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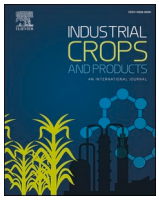

Biotic stress caused by *Tetranychus urticae* mites elevates the quantity of secondary metabolites, cannabinoids and terpenes, in *Cannabis sativa* L.

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ABSTRACT

Secondary metabolites are known to play a role in the plant's defense system, which can be triggered by biotic or abiotic stress. Cannabis (*Cannabis sativa* L.) plants and mainly their female flowers, have a variety of bioactive metabolites, predominantly cannabinoids and terpenes, which are synthesized and secreted by the trichomes. Many studies have examined their chemistry and bioactive effects; however, there is insufficient information on the effect of biotic stress on the presence of secondary metabolites in cannabis. The present study examined the effect of a well-known cannabis pest, *Tetranychus urticae,* on the occurrence and concentration of cannabinoids and terpenes in cannabis leaves and flowers. Six cannabis plants were infested with *T. urticae* mites (treatment group), and six plants were used as the control group. Cannabinoids and terpenes were analyzed and quantified by liquid chromatograph mass spectrometer and gas chromatograph mass spectrometer, respectively. The contents of several cannabinoids and terpenes increased significantly in the leaves of the treatment group of plants in their late vegetative phase as the mite population increased, compared with the control group. Significantly increased content of almost all terpenes, and the cannabinoids; Δ^9-tetrahydrocannabinol, cannabichromene, and cannabigerol, was also seen in mature flowers of the treatment group plants, compared with the control group. Thus, cannabis plant infestation has an impact on its secondary metabolites, cannabinoids and terpenes, reflected by an overall increase in these compounds.

1. Introduction

Cannabis sativa L. is used worldwide for medicinal and recreational purposes, due mainly to its unique bioactive compounds, the phytocannabinoids. In addition, cannabis plants that are classified as hemp are used in various industries, including textiles and building construction (Schultes, 1996). Although the benefits of this plant have been well-recognized in eastern cultures for thousands of years, it is only in the last century that cannabis has garnered the interest of western medicine and its scientific community; in recent years, its medicinal uses have considerably expanded. Medicinal cannabis is given worldwide to patients suffering from cancer, multiple sclerosis, Parkinson's disease, Crohn's disease, numerous psychiatric disorders, and more (Hill, 2015; Sexton et al., 2016). There is strong evidence of its beneficial impact in treating chronic and neuropathic pain, along with the spasticity associated with multiple sclerosis (Hill, 2015). Medicinal cannabis has the potential to treat nausea and stimulate appetite in patients suffering from HIV infection (Woolridge et al., 2005).

Cannabis contains a rich spectrum of phytochemicals, including cannabinoids and terpenes, which are synthesized in the disk cells and stored in the secretory cavity in glandular trichomes, which are present on the surface of leaves and flowers but are most abundant in mature female flowers (Mahlberg and Eun, 2004). Cannabinoids and terpenes are biosynthesized in the trichomes from the mutual parent compound geranyl pyrophosphate. Terpenes are derived from the mevalonate pathway, which is active in the cytosol, or from the plastidial deoxyxylulose phosphate/methylerythritol phosphate (DOXP/MEP) pathway (Flores-Sanchez and Verpoorte, 2008). Both pathways form isopentenyl diphosphate and dimethylallyl diphosphate. The mevalonate pathway is generally considered to provide precursors for the synthesis of sesquiterpenes and triterpenes, whereas the DOXP/MEP pathway supplies precursors for monoterpenes and diterpenes. In cannabis, both pathways can be present, and the DOXP/MEP pathway supplies the geranyl pyrophosphate precursor for the biosynthesis of cannabinoids as well

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(Flores-Sanchez and Verpoorte, 2008).

Cannabinoids play a role in the cannabis plant's defense against pathogens, and they are known to be efficient against insects, fungi, viruses and bacteria (McPartland, 2000). These unique compounds have an effect on mammalian cells due to their ability to interact with membrane receptors of the endocannabinoid system, in particular with the cannabinoid receptor CB1, which is present mainly on cells of the central and peripheral nervous system, and CB2, which is present mainly on immune cells (Mackie, 2008). More than 100 cannabinoids are known; all are biosynthesized from cannabigerolic acid (CBGA) or cannabigerivarinic acid (CBGVA) as carboxylic acids and can be found in the plant usually with the carboxyl group, which is dissociated in the presence of heat or light (Russo, 2011).

Terpenes' structure and classification are based on the linking of numerous isoprene units. These compounds are responsible for the aroma of the plant and among others, have a role in its defense system, serving in a range of defense strategies against insects, fungi, and bacteria (Gershenzon and Dudareva, 2007). In addition, the oily content of the trichomes makes them sticky, creating a trap for insects. The repellent properties of terpenes and the insecticidal properties of cannabinoid acids combine to generate a weapon against arthropod pests. Due to their aroma, terpenes are widely used in the perfume and food industries and are well-tolerated by humans. Many terpenes exhibit medicinal potential and are believed to contribute to the immunological and neurological effects of cannabinoids; in fact, a possible synergy between cannabinoid and terpene activities has been suggested, and it is believed that the appropriate selection of such compounds could have a promising medicinal impact in antibacterial and psychopharmacological applications (Russo, 2011).

Even though cannabis produces and secretes efficient antipathogenic factors such as cannabinoids and terpenes, it is known to be affected by numerous pests that can destroy its yields. Most of these are arthropods in the classes Insecta, Crustacea and Arachnida (mostly mites). Other pests, predominantly rodents, birds, and mollusks such as slugs and snails, are also known to be harmful to cannabis, mainly in outdoor crops (McPartland 1996). The most abundant pests of cannabis worldwide, and in Israel in particular, are spider mites, whiteflies, thrips and the fungus *Botrytis cinerea* (Alchimia blog, 2017). The well-known pest *Tetranychus urticae* (the two-spotted spider mite) has a destructive effect on various crops, including cannabis. *T. urticae* inhabits and feeds mainly on the abaxial leaf surface, causing the appearance of small white or yellow spots on the adaxial leaf surface. Most leaf parts turn yellow as the feeding continues. *T. urticae* punctures individual cells of the plant leaf and causes loss of chlorophyll, thereby reducing net photosynthetic rate. In addition, the wide-ranging damage can cause some water loss in the infested plant (Park and Lee, 2002).

In general, environmental conditions, including different types of stresses, are known to affect secondary metabolism in plants. Numerous studies, generated on various plants, have shown that terpenes are affected by various environmental factors such as drought, temperature fluctuations or pathogen attack; which rearrange the biosynthesis and emission of the terpenes (Block et al., 2019; Kopaczyk et al., 2020; Mahdavi et al., 2020; Zhou et al., 2020). The effect of abiotic environmental changes on cannabinoids and terpenes in cannabis plants was relatively studied (Gorelick and Bernstein, 2017; Landi et al., 2019; Magagnini et al., 2018), however the effect of biotic stress on cannabinoid and terpene composition in cannabis plants was not studied yet. The present study aimed to examine the effect of *T. urticae* on the composition and quantity of cannabinoids and terpenes (including terpenoids) in cannabis leaves and flowers.

