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Cannabinoid composition and gland distribution in clones of *Cannabis sativa* L. (Cannabaceae) *

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Introduction

Glandular trichomes covering the plant surface have been implicated as the source at cannabinoids in *Cannabis sativa* L. (DePasquale, 1974: Malingré *et al.*, 1975). Hammond and Mahlberg (1973, 1977), in their scanning electron microscope study of these trichomes, described three gland types: the bulbous gland which possesses a very short stalk and a small head, the capitate-sessile gland which has a large globular and multi-cellular head, and the capitate-stalked gland which consists of a large multi-cellular head, that terminates a stalk of variable height. Non-glandular trichomes are also present in abundance on the plant epidermis (Ledbetter and Krikorian, 1975). Fairbairn (1972) reported the presence of cannabinoids in both capitate-sessile and capitate-stalked glands and indicated that capitate-stalked glands were the major cannabinoid containing glands. DePasquale (1974), in an ultrastructural-study of the capitate-stalked glands, interpreted the secretory part of the gland to be a combination of the gland head and perhaps apical stalk cells. Malingré *et al.* (1975) concluded that cannabinoids were present mainly in the epidermal glands and not in the mesophyll cells or non-glandular trichomes, although there was some indication of cannabinoid content in the leaf mid-rib.

The purpose of this investigation is to determine whether a correlation exists between glandular trichomes and cannabinoid content in cannabis. If specific glands are associated with cannabinoid content, a correlation should exist between the gland number present on a particular plant part and the cannabinoid content of that part. Furthermore, it should also be possible to establish a correlation between the numbers of each gland type and cannabinoid content.

Materials and methods

Clones

Plants of three cannabis strains, which are typically annuals, were cloned to provide material of genetic homogeneity. These strains include a high Δ^9-tetrahydrocannabinol (Δ^9-THC) strain (152), a low Δ^9-THC strain (79), and a fibre strain (87). Plants were grown in a loam-vermiculite-sand mixture (6:2:1) under

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ambient greenhouse conditions. Cuttings were taken from the parent pistillate plant of each strain, treated with Rootone, and rooted in perlite. Three-week-old rooted cuttings were transplanted into the above soil mixture. Cuttings derived from vegetative or flowering plants were made at regular intervals from the parent or previous cuttings to provide a continuous population of each clone throughout the year.

Plant parts sampled

Selected plants parts from each strain were analysed for their cannabinoid content and gland number. Young pistillate bracts were approximately 3 mm in length and enclosed an unpollinated flower. Mature bracts were approximately 7 mm in length and enclosed a seed. Floral leaves, generally monoleaflet leaves, subtending young and mature pistillate bracts were analysed as were mature compound leaves from vegetative regions of the axis.

Gas chromatography

Plant parts to be analysed were collected at a given time (3 p.m.) and immediately oven dried at 60 °C for 12 h. Weighed samples were extracted in covered test tubes with spectograde chloroform (3 X) for at least 1 h/extraction at 4 °C. Plant material was removed by filtration and the combined aliquots were concentrated to dryness by gentle nitrogen evaporation. Cannabinoids were dissolved in 1 ml ethanol containing the internal standard Δ^4-androstene-3, 17-dione (1 mg/ml; w/v) Doorenbos *et al.*, 1971). A 0.5 µl sample of the test solution was injected into the chromatograph.

Analyses were performed on a Hewlett-Packard 5710A chromatograph equipped with hydrogen flame-ionization detectors and programmed from 180 to 240 °C at 4 °C min with an additional 8 min isothermal period (240 °C). The inlet temperature was 250 °C and the detector temperature was 300 °C. Glass columns (2 mm, id x 2.43 m) were treated with 5 per cent dimethyldichlorosilane in toluene and packed with 3 per cent OV-1 on 80/100 mesh Supelcoport. The head pressures for hydrogen and compressed air were 15 and 40 lb/sq. in., respectively. Nitrogen (carrier gas) flow rate was 20 ml/min. Peak area of each cannabinoid was compared with the peak area of the internal standard for cannabinoid quantitation. Data in tables are mean values of three replicates.

