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INVESTIGATION OF CAPITATE-SESSILE GLANDULAR TRICHOMES ON LEAF SURFACE OF *IN VITRO Cannabis sativa* L.

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Abstract

In this study, microscopic characteristics and distribution of capitate-sessile glandular trichomes on 4-week-old leaves collected from *in vitro Cannabis sativa* L. was investigated in order to demonstrate the possibility of using this microscopic characteristic of capitate-sessile glandular trichomes on *C. sativa* leaves as a non-destructive tool in forensic application. The results showed that there were transparent and translucent capitate-sessile glandular trichomes, which the structure composed of a sphere head without stalk was found on the leaf surface. The number of capitate-sessile glandular trichome on adaxial leaf surface was significantly higher than that on abaxail leaf surface, and the highest total gland number per area was obtained from leaves collected from the second position of the plantlet. Chemical analysis of capitate-sessile glandular trichomes also confirmed the presence of cannabinoids, but not in the non-gland tissue. The investigation was also extended to leaf materials cultured on MS medium containing 0.26, 0.84 (control), and 4 g/l of total nitrogen. Total gland number of capitate-sessile glandular trichomes significantly decreased when cultured plantlet in nitrogen-elevating MS medium. In contrast, the number significantly increased in nitrogen-depleting condition. Results from this study demonstrated the possibility of using the microscopic characteristic of capitate-sessile glandular trichomes on vegetative leaves of *C. sativa* as a non-destructive tool in forensic application and could possibly contribute to the control and monitoring of *C. sativa* cultivation for industrial fiber in the future.

Keywords: *Cannabis sativa* L., capitate-sessileglandular trichomes, Fast Blue B salt test, thin layer chromatography (TLC), nitrogen content

1. Introduction

Cannabis sativa L. is a dioecious annual crop plant, which has been long cultivated worldwide for industrial fiber , food (seed and oil), medicinal use to treat labor pain, nausea, and rheumatism, and illegal narcotic drug (1, 2). *C.sativa* can be divided into two types according to its use as narcotic and non-narcotic purpose, *i.e.*, “drug-type” and “fiber-type” respectively (2). The major psychoactive ingredient reported to be unique in *C.sativa* is called cannabinoids. (3-5). The three major constituents of cannabinoids are cannabidiol (CBD), cannabinol (CBN) and tetrahydrocannabinol (THC) (6, 7). Drug-type *C. sativa* contains high THC content, while the fiber-type contains low, or no THC (2). Because *C. sativa*. is listed as a narcotic plant in the Thai Narcotic Law B.E. 2522, cultivation of *C. sativa* for industrial use is under authority control. According to UNODC recommendation (7), methods for the detection and identification of *C. sativa* from other plants are divided into 3 groups, physical, chemical, and genetic examinations. Physical examination of *Cannabis* material is considered non-destructive. The examination are conducted at macroscopic and microscopic level to investigate morphological and anatomical characters of the plant. Chemical examination is conducted for the analysis of cannabinoid content,

focusing on THC. THC content of fiber-type, or non-narcotic-type, *C. sativa* has been limit to less than 0.2 – 0.5% (7, 8) depending on law of each country. Lastly, a number of genetic markers have been developed to indicate the presence of cannabis species, determine cannabis type, as well as individualization and linkage study (2, 9, 10). Although different levels of information would be obtained from these 3 different groups of examinations, both chemical and genetic analysis methods are considered as destructive examination methods because the plant material being analyzed would be destroyed. In addition, analysis cost of these later 2 groups of examination methods are considered high, and they also require complex laboratory instruments.

