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A TEMPORAL STUDY OF CANNABINOID COMPOSITION IN CONTINUAL CLONES OF CANNABIS SATIVA L. (CANNABACEAE)[1]

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Genetically and developmentally defined vegetative samples of three clones of *Cannabis sativa* L. were grown in a common environment and analyzed for cannabinoid production. Significant variations occurred in cannabinoid levels in each clonal population. Throughout the 2-yr study, the cannabinoid fluctuations were random rather than cyclic. Although within each clone all cannabinoids increased or decreased simultaneously, fluctuations in cannabinoid levels occurred independently from clone to clone. In addition, each clone retained its distinctive morphology and cannabinoid profile throughout the study.

Introduction

Cannabinoid production in *Cannabis sativa* L. varies during the growing season (PHILLIPS et al. 1970; LATTA and EATON 1975; TURNER et al. 1975; KUSHIMA et al. 1980). In addition, weekly and monthly rhythmic cycles of individual cannabinoids, where high levels of cannabidiol (CBD) were followed by high levels of Δ^9-tetrahydrocannabinol (Δ^9-THC), reflected steps in the biosynthetic pathway (PHILLIPS et al. 1970; LATTA and EATON 1975). TURNER et al. (1975) reported rhythmic patterns for individual cannabinoids, reflecting some aspects of theoretical cannabinoid biosynthesis but not totally supporting the pathway. TURNER et al. (1975) also reported differences in cannabinoid cycles between staminate and pistillate plants.

The interpretation of these variations in cannabinoid content is complicated by reports that attribute control of cannabinoid production to either genetic or environmental factors. The qualitative cannabinoid profile, generally expressed by a predominance of Δ^9-THC or CBD, is considered to be genetically controlled (DOORENBOS et al. 1971; FETTERMAN et al. 1971; NORDAL and BRAENDEN 1973; FAIRBAIRN and LIEBMANN 1974; LATTA and EATON 1975). However, quantities of cannabinoids produced (reflective of the level of genotypic expression) were reportedly controlled by the environment (HANEY and KUTSCHEID 1973; FAIRBAIRN and LIEBMANN 1974; COFFMAN and GENTNER 1975; LATTA and EATON 1975; VALLE et al. 1978). Plants under stress had increased levels of cannabinoids (HANEY and KUTSCHEID 1973; LATTA and EATON 1975), although stress may only cause the loss of older leaves, which contain low levels of cannabinoids (SMALL et al. 1975), thereby increasing the average cannabinoid content in a plant.

Many reports emphasize a variation in cannabinoid quantity among specific plant parts (DOORENBOS et al. 1971; FETTERMAN et al. 1971; FAIRBAIRN and LIEBMANN 1974; LATTA and EATON 1975; TURNER et al. 1977; HEMPHILL et al. 1980). Cannabinoid variability also has been correlated with the stage of plant development (LATTA and EATON 1975; TURNER et al. 1975, 1977; HEMPHILL et al. 1980), genetic heterogeneity in the population (DAVALOS et al. 1977), time of sample collection (LATTA and EATON 1975; TURNER et al. 1975), and methods of sample preparation (COFFMAN and GENTNER 1974; TURNER and MAHLBERG 1984).

The purpose of this study was to monitor cannabinoid production over an extended period of time for the occurrence of any cycles or rhythms. Possible influence by genetic or environmental factors was controlled by using clones and by sampling leaves of a specific size and comparable developmental stage. The clones were grown together in one greenhouse in a common environment, which was potentially variable but to a much lesser degree than would occur in the field.

Material and methods

PLANT MATERIAL

Three strains of *Cannabis sativa*, maintained as clones in a greenhouse, included (1) a drug strain (clone 152) with a characteristic high Δ^9-THC content, (2) a fiber strain (clone 87) with a characteristic high CBD content, and (3) an intermediate strain (clone 79) that was characteristically high in CBD but was morphologically intermediate between clones 87 and 152. Each clone originated from a single pistillate plant of the strain and was maintained by vegetative cuttings rooted for 6 wk in perlite. The rooted cuttings were transplanted into 6-inch clay pots with 6:2:1 loam:sand:vermiculite and were grown for a second 6 wk. During

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the third 6 wk the clones provided leaf samples and new clonal cuttings.

