25 ± 0.75[a]
30.	Cannabinol	1.05 ± 0.35[b]	1.09 ± 0.31[b]	2.37 ± 0.23[a]

Values are means of three replications ± SD. Different letters indicate significant differences ($P < 0.05$) from the control according to Duncan tests

MEP pathways. GA_3 treatment caused a decrease in DXS activity and biosynthesized primary terpenoids from MEP pathway, but this treatment increased HMGR activity and phytosterols from MVA pathway. Whereas secondary terpenoids showed different response to GA_3 treatment and it could be because of interference of two biosynthetic pathways in their formation.

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