2. Materials and Methods

2.1. Materials

Analytical standards of terpenes were purchased from Merck (Sigma-Aldrich, Israel); terpene mix A (CRM40755) and B (CRM40937) containing in sum 34 abundant cannabis terpenes, at concentration of 2000 mg/L in methanol each. Additional 5 single terpene standards were purchased as well; (-)caryophyllene oxide (CRM40928), (-)guaiol (CRM40917), valencene (CRM40934) and *cis/trans*ocimene (CRM40748), each at concentration of 2000 mg/L in methanol, and myrcene (64643–100MG-F), from which a stock solution of 2000 mg/L in methanol was made. A total amount of 14 cannabinoid standards were purchased from Merck: cannabidivarin (CBDV, C-140), cannabigerol (CBG, C-141), cannabidiol (CBD, C-045), tetrahydrocannabivarin (THCV, T-094), cannabinol (CBN, C-046), Δ^{9}-tetrahydrocannabinol (Δ^{9}THC, T-005), Δ^{8}- tetrahydrocannabinol (Δ^{8}THC, T-032), and cannabichromene (CBC, C-143), all at concentration of 1000 mg/L in methanol. In addition to cannabidiolic acid (CBDA, C-144), cannabigerolic acid (CBGA, C-142), cannabinolic acid (CBNA, C-153), cannabicyclol (CBL, C-154), Δ^{9}-tetrahydrocannabinolic acid (THCA, T-093), and cannabichromenic acid (CBCA, C-142), which were at 1000 mg/L in acetonitrile. For more accurate quantitation, internal standards were purchased from Merck as well: deuterated cannabidiol (CBD-D_3, C-084) at concentration of 100 mg/L in methanol for all cannabinoids quantification, and deuterated toluene (toluene-D_8, 233382–1 G), from which a stock solution of 100 mg/L was made in methanol, for terpenes quantification. A mix of all terpene standards was prepared in methanol solution at concentration of 100 mg/L and used as a stock standard solution. The concentration of toluene-D_8 in the standards mixture and sample solutions was 1 mg/L. All cannabinoid standards were mixed into a stock solution of 50 mg/L in methanol, and the concentration of CBD- D_3 was 0.1 mg/L in all the solutions. For the calculation of retention indices, a mixture of *n*-alkanes (C7-C40) was purchased from Merck (49452-U). Additional materials, including solvents, were purchased from Avantor (Israel).

2.2. Cannabis plants growth

Cannabis plants were cloned by cuttings of the strain RCK23 and grown in a polycarbonate-roofed greenhouse, located at RCK cannabis company in kibbutz Ruhama, Israel. The average temperature was 25–27 °C with 45% relative humidity. The vegetative (16 h light/day) and reproductive (12 h light/day) phases lasted 2 months each, generated by automatic darkening. The control group consisted of six plants that were spray-treated in the beginning of the study with Xmite acaricide, purchased from Rimi, to prevent mite development. In parallel, at the same time, the treatment group consisted of six plants that were infested with *T. urticae* eggs, purchased from Biobee. Two leaves from the lower part of each of the plants were collected during the vegetative phase, at nine sampling times (Supplementary Table S1), and kept at − 80 °C until analysis. The first three sampling times were prior to mite infestation and Xmite acaricide treatment. At sampling times 6–9, three plants died (two and one from the treatment and control group, respectively). Plants from the treatment group were treated with acaricide in their late vegetative phase (after the eighth sampling time), to prevent the remaining plants from dying prior to flower collection. The mature flowers, terminal and several axillary, were collected at the end of the cycle (Supplementary Table S1) and kept under the same conditions.

The study was approved by Israel medicinal cannabis agency, ministry of health (license number REQ277).

2.3. Metabolite extraction

An extraction method was developed based on the analytical results under different conditions. The effects of drying time and temperature, the nature of the solvent and its volume, and the extraction time, were examined. The final optimized extraction protocol included weighing each sample of wet plant material (100 mg for leaf and flower samples) into a 15-mL plastic tube. The samples were ground in liquid nitrogen

and mixed with 5 mL methanol. After short vortexing, the samples were incubated for 90 min at 30 °C and 200 rpm in a shaking incubator. Then the samples were centrifuged at 3150 g for 10 min and the upper liquid layer was collected and stored until analysis. The extracts of the leaf samples were injected directly into the liquid chromatograph mass spectrometer (LC–MS) without dilution or with a 10-fold dilution in methanol, whereas the extracts from the flower samples were diluted by 10- and 100-fold. For the gas chromatograph mass spectrometer (GC–MS) analysis, the samples were injected directly without any dilution. For chlorophyll measurement, samples were diluted by 2-fold.

In the leaf and flower samples, eight cannabinoids were detected THCA, CBCA, CBDA, CBNA, Δ^9THC, CBC, CBD, and CBG, and another one, CBGA was detected just in the flower samples. The terpenes detected in both the leaf and flower samples are; β-caryophyllene, humulene, and (-)guaiol, while other 14 terpenes were detected just in the flower samples, α-pinene, β-pinene, myrcene, limonene, ocimene, fenchone, terpinolene, fenchol, borneol, α-terpineol, valencene, *trans*-nerolidol, caryophyllene oxide, and β-eudesmol.

2.4. Analysis of cannabinoids using LC–MS

LC–MS method was developed to quantify cannabinoids using the Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) equipped with a heated electrospray ionization source (HESI-II) connected to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (MS). Identification was based on retention time and molecular mass of the compounds. Calibration curves were obtained for each compound using a mixture of analytical standards at concentrations of 0.019–10 mg/L with an internal standard (CBD-D_3) at concentration of 0.1 mg/L, analyzed under the same conditions as the samples.

A 5-μL aliquot of sample was injected into the UHPLC connected to a Poroshell 120 EC-C18 (3.0 ×150 mm, 2.7 μm) column (Agilent Technologies). The mobile phase consisted of (A) 0.1% formic acid in double distilled water and (B) 0.1% formic acid in acetonitrile. Separation was achieved by gradient elution as follows: 0–3 min 60% B, 3–7 min from 60% to 80% B, 7–12 min from 80% to 90% B, held at 90% B for 12–20 min, 20–22 min from 90% to 60% B, and held at 60% B for 22–25 min. The flow rate was 0.4 mL/min. The column temperature was set to 30°C and the autosampler temperature to 10°C.

All cannabinoids were injected to the MS in a positive ion mode and the mass value used for their quantification was $[M+H]^+$. The capillary voltage and temperature were set to 3.5 kV and 350 °C, respectively. The sheath, aux and sweep gas flow rates were set to 35, 10 and 1 arbitrary units, respectively. Data were acquired using X-calibur and Freestyle software. The mass spectrometer was operated in full-scan mode in the m/z range 150–800 with a resolution of 70,000 and ACG target of 1e6.