Plants were intentionally grown in the vegetative state under long-day conditions to maintain a common developmental stage in all clones. The 20 h of daily light were provided by sunlight and by incandescent light during the evening. Plants were watered daily and fertilized monthly with Peters 20-20-20. Temperature and humidity were ambient greenhouse conditions with heating and air-conditioning provided as required seasonally for Indiana.

Leaf samples

Leaf samples were collected every Wednesday at 3 P.M. from October 1979 to March 1981 and then every fourth Wednesday at 3 P.M. until November 1981. Only newly expanded 7.5-cm central leaflets of the compound leaf were harvested. Four replicates were collected from each clone on most sampling dates. Fewer replicates were collected from each clone only when insufficient sampling material was available. Replicate samples from each sampling date were averaged, and standard deviations were determined.

Cannabinoid extraction

Leaf samples were collected and immediately oven-dried at 60 C for 12–18 h. Dry weights were determined and samples extracted at 4 C with "Spectranalyzed" grade chloroform for 1 h; then the extract was removed and filtered. The extraction was repeated twice, and the three filtrates were combined, evaporated under nitrogen, and resuspended in 1 ml chloroform containing 0.25 mg/ml eicosane as an internal standard.

Gas-liquid chromatography

Analyses were done on a Hewlett-Packard 5710A chromatograph equipped with a hydrogen flame ionization detector and a Hewlett-Packard 3380A integrator. Cannabinoid standards provided by the National Institute on Drug Abuse were chromatographed, and the column was calibrated by the integrator, using the internal standard method. Glass columns (2 mm i.d. × 2.43 m) were cleaned, treated with 5% dimethyldichlorosilane in toluene, dried, and packed with 3% OV-1 on 80/100 mesh Supelcoport. The inlet and detector temperatures were 250 and 300 C, respectively. A 1-μl aliquot of sample was injected and analyzed with a program of 200–240 C at 2 C/min with an additional 8-min isothermal period at 240 C. Nitrogen was used as the carrier gas with a flow rate of 20 ml/min.