Scanning electron microscopy

Collections of plant organs were made at a given time (3 p.m.), fixed 18-20 h in 4 per cent glutaraldehyde and 0.7 per cent picric acid in 0.1 M sodium cacodylate (Turner, 1970), washed in 0.1 M sodium cacodylate at pH 7.2, post-fixed in 1 per cent OsO in sodium cacodylate for 1 h, rinsed with water, and dehydrated through a graded series of ethanols into amyl acetate. Samples were dried by the critical point technique using CO_2 (Anderson, 1951; Horridge and Tamm, 1969), mounted on stubs with epoxy glue, coated with a layer of carbon followed by gold palladium utilizing a Rotilt shadower (EBTEC Corp.), and subsequently examined with an ETEC Autoscan SEM operated at 20 kV.

Gland quantitation

Gland number per unit area was determined by counting glands directly on the SEM screen. At a magnification of 360 X with a tilt of 25°, the observed field

was 1/4 mm on a side (1/16 mm²). All glandular and non-glandular trichomes within the field were counted. For both the young and mature bracts, random fields totalling 2 sq. mm were counted. On floral leaves, counts were made at the mid-point of the leaf blade from the mid-rib to the margin. Multiple counts (16 fields, totalling 1 sq. mm) of both adaxial and abaxial vein and non-vein areas were made, and the results were averaged to provide a mean for the sample. On compound leaves, gland counts were made at the tip, mid-point, and base of the leaf along the mid-rib and midway between the mid-rib and the leaf margin. The number and type of fields counted were the same as for the floral leaves. For all samples, the standard deviation was determined and critical values of t were calculated to test for significance when means were compared.

Single gland sample

Cannabinoid content of single capitate-stalked glands was determined by removing glands with a tungsten needle and analysing them by gas chromatography as previously described. Only intact gland heads were taken for the sample. Samples consisted of 20 gland heads, although for 2 samples an additional 100 gland heads were taken to provide a greater concentration of extractable material for ascertaining the cannabinoid spectrum of glands.

Results

Gland quantitation of bracts

The number of capitate-stalked glands per unit area of the abaxial surface of the bract increased with maturity of the bract in all three strains (table 1). Mature bracts of clones 152 and 87 had a similar number of capitate-stalked glands (34 and 37 glands/mm², respectively) while clone 79 had only half that number

TABLE 1

Quantitation of glands on bracts

Strain	*Glands/mm²*		
	Capitate-stalked	*Capitate-sessile*	*Bulbous and immature capitate-stalked* [a]
Clone 152 (drug)			
Young bract	0.6	72.0	48.6
Mature bract	34.2	41.1	8.2
Clone 79 (non-drug)			
Young bract	10.4	66.1	83.5
Mature bract	17.1	32.8	10.4
Clone 87 (fibre)			
Young bract	0	149.3	157.3
Mature bract	37.3	10.0	0.7

[a] Bulbous and immature capitate-stalked glands were scored as one category because of the difficulty in distinguishing between the two types at a young stage in development.

(17 glands/mm²). Capitate-sessile glands were abundant on the young bracts of all strains with twice the number (149 glands/mm²) present on clone 87 than on clones 152 and 79. The number of capitate-sessile glands per unit area decreased during bract enlargement and fewer capitate-sessile glands (10/mm²) were present on the mature bracts of clone 87 than on clones 152 (41 glands/mm²) and 79 (32 glands/mm²). Clones also varied in the number of bulbous glands per unit area on mature bracts: 8 to 10 glands were present on clones 152 and 79 while less than 1 per unit area occurred on clone 87. The ratio of the three gland types on mature bracts also varied between clones. In both young and mature bracts, capitate-sessile glands occurred in greater number than capitate-stalked and bulbous glands on clones 152 and 79, but on mature bracts of clone 87 capitate-stalked glands predominate over sessile glands, while bulbous glands are few in number.

Cannabinoid content of bracts

Determination of cannabinoid content in young and mature bracts, which corresponded to samples analysed for gland distribution, revealed that only clone 87 had an increase in all cannabinoids investigated as the bract matured (table 2). Clones 152 and 79 both showed a decrease in cannabichromene (CBC) and Δ^9-THC content with maturation of the bract. However, 152 showed an increase in cannabidiol (CBD) and cannabinol (CBN) content with maturation while clone 79 showed a decrease. Relatively low concentrations of CBC and CBN were found in bracts of all strains. A positive correlation between the concentrations of CBC and CBN with the characteristic cannabinoid of each strain was observed with the exception of CBN in clone 152.

