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Studies on Growth and Cannabinoid Composition of Callus Derived From Different Strains of *Cannabis sativa*[1]

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ABSTRACT.—Callus derived from different plant parts of various strains of *Cannabis sativa* L. was established and analyzed for growth on different media and for cannabinoid content by thin-layer (tlc) and gas-liquid chromatography (glc). Plant organs which included bracts and anther-calyx complexes of drug (152) and fiber (150) types and maturing leaves of Mexican (drug) and Turkish (fiber) strains were shown to accumulate characteristic cannabinoids and, therefore, were selected as callus source material. Callus initiated from these plant organs demonstrated different growth responses to casein hydrolysate (CH) which were different from its growth response to various amino acid mixtures as media supplements. Numerous roots were formed on newly initiated callus and subcultured tissues of several transfers. Addition of 2,4-dichlorophenoxyacetic acid at 0.2 mg/liter inhibited root formation. In long-term cultures, different concentrations of naphthaleneacetic acid and kinetin did not initiate root and/or shoot differentiation. However, growth responses of these callus strains differed from each other when maintained on various combinations of these growth regulators. Under these experimental conditions, no major cannabinoids were detected in callus extracts, although numerous unknown metabolites were present. One minor cannabinoid was detected as indicated from a positive Fast Blue Salt B test.

Numerous cannabinoids have been reported to occur naturally in *Cannabis sativa* L. (1). These products, known as terpenophenols or C_{21} compounds, represent the most distinctive class of compounds in *Cannabis* (2). Both neutral and carboxylic acid cannabinoids have been detected; however, the cannabinoid acids are believed to be the natural forms in the plant (3, 4, 5). Several studies have shown that there are different types of chemical strains in *Cannabis* recognizable by their cannabinoid content (6, 7). Terpenophenol-containing organs of each specific chemical strain possess a cannabinoid profile characteristic of the strain (8). However, bracts and the leaves which subtend these floral organs contain higher levels of cannabinoids than vegetative leaves (8). Roots have been reported to contain no cannabinoids (2). Even though these differences in cannabinoid content within a single plant are well documented, the factors which influence the accumulation and control of the biogenesis of cannabinoids in the different organs remain to be fully investigated (3, 4).

Tissue culture procedures have been employed by several investigators to study secondary product formation in tissues other than the intact plant. However, the profiles of secondary products isolated from cultured cells usually differ from the natural products. The latter, if present, usually occur in smaller quantities than those present in the parent plant (10). In some instances, the formation of secondary products in cultured tissues has been correlated with organ differentiation on callus (10, 11, 12). In a recent study on cultured cells of *Cannabis,* Veliky and Genest (13) reported that no major cannabinoids were detected in cell suspension cultures derived from root explants.

The purpose of this study was to determine whether callus derived from different cannabinoid-containing shoot organs, as contrasted with the root (13), were capable of synthesizing cannabinoids. Analyses were performed on long-term callus derived from four *Cannabis* strains of different geographical orgins and representing several known phenotypes (14).

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MATERIALS AND METHODS

Seeds of *Cannabis sativa* L. from different geographical sources and chemical types were employed in this study. Seeds of a drug type (152) from Japan and a fiber type (150) from Germany were obtained from E. Small (15), and a fiber type (TU A(2): C–71) from Turkey was obtained from C. Turner (16). A Mexican strain which was a drug type was obtained from a police seizure (17). Plants were germinated from seed and grown under ambient greenhouse conditions before being used as source material for callus initiation. Callus from strains 150 and 152 was initiated from maturing bracts before seed formation, herein termed bract callus, and mature anther-calyx complexes after anthesis, herein termed calyx callus, of each strain. Mexican and Turkish cultures were initiated from vegetative leaves, herein termed leaf callus.