Results

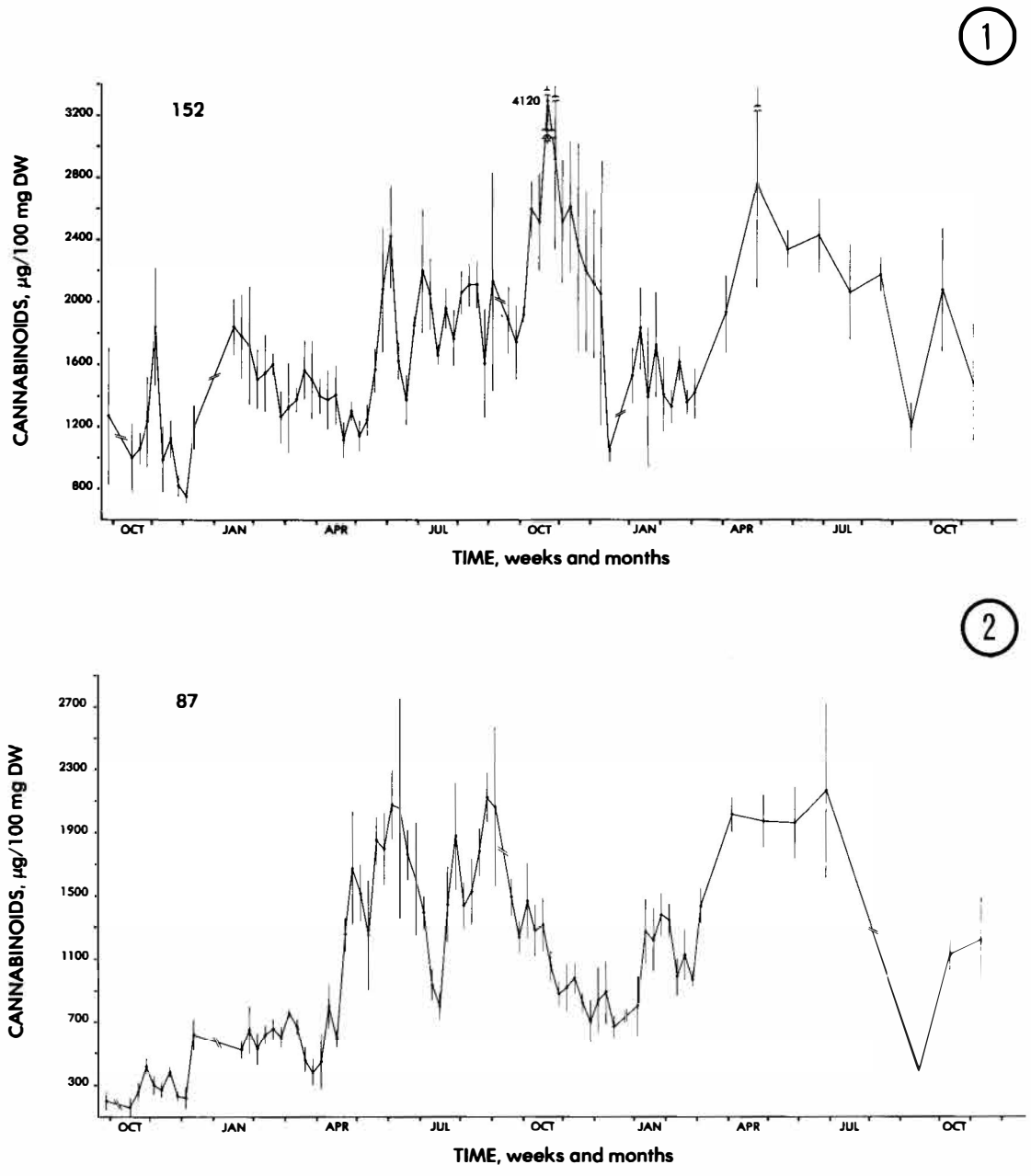
Cannabinoid analysis

Cannabinoid levels in all three clones varied considerably throughout the 2 yr, both within, as indicated by standard deviations, and between sampling dates (figs. 1–3). For individual clones, an analysis of variance was carried out on data collected each month. In some months the samples differed significantly from one another, while in other months samples were not significantly different. In June, July, and August 1980, the *F*-values for clone 152 were 16.66 (significant at the .01 level), 2.70 (not significant), and 4.35 (significant at the .05 level). For clone 87, *F*-values were 1.29 (not significant), 12.87 (significant at the .01 level), and 10.07 (significant at the .01 level). Clone 79 had *F*-values of 3.79 (significant at the .05 level), 2.01 (not significant), and 5.22 (significant at the .05 level). In all three clones, the monthly *F*-values for cannabinoid content were apparently randomly significant, significant only at the .05 level, or nonsignificant.

Total cannabinoid content between clones was compared with the Student's *t*-ratio. For clones 87 and 79, both of which produce CBD as the major cannabinoid, the *t*-ratios between monthly data indicated more months in which the two clones differed significantly than months when no significant differences were found. The reverse was found when comparing Δ^9-THC clone 152 with clone 87. A comparison between clones 152 and 79 revealed few months that were significantly different. However, *t*-ratios were influenced in part by variation among samples during the month. During the second December of the experimental period, clone 152 averaged 1.74 mg cannabinoids/100 mg dry weight (DW). Clone 87 averaged 0.80 mg cannabinoids/100 mg DW. While clone 152 had an average cannabinoid level almost twice as high as clone 87 for that month, the *t*-ratio indicated no significant differences.

In addition to statistical analyses of data collected each month, specific data peaks were also analyzed, again within and between clones. A peak was considered to be real when it encompassed four or more sampling dates. An analysis of variance determined whether the peak reflected a real increase in cannabinoid levels. While the *F*-values showed that many of the peaks reflected real increases in cannabinoid levels, some peaks did not, and others did only at the .05 level. In clone 152 (fig. 1), the peak occurring approximately in August 1980 had an *F*-value of 4.199 and was significant only at the .05 level. The subsequent peak, occurring approximately in September, had an *F*-value of 0.8003 and was not significant. In clone 79 (fig. 3), the two peaks in the first June and July were not significant. Similar results were found throughout the experimental period in all three clones: differences in the statistical significance of the peaks were a result of variation within the samples of the individual peaks.