C. sativa can be identified by the presence of the combination of the following microscopic structures, cystolithic hairs on the leaf upper surface, capitate-stalked gland and capitate-sessile glands on the lower surface, which is unique to *C. sativa* (7). There are two types of trichomes present in *C. sativa*, *i.e.*, non-glandular, and glandular trichomes. There are 2 types of non-glandular trichomes (cystholithic and non-cystolithic trichomes), and 3 types of glandular trichomes (bulbous, capitate-sessile, and capitate-stalked glandular trichomes) found in *C. sativa* (7, 11, 12). Numerous amount of non-glandular trichomes found in the aerial part of *C. sativa* are rigid unicellular curved hairs with a slender pointed apex. If calcium carbonate crystal is present at the trichome's base. This microstructure is called cystolithic trichomes. Glandular trichomes are present in all the aerial part of the *C. sativa*, and are abundant on bracts and leaves (6, 7). More specifically, capitate-sessile glands are present on vegetative leaves, and capitate-stalked glands are found in association with inflorescence (6, 11). Glandular trichomes have been implicated as major reservoir of cannabinoids. There are evidences indicated that quantity of glandular trichomes has positive correlation with cannabinoids content (13, 14) and confirmed the presence of cannabinoids in glandular trichomes (12, 13, 15). Moreover, immunochemical technique revealed that THC was secreted from disc and secretory cells in these glandular trichomes, and accumulated in the glandular trichomes (16, 17). Regarding forensic aspects of the control and monitoring of *C. sativa* cultivation for industrial fiber, harvest period is towards the end of vegetative stage, which only capitates-sessile glandular trichomes would be present on the leaf surface (12). There is a possibility of using this microscopic character of capitate-sessile glandular trichomes on *C. sativa* leaves as a non-destructive tool in this forensic application.

In order to demonstrate this possibility, microscopic characteristic, quantity, distribution, and the presence of cannabinoids in capitate-sessile glandular trichomes were investigated. Investigations on capitate-sessile glandular trichome variations were also extended to *C. sativa* leaf materials collected from plants cultured on medium containing different nitrogen content *in vitro*.

2. Methodology

Microscopic characteristic and distribution of capitate-sessile glandular trichomes on developing vegetative leaf of in vitro C. sativa

Developing vegetative leaf of 4-week-old *C. sativa* plantlets cultured on Murashige and Skoog (MS) hormone free media (18) were examined under stereomicroscope (Olympus®, Japan) to observe microscopic characteristic of glandular trichomes on both adaxial and abaxial surface.

Distribution of glandular trichomes was investigated on 4-week-old leaves collected at 3 different positions, which represents 3 different physiological ages. Average numbers of capitate-sessile glandular trichomes were obtained from the average number of glands directly counted in a 0.25 mm^2 grid from 10 areas on the same leaf (see Figure 2-1).

Leaf area was measured by area meter AM 100 (ScanMan™, UK). The number of capitate-sessile glandular trichome per leaf was estimated. Results were analyzed by paired T-test to compare the number of capitate-sessile glandular trichomes on adaxial and abaxial surfaces.

Figure 2-1. Positions of leaf samples collected from 4- week-old plantlet (a), and 10 areas of 0.25-mm^2 each which capitate-sessile glandular trichomes were counted (b).

Testing for the present of cannabinoids in different tissue of in vitro C. sativa

Capitate-sessile glandular trichomes and non-glandular trichomes were dissected from surface of fully expanded leaf collected from a 4-week-old plantlet by needle number 30 (0.30 mm pore diameter) under stereomicroscope (Olympus®, Japan). Each trichome types, was put on to separate filter papers. The presence of cannabinoids was presumptively determined by color test using Fast Blue B salt reagent. A small amount of the solid Fast Blue B salt, or anhydrous sodium sulphate (1:100 mg) was added to the sample on the filter paper followed with 2 drops of 10% (w/w) sodium bicarbonate. Colors were observed immediately; THC, CBD, and CBN would give red, orange, and purple color, respectively. A diagram is showed in figure 2-2.

Confirmatory testing of cannabinoids was performed by thin layer chromatography (TLC). One hundred of each transparent and opaque capitate-sessile glandular trichomes, with approximately 55 µm diameter, were collected and transferred on to glass slides and smashed. An aliquot of 10 µl of acetone was added to each sample, mixed and transferred by a capillary tube to spot onto the TLC plate (Silica gel 60 F_{254} coated on aluminium sheet; Merck, USA). This step was repeated 3 times to ensure that all complete transfer of the phytochemical. For the non-gland tissue, 12 mm^2 of leaf tissue with no gland was extracted with 100 µl of acetone for 15 minutes in ultrasonic bath. The supernatant was transferred to a new vial and concentrated to 20 µl before spotting on to the TLC plate. Separation was carried out using n-hexane: 1,4-dioxane: methanol in the ratio 7:2:1. TLC plate was developed by Fast Blue B salt reagent.