2.5. Analysis of terpenes using GC–MS

Terpenes were separated and identified using a Thermo Fisher TSQ 8000 EVO GC–MS instrument. A 1-μL aliquot of sample was injected in splitless mode into an Equity-1 (60 m x 0.25 mm I.D., 0.25 μm) column (Supelco). The injector temperature was set to 280 °C, the carrier gas was helium at a flow rate of 1 mL/min, and the oven temperature program was: hold for 2 min at 60 °C, and ramp to 275 °C at 5 °C/min, then hold for 15 min. Peaks were identified using MS spectral matching against reference spectra in the NIST library. Calibration curves were prepared gravimetrically in methanol, using all terpenes mix solution at 20–0.625 mg/L and an internal standard (toluene-D_8) concentration of 1 mg/L. Confirmatory identification was based on retention time, which was calculated for the compounds identified in each sample using the analytical standards analyzed under the same GC conditions. In addition, identification was supported by comparison of retention indices to literature (Bianchi et al., 2007; Cicció and Chaverri, 2008), which were calculated based on Kováts equation: $RI(x) = 100^*z + 100^*\frac{RT(x)-RT(z)}{RT(z+1)-RT(z)}$, where x is the target terpene, z is the number of carbon atoms of the *n*-alkane eluting before the target terpene and z + 1 is the number of carbon atoms of the *n*-alkane eluting after the terpene target.

2.6. Chlorophyll analysis

Chlorophyll content was measured as a marker for pest damage. Total chlorophyll (*a* and *b)* was quantified using equations that were developed and published by Porra et al. (Porra et al., 1989) after extraction in methanol. Spectrophotometric measurement at 652 and 665 nm were performed on an infinite M200 PRO Tecan plate reader.

2.7. Validation of the extraction and quantitation methods

To demonstrate the reliability of the LC–MS and GC–MS methods, validation was carried out based on the European Medicines Agency and World Health Organization guidelines (Kopp, 2016; E.M.A., 2006). Specifically, the method was validated in terms of specificity and selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability and recovery.

2.7.1. Specificity and selectivity

Cannabinoid identification was assessed by comparing accurate (within $\Delta m/z$ = 5 ppm) m/z of $[M+H]^+$ of analytical standards with those obtained by LC-MS of cannabis leaf and flower samples. Terpenes' identification in GC-MS was assessed by the mass spectra profiles of the analytical standards. Comparison of the retention time was performed as well between the analytical standards and those obtained by LC-MS and GC-MS of cannabis extraction samples.

2.7.2. Linearity

Cannabinoid calibration curve was constructed at 10 calibration levels (0.019, 0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5 and 10 mg/L) by diluting a mixture of the cannabinoids in methanol solution containing 0.1 mg/L of CBD-D_3. Terpene calibration curve was constructed at six calibration levels (0.625, 1.25, 2.5, 5, 10 and 20 mg/L) by diluting a mixture of the terpenes in methanol containing 1 mg/L toluene-D_8.

Linearity for cannabinoids and terpenes was assessed by the coefficient of determination (r^2), which should be greater than 0.99.

2.7.3. Recovery

Recovery was evaluated using spiked and non-spiked cannabis extract samples. To the spiked extracts, a mixture of cannabinoid standards was added at a concentration of 5 mg/L and analyzed by LC–MS, and a mixture of terpene standards at a concentration of 5 mg/L and analyzed by GC–MS. Recovery was calculated as % Recovery= 100 × $[(CS-CH)/C_{STD}]$, where *CS* is the concentration of metabolites in the spiked extract, *CH* is the concentration of the metabolites in the extract without spiking, and *CSTD* is the concentration of metabolites added to the spiked extract.

2.7.4. Repeatability

Two cannabis samples: flowers and leaves, were separated and extracted repeatedly, six times in a single day. The accuracy of the repeatability was expressed as the coefficient of variation (RSD) between the six repeats of each sample.

2.7.5. LOD and LOQ

LOD was estimated based on a 3:1 signal-to-noise ratio. LOQ was estimated based on a 10:1 signal-to-noise ratio. All peaks obtained in GC–MS and LC–MS at the lowest calibration curve concentration had a signal-to-noise ratio higher than the LOQ (signal-to-noise ratio of 10).

2.8. Statistical analysis

Statistical analysis was performed with SPSS software using Mann–Whitney *U* test to examine the variance between the groups at each sampling time for leaves, and for flowers. Repeated-measures ANOVA was used to analyze the effect with consideration of the time of the experiment. Linear regression and Pearson correlation were used to characterize the relationships of the damage factors.

3. Results

3.1. Validation of the extraction and quantitation methods

The analytical methods of GC–MS for terpenes and LC–MS for cannabinoids were fully validated with respect to linearity, extraction, recovery and repeatability.

All analyzed terpene calibration curves demonstrated r^2 higher than 0.99. Extraction repeatability was high as well, with RSD below 11%. In terms of recovery, all terpenes demonstrated values in the range of 85–115%, except (-)guiaol, which was lower (Supplementary Table S2, GC–MS chromatogram in Supplementary Fig. S1).

Cannabinoid calibration curves demonstrated r^2 higher than 0.99; extraction repeatability was also high, with RSD below 11%. In terms of recovery, all values were in the range of 100–105%, except THCA, which was higher (Supplementary Table S3, LC–MS chromatogram in Supplementary Fig. S2 and S3).

3.2. Effect of the mites on leaf chlorophyll content

The damage caused by mites to the leaves of the treated plants was examined by measuring their chlorophyll content. The mobile stages of the pest were counted at seven different time points during the plants' vegetative phase (Supplementary Table S4). Cumulative mite days (CMD) was calculated based on the average number of mites per leaf as follows: $\sum\left[\frac{xi+xi+1}{2}*\Delta t\right\}$ where xi is the number of mites at point i of counting, $xi+1$ is the number of mites at the following counting point, and Δt is the days interval between the two counting points (Park and Lee, 2005). In addition, the amounts of chlorophyll *a* and *b* were analyzed after extraction in methanol and total chlorophyll content was quantified as described in materials and methods.

The difference between total leaf chlorophyll content for the control vs. treatment group is visualized in a scatter graph in Fig. 1. Significant differences were obtained at the late leaf sampling times, as mite population increased and the chlorophyll content in the treatment group decreased compared with the control group (by average of 32% and 30% at the eighth and ninth sampling times, respectively). Mite counts and leaf chlorophyll measurements are shown for four time points in Fig. 2. CMD of the treatment group was stable for the first three time points and increased sharply at the fourth time point. Chlorophyll was damaged as the CMD increased, and its concentration decreased in parallel to the rise in CMD. The lowest chlorophyll quantities were obtained when CMD reached 2000 units. Linear regression was used to analyze the correlation between CMD and leaf chlorophyll content. The effect of the CMD on chlorophyll content was found to be significant ($P = 0.02$) with the following estimates: $y = 1.513–1.90e^{-04}*x$ (y = chlorophyll concentration, x = CMD). In addition, Pearson correlation was used to examine the relationship between chlorophyll content and CMD, and a significant negative correlation was found between the two ($r = -0.328$, $P = 0.03$).