Gland quantitation of floral leaves

Capitate-stalked glands were present on mature floral leaves of all three clones with 87 having a considerably higher number per unit area (11 glands/mm²) than either clone 152 or 79 (table 3). Capitate-stalked glands increased with leaf maturity in clone 79, but decreased in clone 152. Capitate-sessile glands increased with leaf

TABLE 2

Cannabinoid content of bracts

Strain	*mg cannabinoids/100 mg dry weight*			
	CBD	*CBC*	*Δ^9-THC*	*CBN*
Clone 152 (drug)				
Young bract	0.02	0.45	6.64	0.17
Mature bract	0.10	0.10	5.41	0.39
Clone 79 (non-drug)				
Young bract	5.38	0.21	0.22	0.11
Mature bract	3.02	0.12	0.18	Trace
Clone 87 (fibre)				
Young bract	3.66	0.16	0.20	Trace
Mature bract	5.33	0.33	0.28	0.28

TABLE 3

Quantitation of glands on floral leaves

Strain	Glands/mm²		
	Capitate-stalked	Capitate-sessile	Bulbous
Clone 152 (drug)			
Young leaf	0.6	9.5	18.8
Mature leaf	0.2	11.7	6.2
Clone 79 (non-drug)			
Young leaf	0	6.5	23.7
Mature leaf	2.3	2.7	13.0
Clone 87 (fibre)			
Young leaf [a]	—	—	—
Mature leaf	11.0	8.3	16.8

[a] Non-glandular trichome density obstructed view of the leaf surface and prevented gland quantitation.

maturity in clone 152 from 9 to 11 glands/mm², but decreased in clone 79 from 6 to 2 glands/mm². Bulbous glands decreased with leaf maturity in both 152 and 79. High densities of trichomes of young floral leaves of clone 87 obstructed the view of the leaf surface and prevented gland quantitation. As in the bracts, the relative numbers of glands varied among the clones. On the mature floral leaves, clone 87 had the highest number of capitate-stalked and bulbous glands, while 152 had the highest number of capitate-sessile glands.

Cannabinoid content of floral leaves

Comparisons of cannabinoid levels in leaves subtending young and mature bracts (table 4) showed that clones 79 and 87 followed the same patterns as found

TABLE 4

Cannabinoid content of floral leaves

Strain	mg cannabinoids/100 mg dry weight			
	CBD	CBC	Δ^9-THC	CBN
Clone 152 (drug)				
Young leaf	0.07	0.68	4.03	0.19
Mature leaf	Trace	Trace	1.92	0.12
Clone 79 (non-drug)				
Young leaf	5.00	0.20	0.22	0.32
Mature leaf	1.79	0.11	0.11	Trace
Clone 87 (fibre)				
Young leaf	3.24	0.16	0.16	0.10
Mature leaf	4.30	0.24	0.94	0.16

in the bracts (table 2). Cannabinoids in clone 79 decreased as the leaf matured, while they increased in clone 87. In contrast, the cannabinoid content in clone 152 decreased with leaf maturation (table 4), whereas in the bract some cannabinoids increased in quantity as the bracts matured (table 2). All three clones showed the same quantitative trend of the characteristic cannabinoid as well as the principal cannabinoids with maturation of both bracts and floral leaves (tables 2 and 4).

Gland quantitation of compound leaves

The large size of compound leaves made it necessary to establish a sampling procedure for quantitating the distribution of glands on the leaf surface. Six areas of the leaf were chosen for sampling. Observations of the mid-point of leaves from clone 152 showed that adaxial surfaces of the leaves as well as vein and non-vein regions were significantly different in gland distribution (table 5). This pattern was found in all six sample areas. No capitate-stalked glands were present. For quantitative comparison of capitate-sessile and bulbous glands, a mean was calculated for each sample area (tables 5 and 6). Data evaluated statistically by means of the *t* test showed no significant difference among the six areas. Clones 87 and 79 were found to follow this sampling pattern as described for clone 152. Therefore, data from all six areas on the leaf were combined to give a mean for each gland type observed on compound leaves in each clone (table 7).

Gland type and number varied with the respective clones. Clone 79 had significantly more capitate-sessile and bulbous glands/mm² than either 152 or 87. Clone 152 had the lowest number of bulbous glands (4.5 mm²), but clone 87 had the lowest number of capitate-sessile glands (2.6 mm²). Gland number and type on compound leaves varied considerably as compared to bracts and floral leaves (tables 1, 3 and 7).