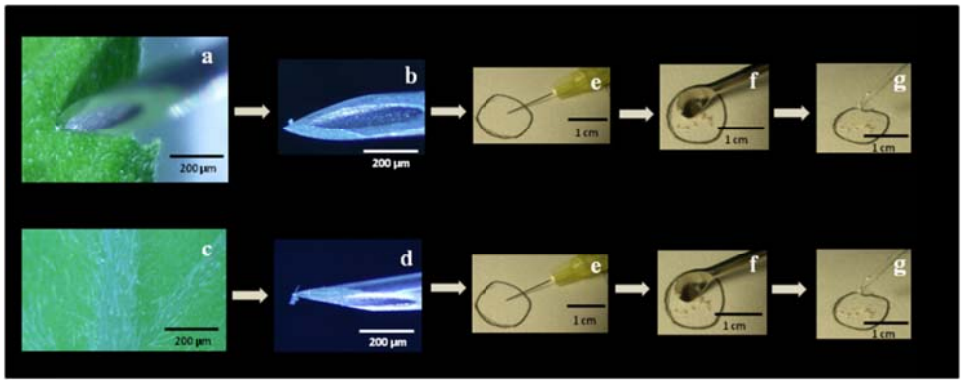

Figure 2-2 Trichomes dissection and color test. Trichomes were collected from leaf surface under stereomicroscope; dissection of capitate-sessile glandular trichome, (a and b), dissection of non-glandular trichome (c and d). The trichome was transferred to filter paper and smashed (e), and fast Blue B reagent was added for color test (f and g).

Investigation of capitate-sessile glandular trichomes on leaves of C. sativa cultured on MS medium containing different nitrogen content

Experimental design was conducted using completely randomized design (CRD). For multiple shoot induction, *C. sativa* were subcultured to MS medium containing 1 mg/l BAP for 4 weeks, then transferred to MS hormone free medium for 4 weeks before starting the nitrogen treatment. These 4-week-old plantlets were subcultured onto 3 different MS medium containing total inorganic nitrogen 0.26, 0.84 (control), and 4 g/l (see table 2-1). There were 53, 43, and 44 plantlets for each of the treatment, respectively. After 4 weeks, capitate-sessile glandular trichomes were investigated as described in previous section.

Table 2-1 Inorganic nitrogen contents in treatment media.

Treatment	N from KNO_3 (g/l)	N from NH_4NO_3 (g/l)	Total N (g/l)
N-depleting	0.26	0	0.26
Control	0.26	0.577	0.84
N-elevating	0.26	3.737	4

3. Results

Microscopic characteristic and distribution of glandular trichomes on developing vegetative leaf of in vitro C. sativa

Glandular trichomes found on leaf of *in vitro C. sativa* were capitate-sessile glandular trichomes, which composed of a sphere head without stalk. These capitate-sessile glandular trichomes found could be further divided into 2 subtypes according to their opacity; the first subtype was transparent and the other was opaque or translucent with a 'milky' look. The 2 types of glandular trichomes were distributed on both adaxial and abaxial surface of fully expended leaves of *in vitro C. sativa* (Fig 3-1).

Results showed that quantity of capitate-sessile glandular trichomes on adaxial surface was higher than that on abaxial surface for leaves of all three different physiological ages. On adaxial surface, the number of capitate-sessile glandular trichomes was

approximately 304, 391, and 287 glands/leaflet of the first to third leaf position from the shoot of plantlet. On abaxial surface, the number of capitate-sessile glandular trichomes increased with the age of leaf, and highest at third position. The numbers were approximately 119, 122, and 276 glands/leaflet, respectively. It was also found that the number of capitate-sessile glandular trichome on adaxial leaf surface was significantly higher than that on abaxail leaf surface (figure 3-2). Therefore, leaf samples at the second position would be collected for investigation of capitate-sessile glandular trichomes on leaf of *C.sativa* plantlets cultured in MS medium containing different level of nitrogen content.