Excised plant parts were surface sterilized by first dipping them into 70% ethanol, 10% autoclaved Clorox (10 min) and then washing them four times in autoclaved distilled water (3 min each). Sterile explants were placed on Miller's medium (18) which contained Murashige's iron source (19) and 2% sucrose. A set of cultures was maintained under a light cycle (12 hr at 700 lx – 12 hr dark) and another set under dark conditions. The callus growth yields were greater when grown in the dark than under light conditions. Therefore, the results presented in this report emphasize the data obtained on dark-grown cultures. *Cannabis* callus cultures were initiated on agar medium (8 gm/liter) containing one of the following hormonal mixtures: No. 1, indoleacetic acid (IAA) (1 mg/liter) and kinetin (1 mg/liter); No. 2, IAA (1 mg/liter) and kinetin (1.5 mg/liter); and No. 3, IAA (0.25 mg/liter), 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5×10^{-6} M), α-naphthaleneacetic acid (NAA) (0.25 mg/liter) and kinetin (1 mg/liter). After several transfers, all stock cultures were routinely maintained on the following hormonal mixture: No. 4, IAA (1 mg/liter), NAA (0.1 mg/liter), 2,4-D (0.2 mg/liter) and kinetin (2 mg/liter). Four small pieces of tissue, each approximately 50 mg fresh weight, were transferred to medium contained in 125 ml Erlenmeyer flasks and covered with sterile polypropylene. Fresh weight (FW) and dry weight (DW) growth values were employed as growth parameters which were determined after a 30-day culture period by the following formulae:

Fresh Weight (mg) Growth Values =
Final Weight-Initial Weight/Piece
Dry Weight (mg) Growth Values =
Total Dry Weight

Materials were oven dried (65°) overnight in preparation for extraction. Plant parts (100 mg DW) and callus (200 mg DW) were extracted (3X) with chloroform (spectranalyzed) for at least 1 hr/extraction at 4°. Combined aliquots were filtered and concentrated to dryness by gentle nitrogen evaporation. Dried residue of the excised plant parts and their respective callus tissue was taken up in 1 ml of the appropriate solvent. Either Δ^4-androstene-3,17-dione (1 mg/ml) or eicosane (0.25 mg/ml) was used as an internal standard.

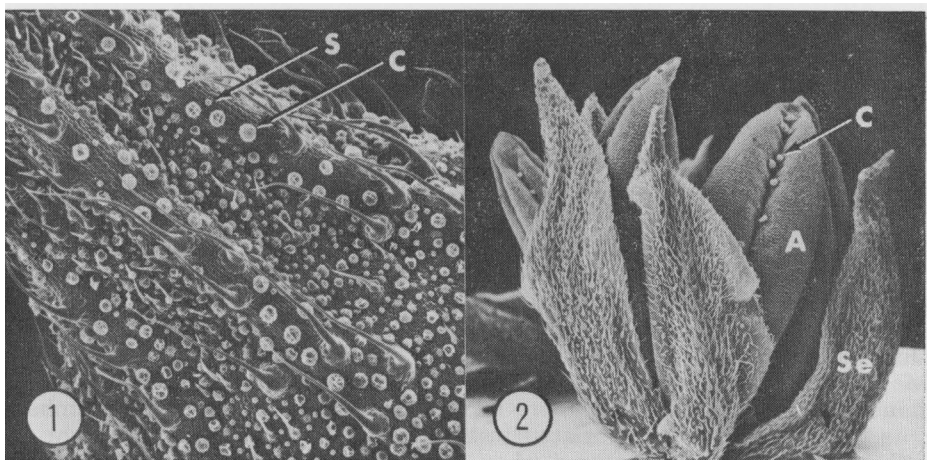
Analyses were performed with a gas-liquid chromatograph (glc) (Hewlett-Packard 5710A) equipped with a hydrogen flame-ionization detector and operated by programming from 200–240 (2/min). Nitrogen (20 ml/min) was used as the carrier gas. The injection port and detector temperatures were 250 and 300, respectively. Glass columns, pretreated with dimethyldichlorosilane in toluene (10%, v/v), were packed with 3% OV 1 Supelcoport (80/100 Mesh). Quantitative data of identified cannabinoids[2] found in excised plant parts were determined with an integrator (Hewlett-Packard 3380A). Relative retention times of unknowns detected in callus tissue and synthetic cannabinoid standards were compared.

For thin-layer chromatography (tlc), silica gel sheets (Eastman No. 6061) were impregnated with N,N-dimethylformamide and carbon tetrachloride (3:2, v/v) and dried before use. After application of the samples, the sheets were developed in a solvent system of petroleum ether and diethyl-ether (4:1, v/v). Chromatograms were developed twice in the solvent, and subsequently sprayed with 0.5% Fast Blue Salt B (20) in methanol-ethanol (3:2, v/v).

RESULTS

PLANT ORGANS.—Both glandular and nonglandular trichomes were present on bracts (fig. 1) and anther-calyx complexes (fig. 2). Two of the three types (17) of glandular trichomes, capitate-sessile and bulbous glands, were present on maturing bracts of the drug 152 strain when used as explants. Capitate-stalked glands are known to appear later in bract development of this strain and are reported to be associated with seed development (21). In comparison, bracts of fiber 150 possessed all three glandular types when used as explants. The anther-calyx complexes of 150 and 152, as well as vegetative leaves of the Mexican and Turkish strains, possessed only capitate-sessile and bulbous glands.