A comparison of peaks between clones indicat-



FIGS. 1,2.—Concentration of total cannabinoids in clone 152 (fig. 1) and clone 87 (fig. 2) from October 1979 to November 1981. Standard deviations are indicated by vertical lines through each sample point.

ed random increases and decreases in cannabinoid levels. While peaks at some points were common to all three clones (figs. 1–3), other peaks were unique to an individual clone. Also, we found peaks common to two clones but with the subsequent peak for each clone substantially different. Although cannabinoid levels increased or decreased significantly, no pattern was apparent. Where a pattern might appear to exist for a short time, examination of the entire experimental period did not substantiate any type of rhythmic cycling of cannabinoid levels.

INDIVIDUAL CANNABINOIDS

Within each clone, an increase or decrease in the major cannabinoid (CBD or Δ^9-THC) was closely paralleled by the other cannabinoid components (figs. 4–6). When CBD was the major cannabinoid in the clone and was high on a particular sampling date, levels of Δ^9-THC and other detected cannabinoids were also high (table 1). When levels of CBD decreased, so did levels of other cannabinoids in the clone. The same pattern was found in the Δ^9-THC clone.

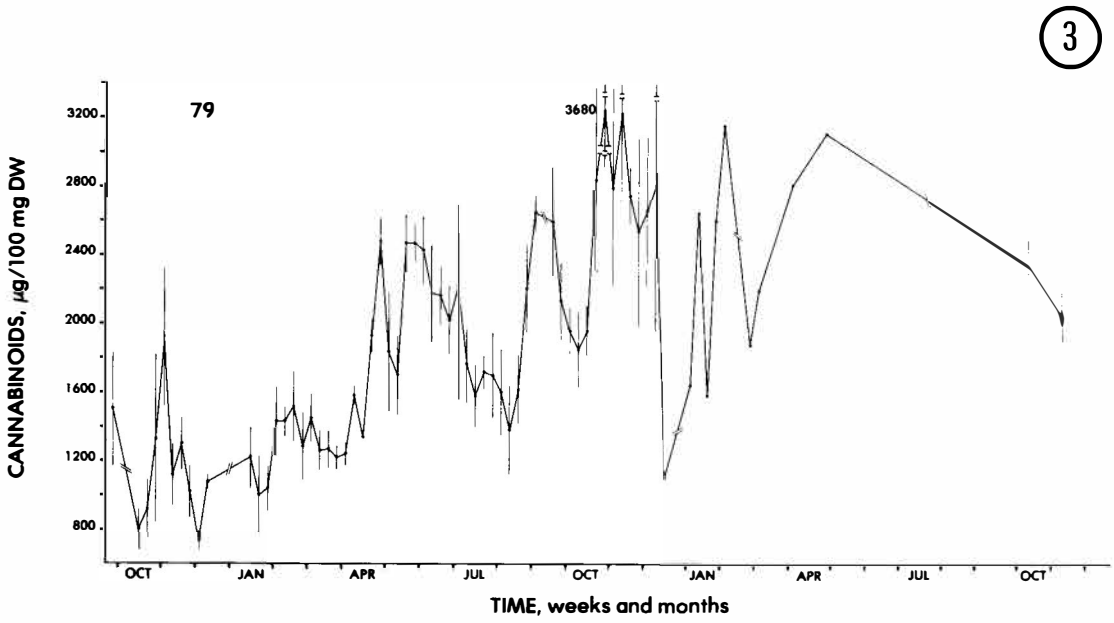


FIG. 3.—Concentration of total cannabinoids in clone 79 from October 1979 to November 1981. Standard deviations are indicated by vertical lines through each sample point.

An increase in a specific cannabinoid in one of the clones at any time of the year was not necessarily reflected in the other clones (figs. 4–6). While CBD is the major cannabinoid in both clones 79 and 87, an increase in levels of CBD did not always occur concurrently in both clones (figs. 5, 6). In addition, an increase in quantities of CBD detected in either clone 79 or clone 87 was not followed in any apparent pattern by increases in levels of Δ^9-THC in clone 152 (figs. 4–6). Cannabinoid levels varied independently and randomly in each clone. However, each clone maintained its distinctive cannabinoid profile throughout the experimental period (table 1).