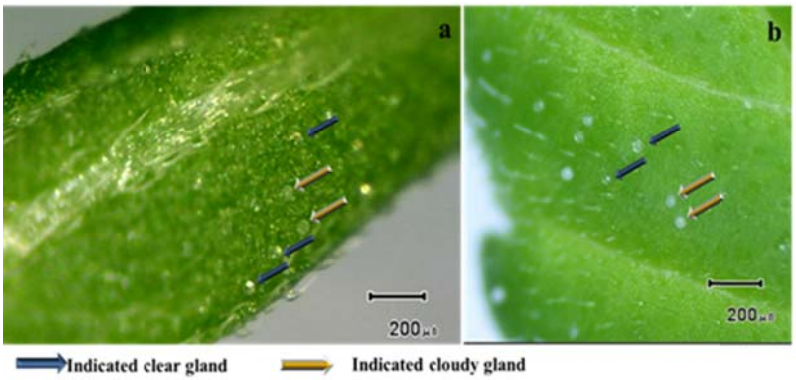

Figure 3-1 Glandular trichomes found on 4-week-old *in vitro C. sativa* leaves: transparent (blue arrows) and translucent (orange arrows) capitate-sessile glandular trichomes on abaxial surface (a), and adaxial surface (b).

and orange arrows indicated cloudy-capitate sessile glands).

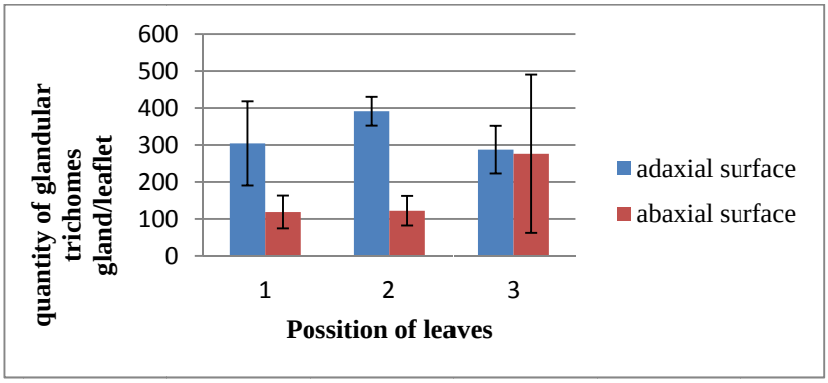

Figure 3-2 Comparison of quantity, or estimated number, of capitate-sessile glandular trichomes on adaxial and abxial surfaces of leaves at different physiological ages. The leaf samples were collected from 3 positions (1-3 was from apex of plantlet). Bars represent S.E. value, and * showed that values were significantly different at the level of 95% confidence interval by paired T-test.

Testing for the present of cannabinoids in glandular trichomes of in vitro C. sativa

Fast Blue B salt color test results showed red color, indicated the presence of THC cannabinoids in capitate-sessile glandular trichome tissue sample tested. No color change was observed from the non-glandular trichome tissue sample and negative control of the color test (see figure 3-3). Confirmatory test of cannabinoids by TLC showed orange, red, and purple color bands, which represented 3 types of cannabinoids (CBD, THC and CBN, respectively), from both transparent and translucent capitate-sessile glandular trichomes extracts (figure 3-4). Although the fingerprint pattern was similar, the red color band intensities were different. Because the extracts were obtained from the same number of 100 glands, results then suggested that the THC content in translucent (or milky color) capitate-sessile glandular trichomes were higher than the transparent glands. This red color band was not observed in the non-gland tissue sample. TLC fingerprint pattern of the non-gland tissue sample, was different from the glandular trichome samples. A sharp red-orange band was clearly showed at the same position of the orange-CBD bands of the 2 glandular trichome samples. Results here confirmed the presence of THC in capitate-sessile glandular tissue type.

Figure 3-3 Result of Fast Blue B salt test in *in vitro C. sativa*: negative control gave no color product (a), non-glandular trichomes gave no color product (b), glandular trichomes gave red-purple product (c).

