HISTOCHEMICAL ANALYSES OF LATICIFERS AND GLANDULAR TRICHOMES IN *CANNABIS SATIVA*

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ABSTRACT.—The unbranched nonarticulated laticifer, including its latex, and capitate glandular trichomes from *Cannabis sativa* L. were analyzed in fresh and cryostat preparations with histochemical procedures for the presence of cannabinoids, alkaloids, and other selected cellular components. A positive response to cannabinoid indicators, Duquenois-Negm, fast blue salt B, Gibb, and Beam reagents occurred in laticifers, as well as exuded latex and in disc cells of epidermal capitate glandular trichomes. No response or only an apparent background response to these reagents was detected in other cells. Alkaloids were detected histochemically in laticifers and exuded latex with Wagner, Dittmar, Ellram, chromic acid, Hager, and Dragendorff reagents. Alkaloid indicators also reacted with capitate glandular trichomes but did not show a positive response in other cells of the plant. Laticifers also contained other specialized contents including the enzymes, cytochrome oxidase, and lipase. Free lipids and storage proteins were not detected in laticifers. Qualitative responses to these histochemical procedures were similar in laticifers and capitate glandular trichomes from various regions and organs of the plant axis. Histochemical indicators can be useful for preliminary surveying of specialized cell types and tissues for the presence of specialized substances.

Recent investigations of the chemistry of *Cannabis sativa* L. have identified a group of terpenophenols, the cannabinoids, which occur only in this genus (1, 2, 3). The major cannabinoids, including Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN), and cannabidiol (CBD), are utilized in the chemical characterization of drug, nondrug, or fiber strains of *Cannabis* (4, 5). These compounds appear to be most abundant in actively growing regions of the shoot system of both pistillate and staminate plants (6). Several recent studies have indicated that these cannabinoids accumulate in epidermal glandular trichomes (7, 8, 9). However, there have been few studies of the relationship of cannabinoids to laticifers, an internal secretory system, or to other specific cells or tissue systems in the plant.

Similarly, the presence of alkaloids, including choline and trigonelline, already noted in the early literature (10) have been reported from *Cannabis* tissues (11, 12, 13). Other investigators have identified a group of cannabimine (14) as well as hordenine, neurine, and spermidine (15, 16, 17, 18) alkaloids from various portions of this plant. The presence of alkaloids has not been related to specific cell types or tissues in this genus, although alkaloids are common constituents in specialized cells such as the laticifer in other plants.

The purpose of this study is to examine whether histochemical procedures can be utilized effectively to survey plant tissues for their general composition. Histochemical techniques are applied to the laticifer and glandular systems as well as other cells to determine sites of accumulation of cannabinoids, alkaloids and other specific compounds in these cells. We will show that these techniques can be useful for preliminary surveying of cells and tissues for the presence of specialized substances.

MATERIALS AND METHODS

Materials of *Cannabis sativa* L. investigated included laticifers, latex exudate, tissue components of fruits, seedlings, leaves, and floral parts of pistillate and staminate axes. Prepared

chemical reagents were applied to freezing microtome or fresh sections of tissues, and fresh latex exudate collected from the petiole.

Major cannabinoids, Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), and cannabinol (CBN), were identified by specific colorimetric reactions. The Duquenois-Negm test is reported to detect Δ^9-THC and other cannabinoids (19), fast blue salt B (FBSB) reportedly identified THC (20), the Beam reagent detected CBD (21), and the Gibb reaction stained all major cannabinoids (1).

Reference standards were obtained from the National Institute on Drug Abuse and included Δ^9-tetrahydrocannabinol, cannabinol and cannabidiol.

Presence of alkaloids in sections was identified with alkaloid-specific reagents: Wagner, Dittmar, Ellram, Dragendorff (modified), and chromic acid (22). Solutions of 1% aq choline hydrochloride, 1% aq trigonelline, and 1% aq hordenine sulfate were employed as reference standards in tests of alkaloid detecting solutions.

Wagner reagent consisted of 1.27 g of iodine and 2 g of potassium iodide in 100 ml of distilled water. Dittmar reagent was prepared with 1 g of potassium iodide, 1 g of sodium nitrite, 30 ml of hydrochloric acid and 30 ml of distilled water. Ellram reagent consisted of 1 g of vanillin in 100 g of 40% sulfuric acid.

Iodine-potassium iodine solution (IKI) and periodic acid-Schiff reagent (PAS) were employed for detection of carbohydrates (23).