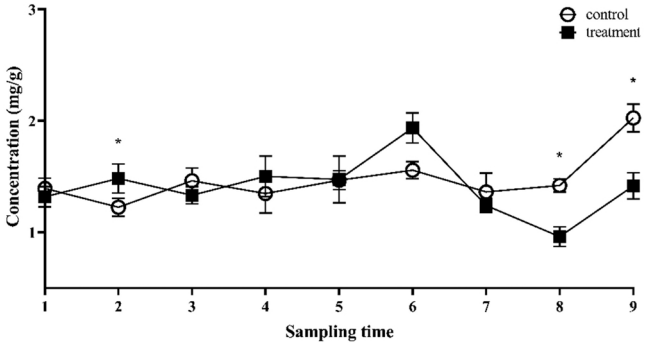

Fig. 1. Total chlorophyll concentration in the leaves with time. Chlorophyll was extracted from leaves of the treatment and control groups and analyzed spectrophotometrically as described in materials and methods. The results were normalized to water-content differences between the groups. Results are presented as mean ± SEM, n = 6 per group (see Supplementary Table S1). * $P \leq 0.05$, * * $P \leq 0.001$ by Mann–Whitney *U* test.

3.3. Effect of the mites on leaf cannabinoid content

The effect of the mites on the presence of cannabinoids in the leaves was examined, and 14 cannabinoids were quantified by LC–MS. Contents of eight of these cannabinoids in the leaves of control and treatment groups at each sampling time are presented in Fig. 3.

At the first six sampling times, the quantities of the eight analyzed cannabinoids were very similar in the treatment and control groups with no significant change in their contents, especially because the first three samples were collected prior to mite infestation. With considerable increasing number of mites and CMD, the quantities of the cannabinoids increased in the treatment group relative to the control group. Some cannabinoids showed a significant increase at the seventh sampling time (CBG by 134%) and some at the eighth sampling time; CBNA, Δ^9THC, CBD, and CBC by 116%, 123%, 125%, and 146%, respectively. The ninth sampling time was subsequent to the acaricide application in the treatment group; nevertheless, some cannabinoids maintained their increased amount compared with the control group.

3.4. Effect of the mites on leaf terpene content

The effect of mites on the presence and quantity of terpenes in the leaves was tested by GC–MS; 39 terpenes were analyzed, 36 based on the calibration curves of the analytical standards and three more based on the mass spectrum compared with the NIST library. Quantitative data of six terpenes in the leaves at each sampling time, for the control and treatment groups, are presented in Fig. 4. Considerable differences in terpene quantities were observed in the treatment group vs. control

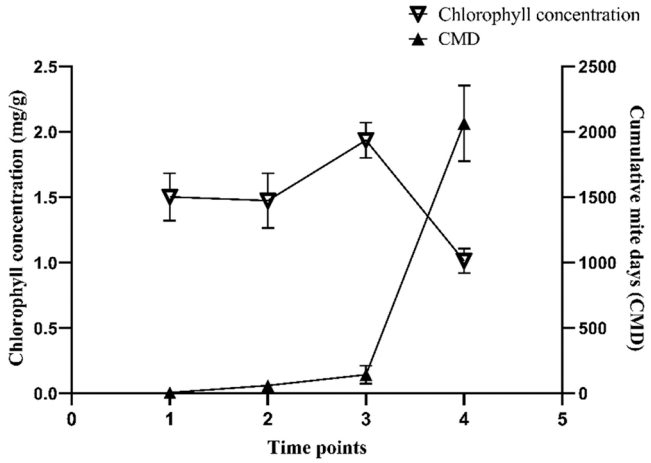

Fig. 2. Scatter plot of total chlorophyll concentration (left Y axis) and cumulative mite days (CMD; right Y axis) in the treatment group plants. Chlorophyll was extracted by methanol and quantified as mg/g wet plant tissue. CMD was calculated. Results are presented as mean ± SEM, for n = 6 per group at sampling points 1 and 2, and n = 5 for points 3 and 4.

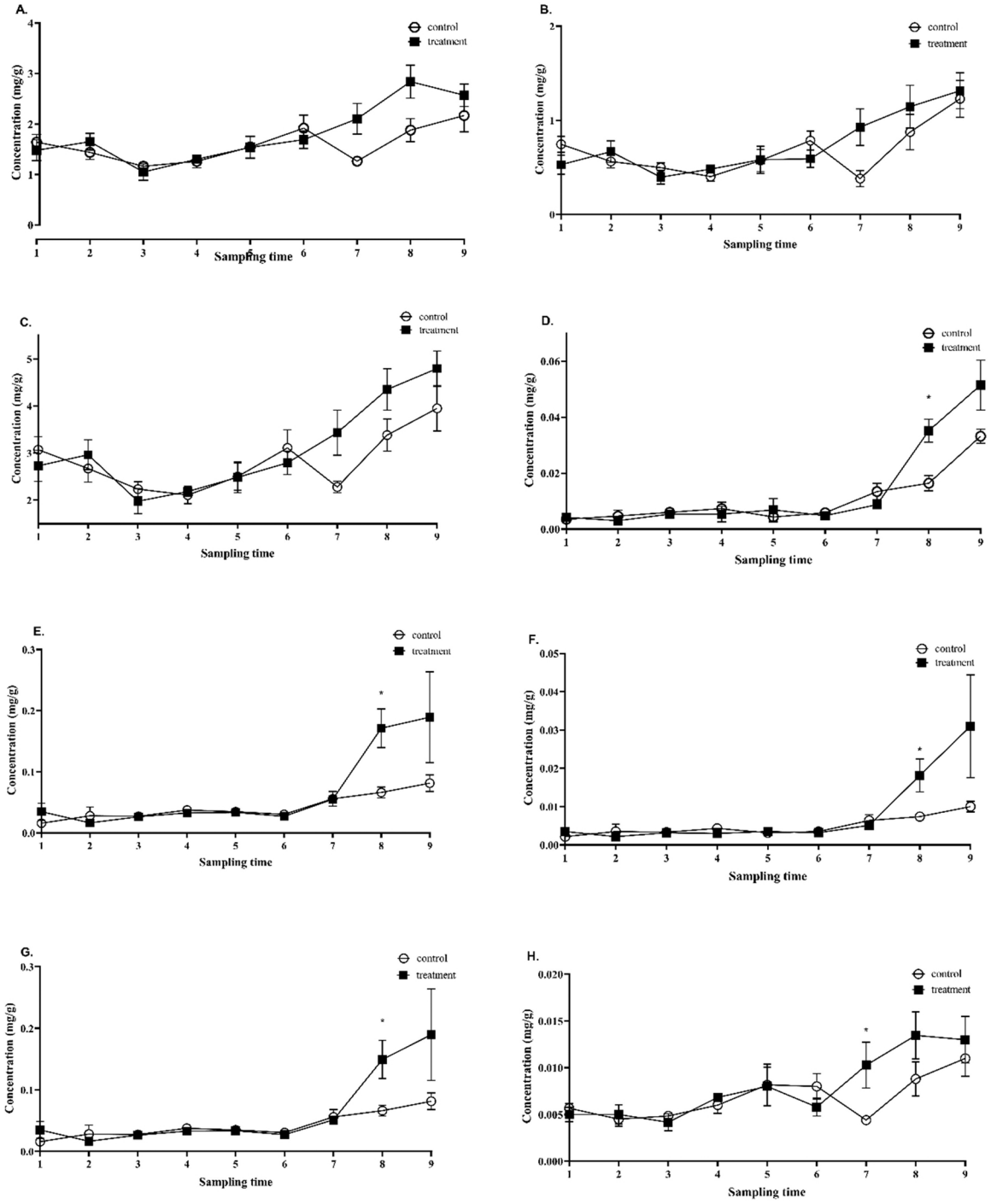

Fig. 3. Cannabinoid concentrations in the leaves with time: THCA (A), CBCA (B), CBDA (C), CBNA (D), Δ^9THC (E), CBC (F), CBD (G), CBG (H). Cannabinoids were extracted from treatment and control leaves and analyzed by LC–MS. All cannabinoids were quantified based on the standard calibration curves of their analytical standards and concentration was calculated in mg/g leaf tissue. The results were normalized to water-content differences between the groups. The results are presented as mean ± SEM, n = 6 per group (see Supplementary Table S1). * $P \leq 0.05$ and * * $P \leq 0.001$ by Mann-Whitney *U* test.