Cannabinoid content of compound leaves

In general cannabinoid content of compound leaves (table 8) was lower than that for bracts (table 2) and floral leaves (table 4). However, as expected, compound

TABLE 5

Quantitation of glands at mid-point of compound leaves of clone 152

	Glands/mm²			
	Mid-rib		*Between mid-rib and leaf margin*	
	Capitate-sessile	*Bulbous*	*Capitate-sessile*	*Bulbous*
Adaxial surface of leaf				
Vein region	0	0	0	0
Non-vein region	1.6	0	2.2	0
Abaxial surface of leaf				
Vein region	1.6	3.9	0.7	0
Non-vein region	14.9	11.4	12.8	13.4
Sample mean	5.5	4.5	4.3	3.6

leaves of clone 152 contained a higher level of Δ^9-THC than either of the other two clones. Clone 87 contained the highest level of CBD as well as the highest total cannabinoids. Clone 79 had approximately the same concentration of Δ^9-THC as clone 87. However, the CBD content was lower in clone 79 than clone 87. Only trace amounts of CBC and CBN were detected in all strains.

Cannabinoid content of single capitate-stalked glands

Three different stages of maturation of capitate-stalked glands could readily be recognized within a population of these glands on mature bracts. Initially, the mature gland possessed a head with a clear liquid content. The second stage was

TABLE 6

Quantitation of glands on compound leaves of clone 152

	Glands/mm²			
	Mid-rib		*Between mid-rib and leaf margin*	
Leaf region	*Capitate-sessile*	*Bulbous*	*Capitate-sessile*	*Bulbous*
Tip	5.3	4.3	5.2	6.3
Mid-point	5.5	4.5	4.3	3.6
Base	2.9	4.6	5.1	3.8

TABLE 7

Quantitation of glands on compound leaves

	Glands/mm²		
Strain	*Capitate-stalked*	*Capitate-sessile*	*Bulbous*
Clone 152 (drug)	0	4.8	4.5
Clone 79 (non-drug)	0	6.0	10.6
Clone 87 (fibre)	0	2.6	8.1

TABLE 8

Cannabinoid content of compound leaves

	mg cannabinoids/100 mg dry weight			
Strain	*CBD*	*CBC*	*Δ^9-THC*	*CBN*
Clone 152 (drug)	Trace	Trace	0.37	0.04
Clone 79 (non-drug)	1.49	0.06	0.06	—
Clone 87 (fibre)	2.38	0.07	0.08	Trace

TABLE 9

Cannabinoid content of capitate-stalked glands

Strain	ng cannabinoids/gland		
	CBD	Δ^9-THC	CBN
Clone 152 (drug)			
Mature gland	—[a]	57	—
Aged gland	—	35	21
Senescent gland	—	9	1
Clone 87 (fibre)			
Mature gland	229	—	—
Aged gland	113	—	—
Senescent gland	29	—	—

[a] A dash (—) indicates the cannabinoid was not detected.

represented by the aged gland which appeared yellow and contained a sticky, more solid content in the head, while the third stage was represented by a senescent gland with a red, dried head. Cannabinoid analyses of these three gland populations on clones 152 and 87 (table 9) indicated that the cannabinoid content of the capitate-stalked glands decreased significantly as the gland head aged. In addition, mature capitate-stalked glands from clone 87 contained higher concentrations of the cannabinoid characteristic of the strain (CBD, 229 ng/gland) than did the glands from clone 152 (Δ^9-THC, 57 ng/gland). With ageing, glands from clone 87 regularly contained higher levels its characteristic cannabinoid than clone 152. During the progressive maturation of glands in clone 152, the CBN content accumulated in the glands and then decreased sharply (table 9). CBC was not detected in the glands of either strain.

Discussion

Floral and vegetative organs of cannabis have been previously analysed for possible sites of cannabinoid synthesis (DePasquale, 1974; Crombie and Crombie, 1975; Malingré *et al.*, 1975). If, as postulated, cannabinoids are synthesized in the glandular trichomes on the plant surface, then gland number should correlate with cannabinoid content. In our attempt to demonstrate this possible correlation, certain sampling difficulties were encountered. Different plant organs vary in their cannabinoid content (Doorenbos *et al.*, 1971; Fetterman *et al.*, 1971) and gland type (Fairbairn, 1972; Hammond and Mahlberg, 1973, 1977; Ledbetter and Krikorian, 1975; Dayanandan and Kaufman, 1976). Therefore, an attempt was made to standardize both quantitative and qualitative analyses for these parameters. Since cannabinoid profiles have been shown to differ among cannabis variants (Small, 1973) as well as within a single strain (Small, 1973; Coffman and Gentner, 1975; Latta and Eaton, 1975), three strains were selected that possessed either a drug, non-drug, or fibre trait and then cloned to insure genetic uniformity within each strain.