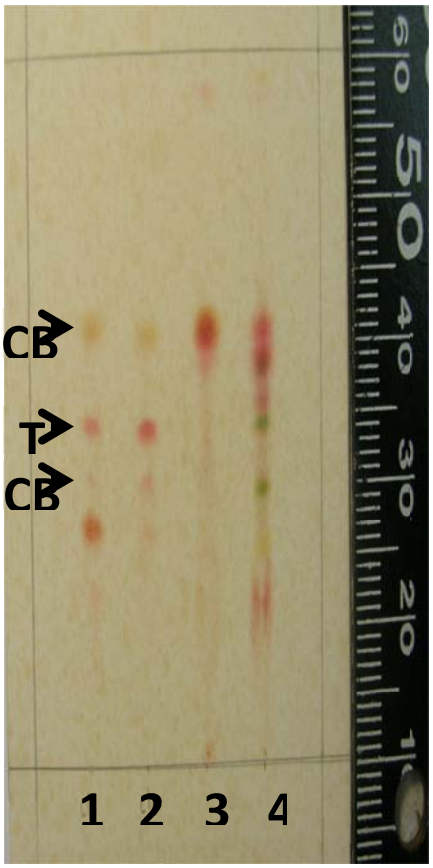

Figure 3-4. TLC fingerprint of extracts from transparent capitate-sessile glandular trichomes (1), translucent capitate-sessile glandular trichome (2), tissue with no glandular trichomes (3), and a positive control *C. sativa* (4), respectively.

Investigation of capitate-sessile glandular trichomes on leaves of C. sativa cultured on MS medium containing different nitrogen content

The numbers of capitate-sessile glandular trichomes after the 4-weeks nitrogen treatment period were investigated. Change of capitate-sessile glandular trichome number per area was calculated from the estimate number before and after nitrogen treatment. Results showed that number of total amounts of capitate-sessile glandular trichomes per 2.5 mm^2 in samples cultured on N-depleting and the standard MS (control) medium significantly increased. The number of capitate-sessile glandular trichomes were 10.41 ± 0.73, and 5.95 ± 1.24, glands/2.5mm^2, respectively. The increased was approximately 8 times when the total nitrogen was reduced from 0.84 to 0.26 g/l. In contrast, total amounts of capitate-sessile glandular trichomes per area significantly decreased (-4.93 ± 0.64 glands/2.5mm^2) when nitrogen content in MS medium was elevated to 4 g/l (see table 3-1).

Regarding the subtype of capitate-sessile glandular trichomes, results revealed that the number of transparent capitate-sessile glandular trichomes significantly increased in *C. sativa* samples cultured on N-depletion medium (7.98 ± 0.77 gland/2.5mm^2), while the number of transparent capitate-sessile glandular trichomes per 2.5 mm^2 significantly

decreased in the *C. sativa* samples cultured on N-elevation medium (-2.27 ± 0.6 gland/2.5mm^2) compare to that cultured on the standard MS medium, or control (3.98 ± 0.78 gland/2.5mm^2). The number of translucent capitate-sessile glandular trichomes per 2.5 mm^2 significantly decreased in *C. sativa* cultured on N-elevation medium (-2.66 ± 0.65 gland/2.5mm^2) compare to that cultured on basal medium; control (1.97 ± 0.68 gland/2.5mm^2). While the comparison of translucent capitate-sessile glandular trichomes per 2.5 mm^2 of *C. sativa* cultured on N-depletion (2.44 ± 0.89 gland/2.5mm^2) was not significantly different when compared to the control (Table 3-1).

Table 3-1 Comparison of total number of capitate-sessile glandular trichomes, transparent capitate-sessile glandular trichome, and translucent capitate-sessile glandular trichomes on leaf samples collected from *in vitro C. sativa* plantlets on MS medium supplemented with different nitrogen concentrations at day 0 (before treatment) and 4-week-old cultures (after treatment).

Nitrogen concentration (g/l)	Delta gland quantity (gland/2.5mm^2)		
	Total gland	Clear-sessile	Cloudy-sessile
0.26	10.41 ± 0.73a[1/]	7.98 ± 0.77a	2.44 ± 0.29a
0.84	5.95 ± 1.24b	3.98 ± 0.78b	1.97 ± 0.68a
4	-4.93 ± 0.64c	-2.27 ± 0.6c	-2.66 ± 0.65b

The values in the table is mean ± S.E.
[1/] values within a column not followed by the same letter differ significantly at the level of 95% confidence interval by LSD.