group at sampling times 7–9 (Fig. 4). Three terpenes (Fig. 4A–C) were quantified based on the calibration curve of their analytical standards; their amounts were elevated from sampling time 7 due to the increased number of mites and CMD. Three additional terpenes (Fig. 4D–F) were identified based on mass spectra and comparison to the NIST library. Their peak areas are presented to indicate their amount, which also increased in the leaves of the treatment group plants from sampling time 7 compared with the control group. Bergamotene's content was significantly increased at the seventh and eighth sampling times by 78% and 114%, respectively. Farnesene showed significant increase by 81% at the seventh sampling time of the treatment group plants, compared with the control group. Three of the six terpenes showed significant differences at sampling time 9 (β-caryophyllene, humulene and (-)guiaol by 81%, 53%, and 39%, respectively), although this sampling was performed after acaricide application to the treatment group plants. Selina-3,7(11)-diene is the only terpene for which the increase in the treatment group was not significant.

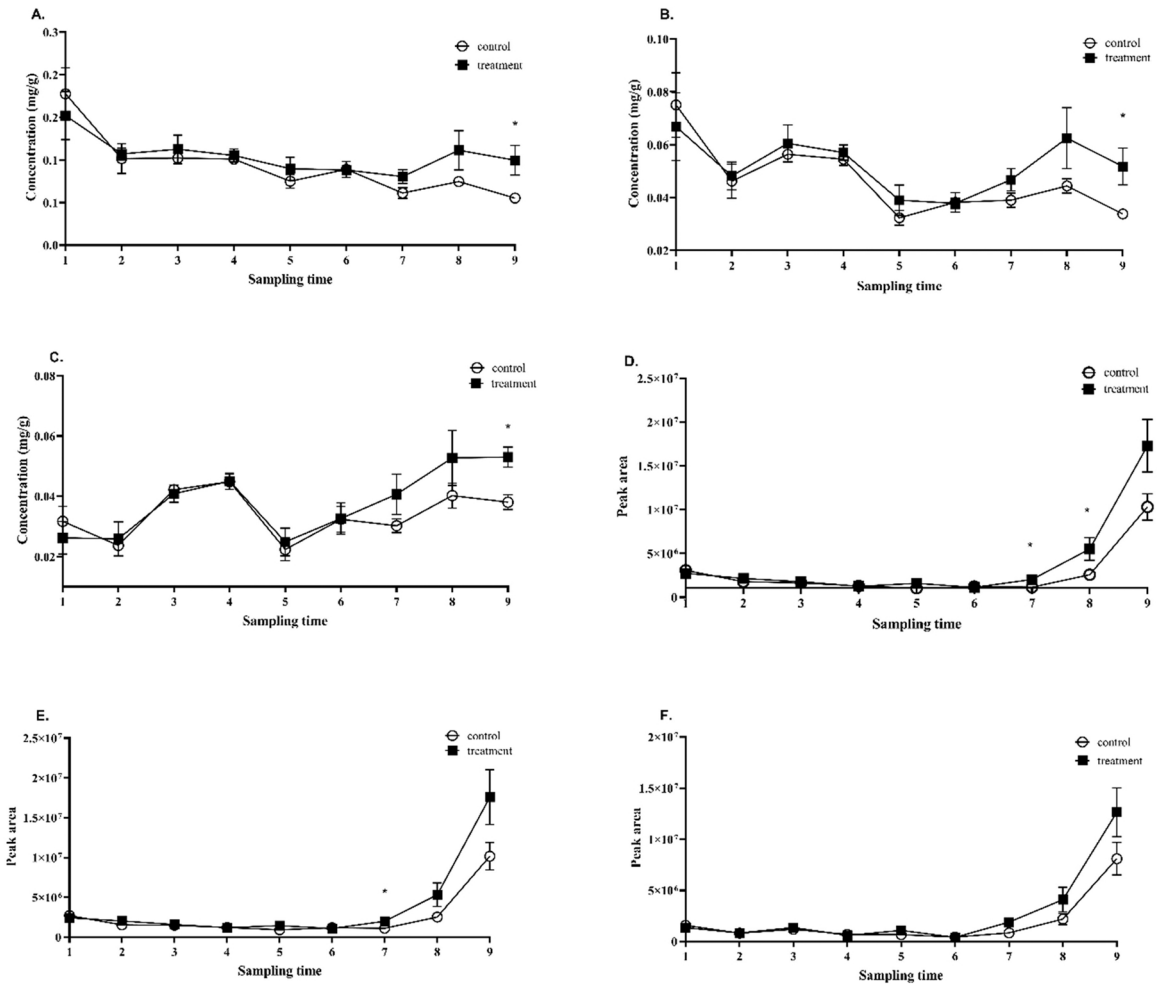

Fig. 4. Leaf terpene concentrations with time. Terpenes in leaves from the treatment and control groups were extracted and analyzed by GC–MS. β-Caryophyllene (A), humulene (B), and (-)guiaol (C) were quantified based on the standard calibration curves of their analytical standards, and concentration was calculated in mg/g leaf tissue. Bergamoetene (D), farnesene (E), and selina-3,7(11)-diene (F) were analyzed without their analytical standards, and are presented as peak areas of the compounds. The results were normalized to water-content differences between the groups. Results are presented as mean ± SEM, n = 6 per group (see Supplementary Table S1). * $P \leq 0.05$, * * $P \leq 0.001$ by Mann–Whitney *U* test.

3.5. Effect of mites on cannabinoid content in flowers

The effect of the mites on cannabinoid content in the female flowers was measured by LC–MS. Flowers from the treatment and control groups were collected during the late reproductive phase, 7 weeks after the acaricide application in the treatment group plants. Quantitative data of the cannabinoids for the control and treatment groups are presented in Fig. 5.

Of the total 14 cannabinoids tested, 9 were quantified. The contents of most of the identified cannabinoids were higher in the flowers of the treatment group vs. control group, and for 3 of them—CBG, CBC, and Δ^9THC— significantly increased by 26%, 41%, and 52%, respectively (Fig. 5). In contrast, CBCA had significantly higher content in the control group (by 15%).