Lipids were identified by 0.1% aq nile blue, osmium tetroxide vapors, or Sudan dyes (Sudan IV and Sudan black) (23).

Proteins and enzymes were detected by a ninhydrin-Schiff reaction medium. Sections of *Ricinus* seeds which contain storage protein served as a positive control. A deamination solution, sodium nitrite and acetic acid or acetic anhydride in pyridine, served as a negative control (23).

Acid phosphatase activity was localized by incubating tissues in a modified Gomori reaction medium in which ammonium sulfide was used to precipitate lead sulfide at acid phosphatase reaction sites. Controls were either pretreated 5 min in boiling water to inactivate the enzyme or incubated in the medium lacking substrate (23).

Presence of lipase activity in tissue sections was identified with a sulfide reaction (23). In the positive test, lead sulfide was precipitated in incubated sections as a reaction product of lead nitrate and hydrogen sulfide water. Controls were incubated in the reaction medium lacking the substrate.

Cytochrome oxidase was identified in sections incubated in alpha naphthol and dimethyl paraphenylene diamine solution (23). Controls were pretreated 5 min in boiling water prior to incubation in the substrate.

The presence of callose was determined with the aniline blue technique and fluorescence microscopy (24).

RESULTS

CANNABINOID TESTS.—Color responses to cannabinoid-detecting reagents were compared individually against cannabinoid standards (Δ^9-THC, CBN, and CBD). CBD and Δ^9-THC were stained red with fast blue salt B, while CBN stained purple with this reagent. The Duquenois-Negm test reacted with Δ^9-THC and CBN to produce a purple color. Colors produced with the Beam test were red-brown for Δ^9-THC and CBN and purple for CBD. The Gibb reaction, similar for all cannabinoids, resulted in formation of yellow color upon addition of 2,6 dibromo-quinone-4-chlorimide and blue-black color upon addition of isopropyl-amine (table 1).

Laticifers and both capitate-sessile and capitate-stalked glandular trichomes on various plant organs were stained when treated with fast blue salt B, Duquenois-Negm, Beam, and Gibb reagents (table 1). These cells when treated with Duquenois-Negm stained red within 1 min of treatment and subsequently changed to blue-purple within 10 min indicating the presence of a cannabinoid content.

The response of various fresh tissues to cannabinoid indicators was examined for plant parts of different ages (table 2). Seed parts, roots, hypocotyl, and cotyledons of 7-day old seedlings contained no detectable quantities of cannabinoids. The epicotyl of seedlings and shoot apices of both staminate and pistillate adult plants showed a positive response for cannabinoids, as did vegetative leaves and floral organs. Freshly exuded latex derived from the petiole contained cannabinoids (table 2).

TABLE 1. Response of specific cellular structures in *Cannabis* to alkaloid and cannabinoid indicators.

Reagents:	Laticifer	Parenchyma	Capitate glandular trichomes
Cannabinoid indicator:			
Fast blue salt B[a]	+++	−	+++
Beam[b]	+++	−	+++
Duquenois-Negm[c]	+++	−	+
Gibb	+++	−	+++
Alkaloid indicator:			
Wagner (pH 4.5)	+++	−	+++
Dittmar (pH 1.5)	+++	−	+++
Ellram (pH 1.0)	+++	−	
Dragendorff (pH 5.5)	++	−	++
Chromic acid (pH 1.5)	+	−	−
Hager (pH 1.0)	+	−	−

+++ intense reaction, ++ moderate reaction, + weak reaction, − no reaction.

[a]A positive reaction is a red color.

[b]A positive reaction is a purple color.

[c]A positive reaction is represented by the color sequence, first pink and then blue-purple color.

Alcoholic extracts of various *Cannabis* tissues also were stained with cannabinoid detecting reagents, including fast blue salt B, Duquenois-Negm, and Gibb reagents. No color response occurred in extracts of roots. The Beam reagent did not react with any tissue extract.

Comparative tests of the cannabinoid reagents, fast blue salt B and Duquenois-Negm, on alkaloid standards (trigonelline, choline, hordenine) indicated that cannabinoid reagents do not produce a color reaction with alkaloids (table 2).

TABLE 2. Reaction of tissues, fresh or cryostat sections, of *Cannabis to* alkaloid and cannabinoid indicators.