[2]Provided by NIMH: cannabidiol, CBD; cannabichromene, CBC; Δ^8- and Δ^9-tetrahydrocannabinol, Δ^8- and Δ^9-THC; cannabinol, CBN.



FIGS. 1–2. *Floral parts of* Cannabis sativa *L., drug 152, utilized to initiate callus.* Fig. 1, portion of a bract with glandular and nonglandular pointed trichomes from a pistillate plant. ×42. Fig. 2, anther-calyx complex from a staminate plant. The numerous nonglandular pointed trichomes obscure the glandular trichomes on the sepal in this figure. ×15. These organs are similar in appearance for all strains employed in this study. Floral parts were prepared for scanning electron microscopy as described previously (8).
KEY TO LABELING: A, anther; C, capitate-sessile gland; S, small bulbous gland; Se, sepal of calyx.

All materials possessed nonglandular trichomes in addition to the glandular forms.

Cannabinoid profiles of bracts, anther-calyx complexes, and leaves showed terpenophenol spectra characteristic of the different *Cannabis* strains (table 1). A high concentration of Δ^9-THC was found in the flower parts of drug 152 and vegetative leaves of the Mexican strain. Except for the high level of CBD in the vegetative leaves of the Mexican strain, the C_{21} compounds (CBD, CBC and CBN) were present only in relatively low quantities. In comparison, a high concentration of CBD was found in the flower parts of fiber 150 and vegetative leaves of the Turkish strain. This fiber strain also contained a high drug (Δ^9-THC) content in its vegetative leaves. In addition to known cannabinoids, the bracts and leaves of *Cannabis* strains used for callus initiation contained

TABLE 1. *Cannabinoid profiles of plant organs from different strains of* Cannabis.

Strain	Organ	Cannabinoids, mg/100 mg dry weight						
		Unknown No. 1	Unknown No. 2	CBD	CBC	Δ^8-THC	Δ^9-THC	CBN
Drug 152								
Pistillate	Maturing bracts	–[a]	+	0.01	0.19	0.10	1.14	0.06
Staminate	Mature anther-calyx	–	–	–	0.29	0.19	1.11	0.08
Fiber 150								
Pistillate	Maturing bracts	–	+	2.16	–	–	0.06	0.01
Staminate	Mature anther-calyx	–	–	1.29	–	–	0.04	0.02
Mexican								
Vegetative	Maturing leaves	++	–	1.36	0.44	–	1.23	0.10
Turkish								
Vegetative	Maturing leaves	+++	–	2.37	+	0.05	0.74	0.08

[a] + or – indicates the presence or absence of a metabolite.

Growth of leaf callus from the Mexican and Turkish strains differed when these cultures were maintained on CH and under various cultural conditions. Maximum growth of both strains that were grown in the dark occurred on medium containing 2 gm/liter CH. However, under a light-dark cycle, the Mexican strain grew best on 1 gm/liter CH, but the Turkish strain developed optimal growth on medium containing 2 gm/liter CH (unpublished results, Hemphill, et al.).

Since most of these strains required CH for callus growth, two amino acid mixtures were incorporated into the basal medium in an attempt to establish a defined medium for these *Cannabis* strains (table 2). Maximum dry weight accumulation for the bract and calyx calli of drug 152 occurred on amino acid mixture No. 2 (23), although the calyx callus tissue grew more slowly than bract callus. For fiber 150, callus of bract and calyx origins showed the highest dry weight yields on amino acid mixture No. 1 (see table 2).

In general, callus maintained on these amino acid mixtures grew slower than their counterparts grown on media containing either 1 or 2 gm/liter CH (table 2). Neither amino acid mixture yielded the dry weight accumulation supported by 1 or 2 gm/liter CH for bract callus of strains 152 and 150, or for calyx callus of drug 152. In contrast, the maximum dry weight accumulation of calyx callus from fiber 150 occurred on a medium containing amino acid mixture No. 1 and exceeded the growth on CH-supplemented media (table 2). Thus, the calyx callus of fiber 150 can be grown on a defined medium in contrast to callus cultures derived from other strains. Fresh weight values, in most cases, from these tissues paralleled those from the dry weight analyses (data not presented). With the exception of the bract callus of fiber 150, low dry weight percentages corresponded with high callus growth (table 2).