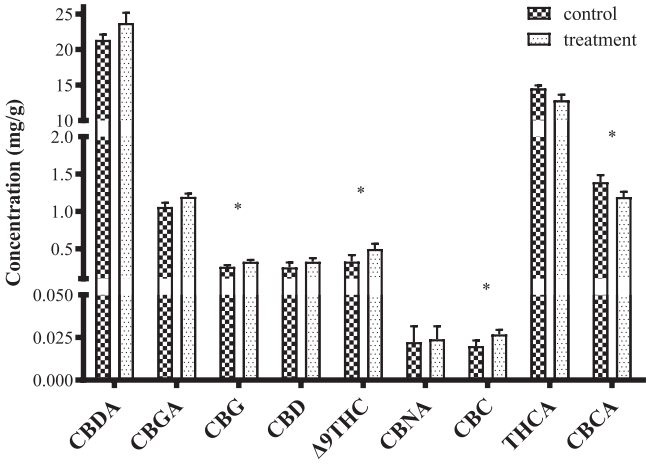

Fig. 5. Cannabinoid concentration in the flowers. Cannabinoids in flowers from the treatment and control groups were extracted and analyzed by LC–MS. All were quantified based on the standard calibration curves of their analytical standards and concentration was calculated in mg/g flower tissue. Results are presented as mean ± SEM, n = 3 for the control group (average of several flowers per plant) and n = 4 for the treatment group (average of several flowers per plant). * $P \leq 0.05$, * * $P \leq 0.001$ by Mann-Whitney *U* test.

3.6. Effect of the mites on terpene content in flowers

The effect of the mites on the presence and quantity of terpenes in the mature female flowers was tested by GC–MS. Quantitative data of the terpenes for the control and treatment groups are presented in Fig. 6.

Interestingly, all terpene quantities were higher in the treatment group compared with the control group (Fig. 6). The most abundant monoterpene in the flowers was myrcene, and the most abundant sesquiterpene was β-caryophyllene. All terpene quantities, except for

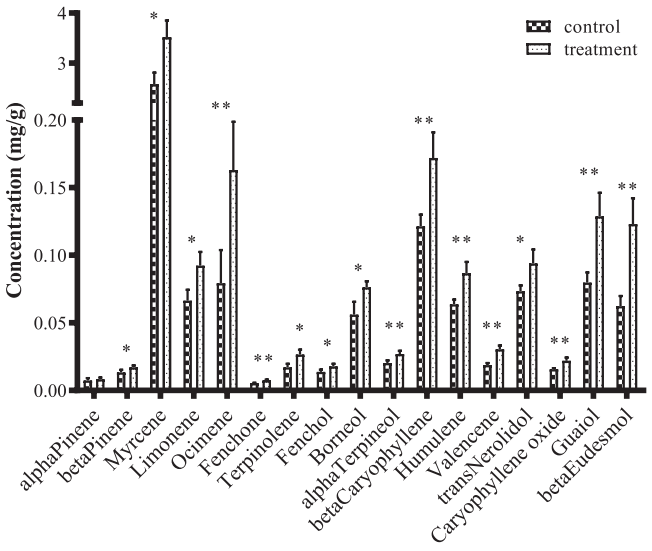

Fig. 6. Terpene concentration in the flowers. Terpenes in treatment and control group flowers were extracted and analyzed by GC–MS. All were quantified based on the standard calibration curves of their analytical standards and concentration was calculated in mg/g flower tissue. Results are presented as mean ± SEM, n = 3 for the control group and n = 4 for the treatment group (average of several flowers per plant). * $P \leq 0.05$, * * $P \leq 0.001$ by Mann–Whitney *U* test.

α-pinene, were significantly higher, even though the mites were treated with acaricide prior to the flowers' late development and collection. Most of the terpene quantities showed a significant increase at $P < 0.001$.

3.7. Distribution of metabolites in flowers of treatment vs. control group

To obtain a more visual assessment of the impact of the treatment on the tested cannabinoid and terpene quantities in the flowers, a heat map (Fig. 7A) and principal component analysis (PCA, Fig. 7B) were generated.

In the heat map, the red color, which represents an increase in compound quantity, dominated in the flowers of the treatment group compared with the flowers of the control group. The latter was predominantly blue in color, representing a decrease in compound quantities (Fig. 7A).

To discriminate the compounds found in the flowers, we carried out PCA based on the group variable. The PCA results, presented in Fig. 7B, show that two components, accounting for 98.6% of the variability in the original dataset, were extracted. The first principal component displayed 88.4% of the total variation. The clustering classified the compounds in the treatment and control groups separately.

4. Discussion

Cultivation of cannabis and the medicinal use of its products have increased rapidly in the last few decades. Cannabis has a wide variety of secondary metabolites, many of which are active in human cells. The best known of these are the cannabinoids and the terpenes, acting in the plant's defense system. Biosynthesis of secondary metabolites can be affected by abiotic or biotic stresses, especially if their role is to protect the plant from pathogens and pests. In this study, we examined the effect of a biotic stress, the common mite pest *T. urticae*, on the presence and quantity of the bioactive secondary metabolites, cannabinoids and terpenes, in cannabis leaves and flowers.

Damage by folivorous mites (Banerjee et al., 2020), and specifically *T. urticae* (Park and Lee, 2005), is reflected in a decrease in the infested leaves' chlorophyll content. The damage caused by the mites to the leaves of the plants in the treatment group was examined and the chlorophyll content was quantified. The content of chlorophyll decreased as the mite population and CMD increased in the leaves (Fig. 2). Since chlorophyll content was lower in the leaves of the treatment group at the late sampling times (Fig. 1) with increasing CMD, and a significant negative correlation was found between the two, chlorophyll content was a valid indicator of the damage done by the mites.

To assess the impact of the mite infestation on cannabinoid content, leaves were collected throughout the experiment from plants of the treatment and control groups, and their cannabinoid content was analyzed by LC–MS. Throughout the beginning of the study, as the mite population did not increase considerably, no differences were seen in the metabolites' content between the leaves of the treatment and the control group. In general, an increase in cannabinoid quantities was seen in leaves of plants from both groups as the reproductive phase begun (see Fig. 3 and Supplementary Table S1), while the increase in the treatment group was more notable. Statistically significant differences between the cannabinoid contents in the two groups was seen in the late vegetative phase (sampling times 7 and 8, Fig. 3), with the growing number of feeding mites. Cannabinoids that showed the highest amounts in the leaves were THCA and CBDA. There are no other reports in the literature on the effect of a biotic stress, specifically from *T. urticae,* on cannabinoid content in cannabis. Nevertheless, cannabinoids are known to have many antipathogenic properties and to play a role in the cannabis defense system, and their content is therefore likely to be affected by biotic stress, as found in this study. The overall increase in the quantities of cannabinoids in the leaves of the infested cannabis plants strengthens their assumed role in the plants' defense system against mites, specifically *T. urticae*.

The effect of the mites on terpene content was determined based on their quantification by GC–MS. Similar to the cannabinoid results, terpene content was higher in the leaves of the treatment group at sampling times 7 and 8, after the considerable growth of the mites. In addition, the last sampling time (9) also showed an increase in the amounts of quantified terpenes (Fig. 4), in contrast to the cannabinoids, which did not show any significant increase at this latest sampling time. The terpene present at the highest amount out of all quantified terpenes in the leaves was β-caryophyllene. To our knowledge, the effect of a biotic stress on terpene content has not been documented in cannabis; however, it has been documented in various other plants (Kopaczyk et al., 2020), revealing increases in terpene concentration under biotic stress, similar to that under mite stress in our study.