Tissue or section	Alkaloid indicator		Cannabinoid indicator			
	Wagner	Dittmar	Fast blue salt B[a]	Duquenois-Negm	Beam	Gibb
Cannabinoid standards:						
Cannabinol	−	−	+	+	−	+
Δ^9-Tetrahydrocannabinol	−	−	+	+	−	+
Cannabidiol	−	−	+	+	+	+
Plant portions:						
Seed and fruit parts	−	−	−	−	−	−
Cotyledon, seedling	−	−	−	−	−	−
Hypocotyl, seedling	−	−	−	−	−	−
Root, seedling, adult	−	−	−	−	−	−
Epicotyl, seedling	+	+	+	+	+	+
Shoot apex, adult	+	+	+	+	+	+
Leaf, green mature	+	+	+	+	+	+
Leaf, senescing	+	+	+	+	+	+
Bract	+	+	+	+	+	+
Carpel	+	+	+	+	−	+
Stamen	−	−	+	+	−	+
Latex, exudate	+	+	+	+	−	+

[a]Fast blue salt B was applied to different samples both as an aqueous and methanolic solution.

Alkaloid tests.—The protoplasm in laticifers was precipitated by Ellram, Wagner, and Dittmar reagents (table 2). Ellram reagent produced a densely red cellular content in laticifers, while Dittmar and Wagner reagents formed red-brown precipitates in these cells. The positive response of laticifer contents to these reagents occurred in these cells located in various regions of both vegetative and flowering axes. No qualitative differences in color response were detected in laticifers of different ages or in laticifers from different plant organs. The initial response to these reagents occurred in laticifers of 5–10 day old seedlings; no response occurred in younger seedlings, nor in any cells of the embryo. Color reactions for alkaloids were formed in laticifers treated with other alkaloid indicators including chromic acid (pink-red color), Hager solution (red-brown color), and Dragendorff reagent (brown color).

The response of exuded latex to alkaloid reagents indicated the presence of alkaloids. Ellram indicator formed a dense precipitate in exuded latex. Wagner and Dittmar indicators produced a brown and olive-brown precipitate with exuded latex (table 1), which corresponded closely with the brown and olive-brown precipitates formed with trigonelline, choline hydrochloride and hordenine sulfate standards, respectively (table 3).

TABLE 3. Reaction of cannabinoid and alkaloid standards to cannabinoid and alkaloid indicators.

Substance	Cannabinoid indicator		Alkaloid indicator	
	Fast blue salt B	Duquenois-Negm	Wagner	Dittmar
Cannabinoid:				
Δ^9-THC	Pink-red	Blue-violet	NR[a]	NR
CBN	NR	Blue-violet	NR	NR
CBD	Light pink	Violet	NR	NR
Alkaloid:				
Trigonelline	NR	NR	Brown	Brown
Choline	NR	NR	Orange-brown	Orange-brown
Hordenine	NR	NR	Brown	Brown

[a]NR indicates no reaction.

Color reactions for alkaloids also occurred in capitate-sessile and capitate-stalked glandular trichomes with Wagner, Dittmar and Dragendorff indicators, whereas no reaction was detected in these structures with chromic acid and Hager indicators (table 1).

Comparative tests of the alkaloid reagents with cannabinoid standards (CBD, CBN and Δ^9-THC) yielded no color reaction. Therefore, the alkaloid and cannabinoid reagents were sufficiently selective to distinguish between these classes of compounds (table 3).

Histochemistry.—Starch was not detected in the protoplasm of laticifers or glandular trichomes. However, starch grains were detected in chloroplasts in the cytoplasm of parenchyma by both the IKI and PAS procedures. Walls of all cells were stained a positive purple-red, indicative of cellulose, with the PAS reagent. Callose, a normal constituent in phloem sieve tube members, was detected with aniline blue on sieve plates by a yellow fluorescence; callose was not detected in laticifers or disc cells of glandular trichomes (table 4).

Lipids were present in the protoplasm of laticifers as well as in the disc cells of the glandular trichomes and were stained black with osmium tetroxide and dark blue with nile blue (table 4). Latex exudate also contained lipid bodies which stained upon exposure to osmium tetroxide. However, lipids in the exudate did not respond to Sudan dyes (table 4).

A general protein reaction to the ninhydrin-Schiff reagent occurred as a faint pink color throughout the protoplasm of laticifers and disc cells of glandular trichomes. No large protein bodies were evident in these cells (table 4).