PLANT MORPHOLOGY

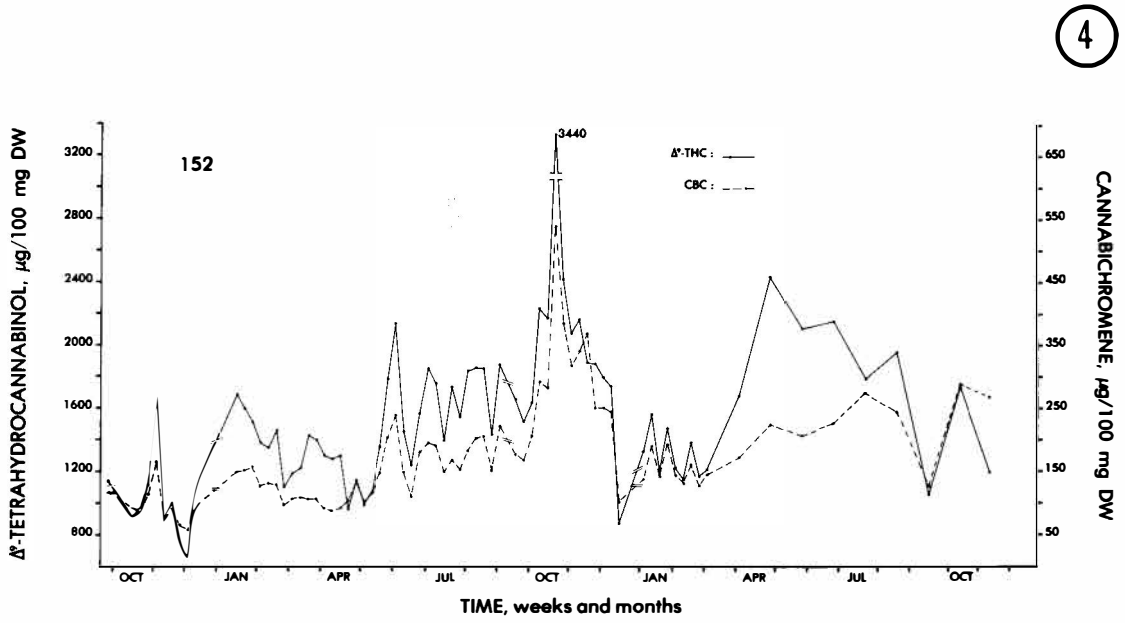
As with cannabinoid profiles, each clone had a distinctive morphology that was maintained throughout the experiment. Drug clone 152 was generally short and multibranched with short internodes. Fiber clone 87 was tall and conservatively branched with long internodes. Clone 79, the high CBD but nonfiber clone, had a morphology approximately intermediate between clones 87 and 152. None of the clones altered its morphology in any way during the experimental period.