Callus of these *Cannabis* strains grown as long-term cultures were transferred to media supplemented with different concentrations of NAA and kinetin to determine whether a correlation existed between cannabinoid production and organogenesis in *Cannabis,* as has been reported for secondary metabolite production in other culture systems (11, 12). Under these cultural conditions, root differentiation was minimal and unrelated to the concentrations of exogenous growth regulators (table 3). No shoot formation was observed in these cultures.

TABLE 3. *Effects of hormones on growth of* Cannabis *callus derived from different plant parts.*

Hormones (mg/liter)[a]		Drug 152		Fiber 150	
NAA	Kinetin	Bract	Calyx	Bract	Calyx
0	0	24.4[b] (16.1)	79.1 (6.6)	64.6 (7.6)	155.5 (4.4)
0	1.0	31.3 (18.9)	76.3 (5.6)	72.9 (11.9)	260.3 (3.9)
0.5	1.5	155.0 (7.6)	156.6 (4.8)	166.1 (7.8)	206.7 (4.5)
0.5	2.0	161.8 (7.8)	176.0 (4.7)	118.9 (6.5)	198.4 (3.9)
1.0	2.0	85.6 (7.8)	155.2 (4.9)	108.1 (5.9)	131.7 (5.0)
1.0	0	59.0 (6.8)	158.3 (5.0)	88.7 (6.1)	119.0 (5.0)
1.5	0.5	72.3 (7.2)	171.0 (5.0)	111.7 (6.0)	110.0 (4.6)
2.0	0.5	59.4 (7.0)	156.1 (5.0)	96.6 (5.6)	100.6 (5.2)

[a]Culture medium contained 1 gm/liter CH.
[b]Data expressed as DW (mg) and DW % in parentheses (FW can be calculated from the DW %).

Growth yields varied with hormonal concentrations (table 3). Maximum dry weight values for callus derived from bracts and anther-calyx complexes of drug 152 were obtained for cultures grown on a combination of 2 mg/liter kinetin and 0.5 mg/liter NAA. In comparison, bract callus of fiber 150 required 1.5 mg/liter kinetin and 0.5 mg/liter NAA for maximum growth. Calyx callus of the fiber 150 grew best on kinetin alone (1 gm/liter). This callus strain also grew well in the absence of both kinetin and NAA when compared to other strains grown in the absence of these hormones (table 3). Fresh weight values (data not presented) of these tissues paralleled the dry weight analyses. In general, low dry weight percentages corresponded with high growth yields of these callus strains (table 3).

CANNABINOID ANALYSES OF CALLUS TISSUES.—Callus derived from plant organs of the different strains possessed several unknown metabolites that had relative retention times similar to cannabinoid standards (table 4). Some of these compounds possessed shorter relative retention times than CBD. However, no major terpenophenols were detected in any of the cultures when the callus was screened by glc.

The addition of CH or amino acids to the basal medium did not stimulate the formation of these unknowns in the cultures. Unknowns 1, 3–6 were common, in most cases, to all cultures, whereas unknown 2 occurred less frequently. Callus tissue of bract origin that was grown on medium containing amino acid mixture No. 1 accumulated several of these unknowns. However, when grown on medium containing amino acid mixture no. 2, these unknowns were not detected in bract callus of either the drug or fiber strain (table 4).

A comparable pattern of unknowns was found in callus derived from leaf tissues of the Turkish and Mexican strains (data not presented). Cultures grown on media with or without these organic nitrogen supplements showed numerous unknowns, as described previously. No major cannabinoids were detected in leaf callus (unpublished results, Hemphill, et al.).

Analyses of bract and calyx calli of drug 152 and fiber 150 grown on different combinations of kinetin and NAA showed the presence of these unknowns, which were also found in cultures grown on various organic nitrogen sources (table 4). All combinations of growth regulators described in table 3 supported similar chromatographic patterns as shown for cultured strains grown under optimal growth conditions (table 4). As found in previous experiments, no major cannabinoids were detected in these callus cultures grown on different combinations of kinetin and NAA.

The unknowns were analyzed further with a combination of tlc and glc to determine whether any of these unknowns were cannabinoid homologs. Callus samples from different cultures were extracted and prepared for tlc. The resulting chromatograms of callus extracts when sprayed with Fast Blue Salt B (20) possessed a common blue spot with an R_F of 0.05. Callus samples which accumulated this unknown were processed by preparatory tlc. The silica gel region corresponding to this unknown (R_F 0.05) was scraped from the chromatograms. This unknown plus other possible polar compounds were extracted from the adsorbent and prepared for tlc analyses. Among the unknowns detected, one compound which occurred in the largest quantity possessed a relative retention time similar to unknown 4 in table 4.