Many metabolic pathways in the plant can be triggered and change in response to environmental changes including the use of pesticides (Dubey et al., 2016), which can be accumulated in the plant and affect its secondary metabolism; however we can dismiss the acaricide's effect on the cannabinoid and terpene production in our study based on the results. No notable changes were seen between the groups after the acaricide application in the control group of plants. The elevation of the analyzed metabolites' content in the treatment group was seen only after considerable growth of the mites, which also was weeks before the acaricide application in the treatment group.

To test the differences between the measurements in the leaves of treatment and control groups throughout the experiment and mite growth, repeated-measures ANOVA was performed on the metabolite measurements from sampling times 6–9 (data not shown). For the chlorophyll measurements, the effect of time on chlorophyll content was significant, in addition to the significant interaction between treatment and time effects; this means that time had a different effect on the treatment vs. control group. The effect of time was also significant for all analyzed cannabinoids and terpenes. The treatment was found to be significant for THCA and the interaction between time and treatment was significant for CBNA. The effect of treatment was also found to be significant for all six analyzed terpenes. These results demonstrate that with time and as the plant develops, the amounts of all analyzed secondary metabolites increase. In addition to THCA, the effect of the

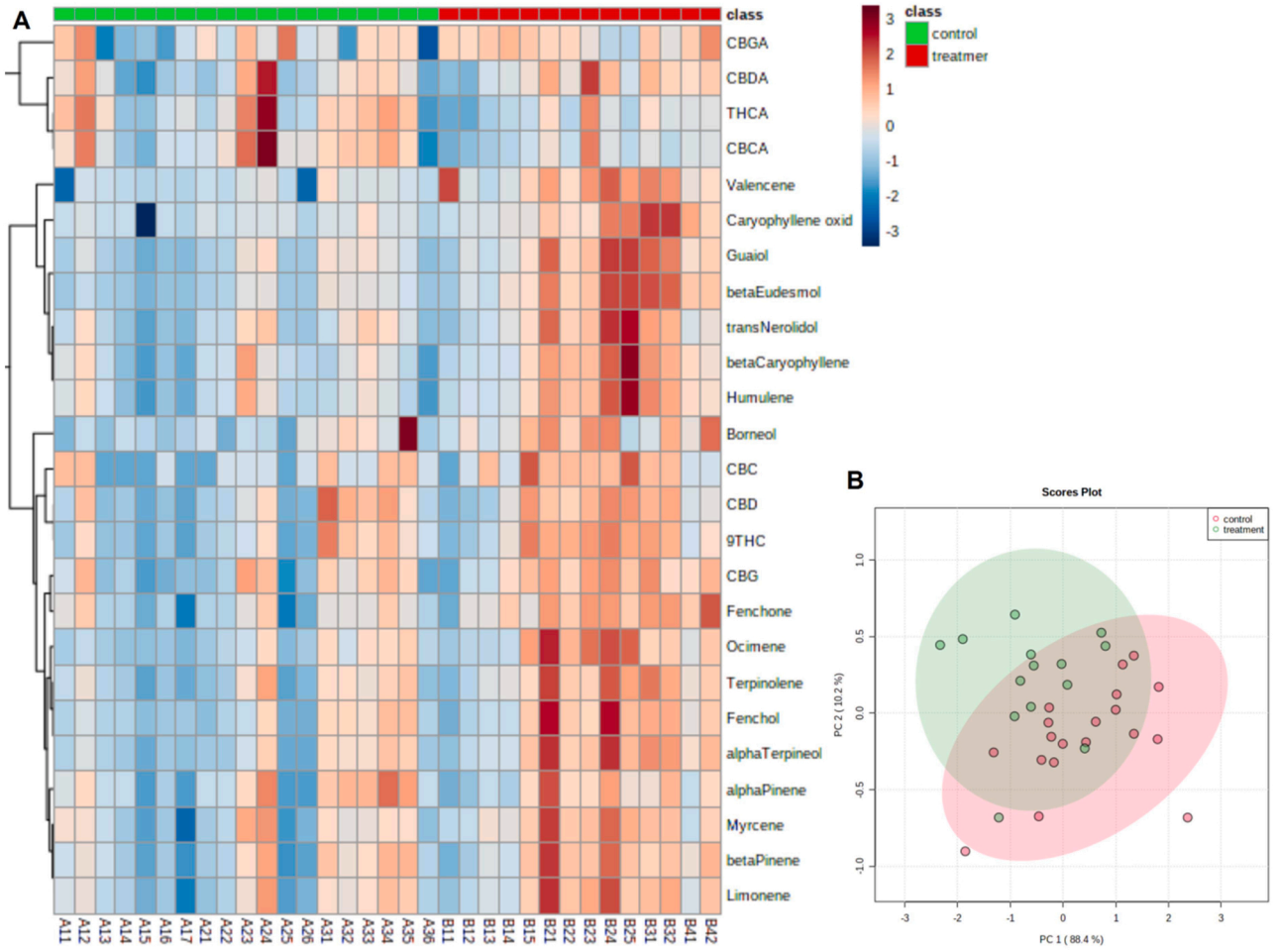

Fig. 7. Heat map (A) and PCA (B) plot of cannabinoids and terpenes in the flowers of the control and treatment groups. Cannabinoids and terpenes were extracted from the treatment and control flowers and analyzed by LC–MS. All were quantified based on the standard calibration curves of their analytical standards and concentration was calculated in mg/g flower tissue. n = 3 for the control group and n = 4 for the treatment group (average of several flowers per plant). The first and second principal components displayed 88.4% and 10.2% of the total variation, respectively.

prolonged growth of mites had a significant effect on all terpene quantities.

Since the medicinal product of cannabis comes from the female flower, it was important to characterize the effect of the early presence of mites on cannabinoid content in the flowers. Analysis of the flowers, which matured without the presence of mites, revealed a relatively long-term effect; the quantities of most of the cannabinoids were greater in the treatment group, and a significant increase was seen in CBG, CBC and Δ^9THC (Fig. 5). Interestingly, while the contents of these cannabinoids were significantly higher in the treatment group flowers, CBCA content was significantly higher in the control group. The most abundant cannabinoids quantified in the flowers were THCA and CBDA, similar to the leaves, albeit in higher quantities.

Flowers from the treatment and control groups were analyzed for differences in terpene content. The mites' effect on flower terpene contents was even greater than that in the leaves; their amounts were statistically higher in the treatment group for all analyzed compounds except α-pinene (Fig. 6). The most abundant monoterpene in the flowers was myrcene, and β-caryophyllene was the most abundant sesquiterpene.

The mites' effect on the content of all analyzed metabolites was well visualized by a heat map and PCA. The heat map of the metabolites analyzed in the flowers demonstrated the differences in their quantities, clearly showing higher metabolite concentrations in the treatment vs. control group (Fig. 7A). PCA clustering of the metabolites in the flowers (Fig. 7B) showed metabolite separation between the treatment and control groups.