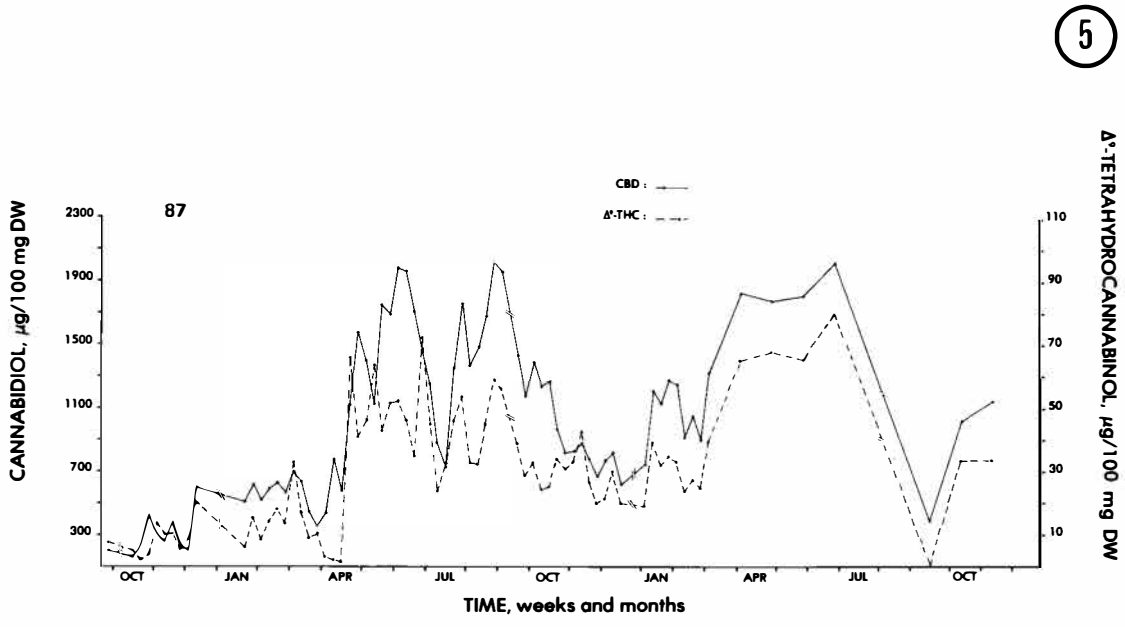
Discussion

Throughout this study, significant increases and decreases in cannabinoid levels occurred in each of three clonal populations. While fluctuating cannabinoid levels were interpreted as weekly to monthly cycles (PHILLIPS et al. 1970; TURNER et al. 1975) or as changing seasonally (LATTA and EATON 1975; KUSHIMA et al. 1980), our study identified the fluctuations as random. Regardless of which of the individual clones was analyzed for cannabinoid production, no repeating cycle occurred. When clones were compared, no common pattern of fluctuation of the cannabinoid content was found. At times, cannabinoid levels increased or decreased simultaneously in some or all of the clones, but not for any extended period of time.

Under the defined conditions of our study, the degree to which cannabinoid levels varied was somewhat surprising. Of the conditions reported to influence cannabinoid levels, such as genetics, environment, or stage of plant development, the greenhouse environment represented the only potential variable in our study. This environment was common for all three clones; yet it did not evoke a common response in levels of cannabinoid production, even for clones 79 and 87, which possessed CBD as the major cannabinoid. Thus, the extent to which the environment influences cannabinoid production on a macro level may be limited. If the environment were a significant factor in determining levels of cannabinoid production by the plant as a whole, simultaneous increases or decreases should have occurred among the clones. In addition, all plants within each clone would be expected to have the same levels of cannabinoids at any particular sampling date. As determined by the standard deviation data, this was not so.

There are several possible explanations for the observed variability in cannabinoid levels. Since cannabinoids are interpreted as secondary products of the plant and regulatory mechanisms of second-





FIGS. 4,5.—Comparison of individual cannabinoids in clone 152 (fig. 4) and clone 87 (fig. 5) from October 1979 to November 1981.

ary metabolism are not well understood (LUCKNER 1972), secondary products may undergo random variations in production. Fluctuations of levels of cannabinoids may only reflect the lack of a rhythmic phenomenon in the plant. An alternative explanation for cannabinoid variability concerns the glandular trichomes. Leaves have populations of glands, and while leaves perhaps may vary in the number of glands on each leaf, the glands also differ in cannabinoid content (TURNER et al. 1977). Variation among leaf samples may reflect variations in cannabinoid content of individual glands in the glandular population of the leaf. Gland cannabinoid content could be influenced by the developmental stage of the gland, the effects of microenvironments on the leaves, loss of glands, or physical damage to glands.

While significant variations were found in levels of cannabinoids, individual cannabinoids in each clone increased or decreased simultaneously. Therefore, parallel variations of each clone's distinctive cannabinoid profile were seen. Throughout