DISCUSSION

Organs utilized in this study included vegetative leaves and floral parts from both pistillate and staminate plants of drug and fiber phenotypes. Micromorphological examination of the leaves, bracts, and anther-calyx complexes of these strains indicated that the character of the glandular and nonglandular trichomes was similar to those described in previous studies (8, 17, 21).

TABLE 4. *Relative retention time of unknown metabolites isolated from* Cannabis *callus derived from different plant parts grown on media containing organic supplements.*

Callus	Organic Supplement	Relative Retention Times										
		1	2	3	CBD (0.74)	CBC (0.76)	4	Δ^8-THC (0.81)	Δ^9-THC (0.84)	5	CBN (0.92)	6
Drug 152												
Bract.....	0	0.37	—	0.69	—	—	0.80	—	—	0.91	—	0.94
	1 CH	0.36	—	0.68	—	—	0.79	—	—	0.91	—	0.93
	2 CH	—	0.57	0.68	—	—	0.80	—	—	0.91	—	0.93
	AA 1	0.37	—	0.68	—	—	0.79	—	—	0.90	—	0.93
	AA 2	—	—	—	—	—	—	—	—	—	—	—
	NAA–KIN*	0.36	0.57	0.68	—	—	0.79	—	—	0.91	—	0.94
Calyx.....	0	0.36	—	0.67	—	—	0.79	—	—	0.91	—	0.94
	1 CH	—	—	0.68	—	—	0.79	—	—	0.90	—	0.93
	2 CH	—	—	—	—	—	—			0.91	—	0.93
	AA 1	—	—	—	—	—	0.79	—	—	0.91	—	—
	AA 2	0.37	—	0.68	—	—	0.79	—	—	0.90	—	0.93
	NAA–KIN*	0.37	0.57	0.68	—	—	0.80	—	—	0.92	—	0.94
Fiber 150												
Bract.....	0	0.36	—	—	—	—	0.80	—	—	0.91	—	0.93
	1 CH	—	0.57	0.67	—	—	—	—	—	0.91	—	—
	2 CH	—	—	—	—	—	0.79	—	—	0.90	—	0.93
	AA 1	0.38	0.57	0.68	—	—	—	—	—	0.91	—	—
	AA 2	—	—	—	—	—	—	—	—	—	—	—
	NAA–KIN*	0.37	—	0.69	—	—	0.79	—	—	0.91	—	0.93
Calyx.....	0	0.37	—	0.68	—	—	0.79	—	—	0.91	—	—
	1 CH	0.37	—	—	—	—	—	—	—	0.91	—	—
	2 CH	0.37	—	0.69	—	—	0.79	—	—	0.90	—	0.93
	AA 1	—	—	0.68	—	—	0.79	—	—	0.91	—	—
	AA 2	—	—	0.68	—	—	0.79	—	—	0.91	—	0.94
	NAA–KIN*	—	0.57	0.69	—	—	0.80	—	—	0.92	—	0.94

Hormone concentrations: 2,4-D (0.2 mg/l), IAA (1 mg/l), NAA (0.1 mg/l) and kinetin (2 mg/l).

*Medium contained the hormonal combinations (NAA–KIN) which supported optimal growth conditions for each strain as described in table 3. Amino acid (AA) mixtures: No. 1, see table 2: No. 2, Gambor's mixture (23).

Small, in an extensive study of different chemical phenotypes (14), adopted the concentration of 0.3% Δ^9-THC (dry weight basis) to distinguish between drug and nondrug classes of plants (6). This level represented data obtained from "manicured" plant samples which consisted of leaves, flowers, small fruits and twigs (14). In the present study, the bracts and anther-calyx complexes of both drug 152 and fiber 150 possessed cannabinoid profiles characteristic of these strains but contained higher cannabinoid concentrations than those reported by Small (6, 14, 15) for these strains. Except for the levels of CBD and CBC in the vegetative leaves of the Mexican strain, the quantities of major cannabinoids in maturing leaves of the Turkish and Mexican strains were comparable to those reported for 'manicured' samples by Holley, et al. (16). These results re-emphasize the importance of selecting specific plant organs for cannabinoid analyses when determining the chemical phenotype of a *Cannabis* strain (8).