Under the experimental conditions employed, these strains retained the cannabinoid character of their seeds. Strain 152 (drug) could be distinguished by a high level of Δ^9-THC with a low CBD level. Strain 79 (non-drug) contained low levels of Δ^9-THC but high levels of CBD, and strain 87 (fibre also had low levels of Δ^9-THC and a high level of CBD. CBC and CBN were found in all three strains, but in varying amounts while Δ^8-THC was not detected. Similar results were reported by Small (1973) and others (Fetterman *et al.*, 1971; Latta and Eaton, 1975) which de-emphasizes the environmental influence on total cannabinoid biosynthesis.

In comparison, the distribution of the three gland types varied from clone to clone and subsequently could not be shown to fit any pattern that might correlate with cannabinoid content. The only trend observed was a decrease in both gland number and cannabinoid content as the plant organ matured, but this was found in all strains and in varying degrees. Comparison of the analyses of particular plant organs such as bracts and leaves for each of these clones revealed a negative correlation between gland number and cannabinoid content. Compared to the bract, floral leaves subtending the bract have considerably fewer glands per unit area, but only a slightly lower cannabinoid content. Mature compound leaves lacked capitate-stalked glands as is the case for most of the leaves on cannabis except the floral leaves subtending the bract. However, comparison of compound leaves with floral leaves showed no outstanding differences that might implicate capitate-stalked glands as being solely responsible for cannabinoid content.

There are at least two explanations for the lack of correlation between gland number and cannabinoid content. First, cannabinoids could possibly be present in other plant cells besides glandular trichomes. A similar conclusion was suggested by Malingré *et al.* (1975) when he found that leaf veins and pollen grains were stained by Fast Blue Salt B, a cannabinoid specific strain (deFaubert Maunder, 1969).

Second, cannabinoid content of the individual gland types could vary from one plant part to another in response to cannabinoid character, environmental conditions, gland ageing, or any combination of these. Different amounts of cannabinoids have been reported to be present in the capitate-stalked glands (Fairbairn, 1972; Malingré *et al.*, 1975). We found that mature, aged, and senescent glands had significantly different cannabinoid levels. However, at each stage of gland maturity the cannabinoid character of the clone was maintained. Furthermore, ratios of mature, aged, and senescent glands were found to vary on each bract examined. It is possible that the other gland types also may be subject to a similar variability in their concentration on the bract.

This investigation has demonstrated a negative correlation between cannabinoid content and gland number in a comparison or different plant organs from three phenotypically different strains maintained as clones. Variation in number and distribution of glands, as related to plant parts and the age of those plant parts, emphasizes a need for caution in employing these structures in cannabis systematics. These results also emphasize that stringent sampling procedures must be employed as related to plant parts selected, age of plant parts, and presence and age of glands on the plant parts, if valid comparative data are being sought for cannabinoids not only between different strains or within a single strain, but even within a single plant. Taking these factors into consideration, further research can be designed to elucidate the site of biogenesis of cannabinoids to determine whether synthesis occurs solely in the glands or in other cells of the plant.

Conclusions

The relationship between glandular trichomes and cannabinoid content in *Cannabis sativa* L. was investigated. Three strains of cannabis, which are annuals, were selected for either a drug, a non-drug, or a fibre trait and then cloned to provide genetically uniform material for analyses over several years. The distribution of the number and type of glands was determined for several organs of different ages including the bract, and its subtending monoleaflet leaf, and the compound leaf on pistillate plants. Quantitation of glands on these structures was integrated with gas chromatography analyses to determine their cannabinoid content. A negative correlation was found between cannabinoid content and gland number for each of the three clones. Isolated heads of the capitate-stalked glands also were analysed for cannabinoid content and found to vary in relation to clone and gland age. These studies indicate that cannabinoids may occur in plant cells other than glandular trichomes. The results of these studies emphasize the need for stringent sampling procedures in micromorphological studies on trichome distribution and analytical determinations of cannabinoid content in cannabis.

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