4. Discussion and Conclusion

In this study, only the capitate-sessile glandular trichomes, or sphere structure with no stalk, were found on the 4-week-old leaf surface of *C. sativa* plantlets cultured *in vitro.* This is consistent with previous reports which capitate-sessile glandular trichomes are generally present on flower, stem, and both abaxial and adaxial surfaces of leaf, while capitate-stalked glandular trichomes are only present on inflorescence during flowering period (6, 11, 12). No bulbous gland (a sphere head with unicellular stalk) nor capitate-stalked glandular trichomes (sphere head with multicellular stalk) were found (7, 11). In addition, the capitate-sessile glandular trichomes found were either transparent or translucent. No amber color capitate-sessile glandular trichomes were found. The color of capitate-sessile glandular trichomes indicated the maturity stage of *C. sativa* (19, 20) The presence of transparent capitate-sessile glandular trichomes indicated that the plants were immature, while the presence of large number of translucent capitate-sessile glandular trichomes indicated that the plant reached maturity stage, and the amber color of capitate-sessile glandular trichomes indicated that the plant has passed the maturity stage, or aged, and THC would be degraded into CBN. It was suggested that medical cannabis should be harvested when translucent capitate-sessile glandular trichomes are present with some amber glands (19, 20). The presence of only 2 types of capitate-sessile glandular trichomes in this study might be that plant materials were becoming mature. In addition, the study of *C. sativa* plants grown in field revealed that glandular trichomes are found in upper leaf more than lower leaf. But gland density on abaxial surface were more than that on adaxial surface (21). This may be because the variation of number (or quantity) of glands resulted from leaf damage when getting mature.

The research work confirmed the presence of cannabinoids, specifically THC, in capitate-sessile glandular trichomes. This agreed with previous study that used immunochemical technique to reveal that THC was secreted from disc cell and secretory cell of glandular trichomes, and accumulated in glandular trichomes (16, 17). Fast Blue B salt reagent reacts with cannabinoids and showed colors ranging from orange to red, and purple. TLC fingerprints of the non-gland tissue extract showed bands of these colors at positions different from the 3 major cannabinoids (different R_f value). These suggested the presence of other cannabinoids which reacts with the Fast Blue B salt reagent. Petri *et a.l* found that leaves tissue without gland of *C. sativa* present THC (13). Fairbairn revealed CBD was present in bract tissue without glands (22). From these, it suggested that the 3 major cannabinoids were present in capitate-sessile glandular trichomes and there may be other cannabinoids present in the non-gland tissue. There is also report on the presence of "laticifer", a type elongated secretory cell found in the leaf stem that produce latex and rubber as secondary metabolite (23) in *C. sativa*, which could give positive color test results with Fast Blue B , Duquenois-Negm, Beam, and Gibb reagents when test for cannabinoids (24). Results also suggested that cannabinoids of different amounts and may also be types were present in transparent and translucent capitate-sessile glandular trichomes as there were difference in intensities, numbers, positions and colors of bands that were present. Intensity of the red color band in the TLC fingerprint in this work also suggested that higher concentration of THC was present in translucent capitate-sessile glandular trichomes than the transparent capitate-sessile glandular trichomes, but not in non-glandular trichomes tissue.

This study showed that the level of nitrogen content in the growth medium had an effect on the total gland number and density (gland number per area) of capitate-sessile glandular trichomes on leaf at the second position of the *C. sativa* plantlet. Previous researches demonstrated analysis of cannabinoids in glandular trichomes by LCMS (12) and localization of cannabinoids in glandular trichomes by immunochemical technique. It could then be implied that the number of glandular trichome present would reflect the amount of cannabinoids. Results in this showed that the total number of capitate-sessile glandular trichomes on *C. sativa* leaves significantly increased when cultured plantlets in Nitrogen-depleting MS medium. In contrast to the total gland number of capitate-sessile glandular trichomes significantly decreased when cultured plantlet in Nitrogen-elevating MS medium. These suggested that the presence of high level (4 g/l) nitrogen in MS medium would possibly reduce the cannabinoid content in the leaf tissue, as the total gland number of capitate-sessile glandular trichomes was decreased. This agreed with a previous study which demonstrated relationship of nitrogen and cannabinoids content in field-grown plants that cannabinoids extracted from leaf decreased with increasing nitrogen content (25).

This study demonstrated the possibility of using the microscopic character of capitate-sessile glandular trichomes on vegetative leaves of *C. sativa* as a non-destructive tool in forensic application. Results from this study could possibly contribute to the control and monitoring of *C. sativa* cultivation for industrial fiber. It can be suggested that by maintaining high nitrogen level in the cultivation could possibly limit the cannabinoid content in the plant in the vegetative stage. However, further work regarding density of capitate-sessile glandular trichomes per area is needed to establish criteria for monitoring plants at different ages through the life cycle, and phytochemical analysis is needed to confirm cannabinoid level.

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