TABLE 4. Reaction of cellular structures in *Cannabis* to histochemical indicators.

Reagents	Laticifers	Parenchyma	Capitate glandular trichomes
Carbohydrates (protoplasm):			
I-KI	−[a]	+	−
PAS	−	+	−
Aniline blue	−	−	−
Lipids:			
Sudans III and Black	−	−	−
Nile blue	+	−	+
Osmium tetroxide	+	−	+
Storage proteins:			
Ninhydrin-Schiff	+	−	−
Dinitrofluorobenzene	−	−	−
Enzymes:			
Cytochrome oxidase	+	−	+
Lipase	+	−	+
Acid phosphatase	−	+	−

[a]Symbols indicate response upon comparison with control reactions described in Materials and Methods.

Lipase and cytochrome oxidase reaction products were detected throughout the laticifer protoplasm as an evenly distributed gray-brown and blue reaction product, respectively. Disc cells of glandular trichomes developed a red color in the lipase test and a dense blue color in the cytochrome oxidase test (table 4).

Acid phosphatase activity was localized within distinct bodies or granules in parenchyma cells, but was not detected in laticifers or disc cells of the glandular trichomes (table 4).

DISCUSSION

These analyses indicate that cannabinoids, alkaloids, and other specialized substances typically extracted from tissues by chemical procedures for subsequent analysis can be identified in situ in *Cannabis* tissues.

Fast blue salt B, frequently used in thin layer chromatography to identify cannabinoids (20) and reported to be relatively specific for cannabinoids (3, 25), readily stained contents of laticifers and capitate glandular trichomes. Other investigators (26, 27) also reported this reagent to stain contents of stalked glands on fresh tissues. Our application of other histochemical indicators, including Beam, Gibb, and Duquenois-Negm, further confirmed that these reagents can identify cannabinoids in fresh tissue as well as in prepared extracts of tissues.

Choline, hordenine, trigonelline, spermidines, cannabamines and other alkaloids have been reported from *Cannabis* (11, 14, 15–18), but previously these substances have not been associated with a particular cell or tissue. The present study

indicates that alkaloids accumulate in both laticifers and capitate glandular trichomes. The pattern of alkaloid distribution is similar to that for cannabinoids. Alkaloid reactions are distinct from cannabinoid responses both in laticifers and trichomes as shown by the differential histochemical reactions for these two classes of compounds.

Both quantitative and qualitative differences for cannabinoids and alkaloids, as reflected in the intensity and shade of color of the reactions associated with indicators, were detected in different cells and tissues of developing plants. Cannabinoids and alkaloids initially appear in detectable quantities in the first true leaves of seedlings and in various organs of the shoot. The seed, root, cotyledons, and hypocotyl of seedlings lack detectable quantities of these substances. De Faubert Maunder (20) also reported a similar distribution for cannabinoids in the seedling. Reports in the literature indicate that more sensitive techniques, such as gas-liquid chromatography, have identified cannabinoids in roots and seeds (4) or in the hypocotyl (28). The differences for cannabinoid distribution as observed from the present study may reflect changes in the accumulation or synthesis of cannabinoids in the protoplasm of laticifers and disc cells of glandular trichomes during their development. Laticifer differentiation and distribution coincided only in part with the accumulation and profile of cannabinoids, which first become identifiable in the epicotyl of 5-day seedlings.

Storage carbohydrates do not appear to accumulate in quantity in either the laticifers or glandular trichomes of *Cannabis*. Laticifer plastids (29, 30) and glands, in general, appear not to accumulate large reserves of starch, although the laticifer plastids of some plants, such as *Euphorbia* (32), do synthesize large quantities of this carbohydrate.

Storage proteins and free lipids, either acidic or phospholipid forms, apparently do not accumulate in histochemically detectable quantities in the cytoplasm of laticifers or glandular trichomes of *Cannabis*. The enzymes lipase and cytochrome oxidase were abundant in both structures. Neither enzyme has been associated as yet with particular functions in these structures, although it has been suggested that cytochrome oxidase in the laticifer of *Catharanthus* may be related to the differentiation of this cell (33).

Localization of cannabinoids, alkaloids, and other specialized substances with histochemical indicators in the laticifers and glandular trichomes of *Cannabis* indicate that these reagents can selectively identify specific substances within intact cells and tissues. Such techniques are useful, therefore, when employed in conjunction with other specific analytical procedures, for identifying extracted products derived from particular tissues or cells.

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