Newly initiated callus tissue derived from leaves and floral organs, including bracts of pistillate and anther-calyx complexes of staminate plants of these *Cannabis* strains, formed roots in abundance when maintained on several combinations of IAA and kinetin. The capacity for these cultures to form roots progressively decreased during subsequent transfers. In comparison, root formation was inhibited when these cultures were grown on medium containing 2,4-D (0.2 mg/liter). Long term cultures, which were subcultured routinely on Medium 4 for two years, were shown to have lost the capability for organ differentiation when subsequently grown on media containing combinations of hormones reported to induce organ development in other callus culture systems. Similar results have been demonstrated for *Pisum sativum* L. root-derived callus tissue (24) and other tissues (25).

These long-term callus cultures, in general, possessed a CH requirement as a supplement in the culture medium. The results of our studies, therefore, parallel those of Veliky and Martin (22), who reported a CH growth requirement for cell suspension cultures derived from roots of *Cannabis*. Amino acid mixtures did not substitute for CH in the callus cultures that required CH for growth. In other culture systems, several workers (23, 26) have reported that amino acid mixtures can be substituted for the CH growth requirement of callus tissues. Recently, a salt medium (27) developed by Veliky was shown to support *Cannabis* cell growth in suspension cultures (personal communication).

Callus cultures derived from the calyx of fiber 150 differed from those of other strains in showing maximum growth on medium lacking CH or on medium containing amino acid mixture No. 1. This growth pattern, in part, could be the result of a selection process of a limited number of cells present within a large cell population which were capable of multiplying rapidly on a defined medium. Divergent growth responses of closely related strains of cultured tissues have been reported in the literature (28, 29).

Callus tissues derived from different cannabinoid-containing organs of these *Cannabis* strains did not possess the capacity to synthesize terpenophenols when maintained as long-term cultured tissues. Similar results were found by Veliky and Genest for *Cannabis* cell suspension cultures derived from roots (13) which were reported to lack cannabinoids (2). Therefore, callus tissue derived from shoot explants or cell suspension cultures derived from root explants (13) have not as yet been shown to possess the capability to synthesize major cannabinoids which are prominent in the intact plant.

The formation of secondary metabolites in other cultured tissues has been reported to be correlated with organ differentiation (10, 11, 12). However, these long-term callus cultures of *Cannabis* appear to have lost the capacity to form organs under cultural conditions. Therefore, we were unable to evaluate the effects of shoot differentiation on cannabinoid production because shoots were not formed on any cultures grown on the various hormonal combinations em-

ployed in these studies. In comparison, root differentiation did not stimulate cannabinoid formation in newly initiated callus cultures of these *Cannabis* strains (unpublished results, Hemphill, et al.). It has been reported for other cultural systems that 2,4-D suppressed both organ differentiation (12) and alkaloid formation (12, 30, 31). The presence of this synthetic auxin (2,4-D) at 0.2 mg/liter in the medium may have altered the genetic mechanism for organ differentiation and/or cannabinoid metabolism in these long-term cultures. However, conflicting reports have been published on the role of 2,4-D in the formation of chromosomal abnormalities (32). Therefore, the production of cannabinoids in long-term cultures may depend on the determination of specific physiological conditions which regulate cellular and organ differentiation.

Glc analyses of these long-term callus tissues showed the presence of unknown compounds with relative retention times similar to those of cannabinoid standards. These unknown compounds were detected in cultures grown on different media except for bract callus from both strains grown on Gamborg's amino acid mixture. The basis for the difference between bract and calyx calli of both strains grown on this medium, or for the effect of this amino acid mixture as contrasted to amino mixture No. 1, or CH supplements, was not evident. Further studies are necessary to determine whether these cultures can be induced to form cannabinoids when grown on media containing other metabolites or when are exposed to other cultural conditions.

Preparative tlc followed by glc indicated that one component from the callus extract gave a blue reaction to a cannabinoid indicator, Fast Blue Salt B (20), and appeared to possess a relative retention time similar to unknown 4. This unknown was present in many of the long-term callus cultures and could represent a cannabinoid homolog. The presence of these unknown compounds indicates that long-term callus cultures possessed only a limited capacity to synthesize minor terpenophenolic compounds.

In summary, the growth of callus from different organs of several *Cannabis* strains varied depending on the culture medium. Cannabinoids prominent in organs of intact plants of these strains were absent in the derived callus tissues. However, a series of unknowns, one of which may be a minor cannabinoid, was present in callus grown on most cultural media. Additional studies are now in progress to find an *in vitro* system in which callus tissues will synthesize major cannabinoids under cultural conditions.

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