5. Conclusions

The effect of a biotic stress, *T. urticae* infestation, on the content of cannabinoids and terpenes in the leaves and flowers of cannabis, was characterized. The tested biotic stress had an impact on these secondary metabolites in cannabis. All the analyzed metabolites were present in both treated and control plants, but in different amounts. In general, there was an increase in cannabinoid and terpene quantities in the leaves and flowers due to the presence of the pest. The metabolite increase in the leaves was significant at the late vegetative phase of the plant's development, aligned with the growth of the mites and the increase in CMD, and the decrease in chlorophyll content. In the flowers of the post-infested plants, a significant increase was seen as well, especially in the content of terpenes. The overall results reveal that cannabis plants do react to the biotic stress caused by *T. urticae*, in terms of amounts of cannabinoids and terpenes in their leaves and flowers. More research into the effects of abiotic and biotic stresses on the content of secondary metabolites in cannabis is required to better understand this plant's defense mechanisms, and to develop a standardized method for treating the infested plant in relation to the level of damage. Executing another analogous study with a bigger sample size, to avoid as much as possible the statistical limitations, is also recommended.

CRediT authorship contribution statement

Elizabeth Kostanda, methodology, writing the original draft, analysis, validation., **Soliman Khatib,** corresponding author, supervisor, Writing - Review & Editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2021.114331.

References

Alchimia blog, 2017. Cannabis Introduction pests and diseases.
Banerjee, P., Islam, M.M., Laha, A., Biswas, H., Saha, N.C., Saha, G.K., Sarkar, D., Bhattacharya, S., Podder, S., 2020. Phytochemical analysis of mite-infested tea leaves of Darjeeling Hills, India. Phytochem. Anal. https://doi.org/10.1002/pca.2893.
Bianchi, F., Careri, M., Mangia, A., Musci, M., 2007. Retention indices in the analysis of food aroma volatile compounds in temperature-programmed gas chromatography: Database creation and evaluation of precision and robustness. J. Sep. Sci. https://doi.org/10.1002/jssc.200600393.
Block, A.K., Vaughan, M.M., Schmelz, E.A., Christensen, S.A., 2019. Biosynthesis and function of terpenoid defense compounds in maize (Zea mays). Planta. https://doi.org/10.1007/s00425-018-2999-2.
Cicció, J.F., Chaverri, C., 2008. Volatile constituents of the oils from Povedadaphne quadriporata (Lauraceae) from "Alberto M. Brenes" biological preserve, Costa Rica. Quim. Nova. https://doi.org/10.1590/S0100-40422008000300027.
Dubey, G., Mishra, N., Prasad, S.M., 2016. Metabolic responses of pesticides in plants and their ameliorative processes. Plant Responses Xenobiotics. https://doi.org/10.1007/978-981-10-2860-1_4.
European Medicines Agency, 2006. Validation of analytical procedures: text and methodology Q2 (R1),, 1–15.
Flores-Sanchez, I.J., Verpoorte, R., 2008. Secondary metabolism in cannabis. Phytochem. Rev. https://doi.org/10.1007/s11101-008-9094-4.
Gershenzon, J., Dudareva, N., 2007. The function of terpene natural products in the natural world. Nat. Chem. Biol. https://doi.org/10.1038/nchembio.2007.5.
Gorelick, J., Bernstein, N., 2017. Chemical and physical elicitation for enhanced cannabinoid production in cannabis. Cannabis Sativa L. - Bot. Biotechnol. https://doi.org/10.1007/978-3-319-54564-6_21.
McPartland, J.M, 2000. Hemp Diseases and Pests Management and Biological Control. http://dx.doi.org/10.1079/9780851994543.0000.
McPartland, J.M, 1996. Cannabis pests. J. Int. Hemp Assoc. 3(2) 49,52–55.
Hill, K.P., 2015. Medical marijuana for treatment of chronic pain and other medical and psychiatric problems: A clinical review. JAMA. J. Am. Med. Assoc. 313, 2474–2483. https://doi.org/10.1001/jama.2015.6199.
Kopaczyk, J.M., Warguła, J., Jelonek, T., 2020. The variability of terpenes in conifers under developmental and environmental stimuli. Environ. Exp. Bot. https://doi.org/10.1016/j.envexpbot.2020.104197.
Kopp, S., 2016. GUIDELINES Valid. – APPENDIX 4, 1–11.
Landi, S., Berni, R., Capasso, G., Hausman, J.F., Guerriero, G., Esposito, S., 2019. Impact of nitrogen nutrition on Cannabis sativa: An update on the current knowledge and future prospects. Int. J. Mol. Sci. https://doi.org/10.3390/ijms20225803.
Mackie, K., 2008. Cannabinoid receptors: Where they are and what they do. J. Neuroendocrinol. https://doi.org/10.1111/j.1365-2826.2008.01671.x.
Magagnini, G., Grassi, G., Kotiranta, S., 2018. The effect of light spectrum on the morphology and cannabinoid content of Cannabis sativa L. Med. Cannabis Cannabinoids. https://doi.org/10.1159/000489030.
Mahdavi, A., Moradi, P., Mastinu, A., 2020. Variation in terpene profiles of Thymus vulgaris in water deficit stress response. Molecules. https://doi.org/10.3390/molecules25051091.
Mahlberg, P.G., Eun, S.K., 2004. Accumulation of cannabinoids in glandular trichomes of Cannabis (Cannabaceae). J. Ind. Hemp. https://doi.org/10.1300/J237v09n01_04.
Park, Y.L., Lee, J.H., 2002. Leaf cell and tissue damage of cucumber caused by twospotted spider mite (Acari: Tetranychidae). J. Econ. Entomol. https://doi.org/10.1093/jee/95.5.952.
Park, Y.L., Lee, J.H.O., 2005. Impact of twospotted spider mite (Acari: Tetranychidae) on growth and productivity of glasshouse cucumbers. J. Econ. Entomol. https://doi.org/10.1093/jee/98.2.457.
Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. BBA - Bioenerg. https://doi.org/10.1016/S0005-2728(89)80347-0.
Russo, E.B., 2011. Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br. J. Pharm. 163, 1344–1364. https://doi.org/10.1111/j.1476-5381.2011.01238.x.
Schultes, R.E., 1996. The Great Book of Hemp: The complete guide to the environmental, commercial and medicinal uses of the world's most extraordinary plant by Rowan Robinson. Environ. Conserv. (illustr. 1996), 294. https://doi.org/10.1017/s0376892900039047.
Sexton, M., Cuttler, C., Finnell, J.S., Mischley, L.K., 2016. A cross-sectional survey of medical Cannabis users: Patterns of use and perceived efficacy. Cannabis Cannabinoid Res. https://doi.org/10.1089/can.2016.0007.
Woolridge, E., Barton, S., Samuel, J., Osorio, J., Dougherty, A., Holdcroft, A., 2005. Cannabis use in HIV for pain and other medical symptoms. J. Pain. Symptom Manag. https://doi.org/10.1016/j.jpainsymman.2004.07.011.
Zhou, H.C., Shamala, L.F., Yi, X.K., Yan, Z., Wei, S., 2020. Analysis of terpene synthase family genes in Camellia sinensis with an EMphasis on Abiotic Stress Conditions. Sci. Rep. https://doi.org/10.1038/s41598-020-57805-1.