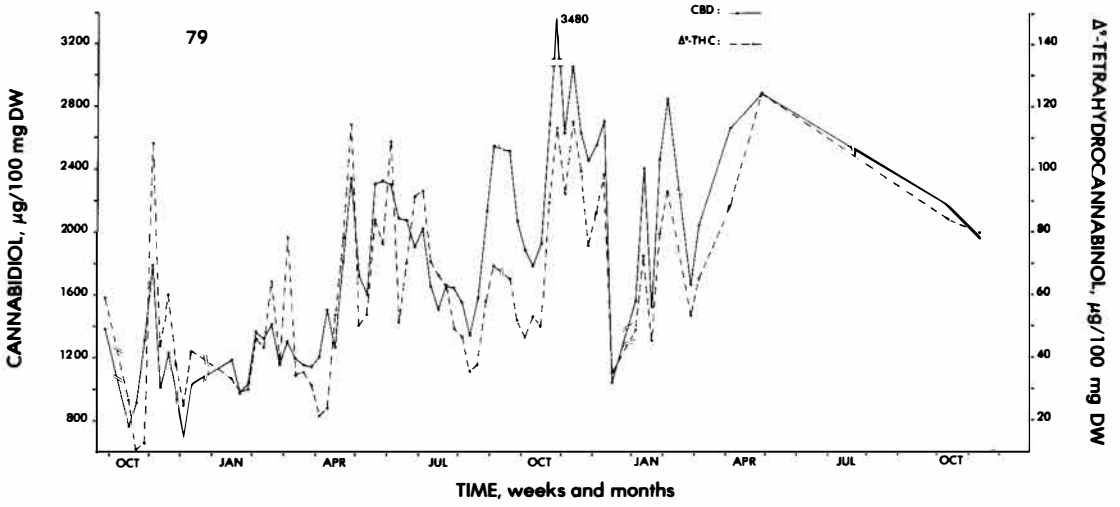


FIG. 6.—Comparison of individual cannabinoids in clone 79 from October 1979 to November 1981.

the experiment, there was no indication of cyclic spiking of individual cannabinoids reflecting the biosynthetic pathway. Most of the reported cyclic spiking has been interpreted from data on flowering plants. However, a close examination of the data during vegetative growth and early flowering (PHILLIPS et al. 1970; LATTA and EATON 1975; TURNER et al. 1975; KUSHIMA et al. 1980) reveals parallel variations of the cannabinoid profile. Offset peaks of individual cannabinoids were not evident in these reports until the plants were flowering. Only plants in the vegetative state were used in our study; therefore, plant developmental stage or, more specifically, the flowering condition may influence the qualitative cannabinoid profile. The results of TURNER et al. (1975) and HEMPHILL et al. (1980) also indicated that flowering may influence quantities of specific cannabinoids.

TABLE 1

CANNABINOIDS DETECTED IN EACH CLONE AT 5-mo INTERVALS DURING THE 2-yr STUDY

CLONE AND COLLECTION DATE	μg CANNABINOIDS/100 mg DW					
	CBD	CBC	Δ^8-THC	Δ^9-THC	CBN	Total
Clone 152:						
Mar 1980	...[a]	110.1	...	1,193.6	15.9	1,319.7±270.5
Aug	...	185.4	...	1,838.0	50.1	2,074.1±138.4
Jan 1981	...	141.7	24.4	1,328.5	33.0	1,530.7±184.6
Jun	...	225.1	25.8	2,154.0	25.2	2,434.4±235.0
Nov	...	268.4	9.4	1,201.0	8.5	1,494.6±373.1
Clone 87:						
Mar 1980	699.7	...	12.1	32.9	17.3	762.0± 30.9
Aug	1,374.0	...	23.1	33.5	9.3	1,439.8±152.8
Jan 1981	754.4	...	27.4	19.7	3.7	805.3±194.4
Jun	2,014.9	...	60.9	79.9	28.3	2,174.5±557.2
Nov	1,137.9	...	40.0	33.6	16.7	1,228.2±258.4
Clone 79:						
Mar 1980	1,309.8	...	...	78.7	58.9	1,447.4±137.7
Aug	1,548.3	...	...	46.6	.8	1,595.7±244.7
Jan 1981[b]	1,561.3	...	...	48.6	32.9	1,642.8
Jun[c]	...	...	...	...	...	...
Nov	1,956.6	...	...	79.5	15.2	2,051.3±140.4

[a] None detected.
[b] Only one sample collected.
[c] No leaves available to collect.

A further result of this study was the confirmation of genetic control of plant morphology and cannabinoid profile in *Cannabis*. Each of the three clones had a distinctive morphology and cannabinoid profile that were maintained throughout the 2 yr. Although SCHULTES (1970) supported an interpretation that *Cannabis* will acclimatize to a particular environment, in our study there was no indication that growing the clones side by side in a common greenhouse environment resulted in a population of plants with a uniform cannabinoid profile or a similar morphology. SCHULTES may have been reporting the results of hybridization rather than acclimatization. In fact, these particular clones have been cultivated continually for more than 7 yr, and plant morphology, as well as the cannabinoid profile distinctive for each clone, has been unchanged.

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