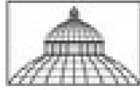
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CHEMOTAXONOMY OF CANNABIS I. CROSSBREEDING BETWEEN CANNABIS SATIVA AND C. RUDERALIS, WITH ANALYSIS OF CANNABINOID CONTENT

JOHN A. BEUTLER[1] AND ARA H. DER MARDEROSIAN

A controlled cross between *Cannabis sativa* L. and *C. ruderalis* Janisch. gave progeny intermediate in both cannabinoid content and morphology. The progeny fell into two distinct populations, those whose tetrahydrocannabinol (THC) content was closer to the *C. sativa* parent (greater than 60% of total cannabinoids) and those whose THC content was closer to the *C. ruderalis* parent (less than 40% of total cannabinoids). The lower THC group was twice as frequent as the other group. Earliness of flowering, number of flowers, and height characteristics were intermediate between the parents.

The taxonomy of *Cannabis* has assumed importance with the spread of marijuana as a drug of abuse, because most state and federal laws are written in terms of only one species, *Cannabis sativa* L. The supposed existence of more than one species in the genus has caused considerable legal difficulty. At least one article (Fullerton & Kurzman, 1974) has been written providing an arsenal of information that has proved successful in achieving acquitals in marijuana possession cases. Generally the Prosecution is forced to prove which species has been confiscated to determine if the law has actually been violated.

Much work has been done from a morphological point of view on *Cannabis* taxonomy. The history of the original botanical literature has been reviewed in detail (Emboden, 1974; Schultes et al., 1974). A large variety of freshly grown specimens have been examined, both for morphology (Small & Cronquist, 1976) and chemistry (Small et al., 1975; Fetterman et al., 1971). Three species have been delineated by Schultes et al. (1974), namely *C. sativa* L., *C. indica* Lam. and *C. ruderalis* Janisch. They have published a key for the differentiation of these three species based on height, branching, seed coat marbling, and seed attachment and its abscission layer.

Other investigators have held to the traditional monotypic concept (Small & Cronquist, 1976), holding that the wide variations in such characters and others are simply due to the inherent plasticity of the species. Most phytochemical studies have revealed no major differences in the content and quality of cannabinoids (other than the ratio of cannabidiol to tetrahydrocannabinol) that could serve to differentiate species. Other chemical markers of taxonomic significance have not been found.

Both chemical and morphological studies have been essentially static studies of a genetically dynamic organism, with little experimental attention given to the genetics on which the taxonomic characters are based.

In an attempt to clarify the taxonomic situation, and to elucidate the genetics of cannabinoid production, we have carried out preliminary cross-breeding experiments between *Cannabis sativa* L. and *Cannabis ruderalis* Janisch. under greenhouse conditions with the plants in reproductive isolation from other strains. Such a cross has been noted in the wild (R. E. Schultes, pers. comm., 1977) but was not included in the work of Small (1972) who intercrossed 38 strains of *Cannabis*, and found all strains to be interfertile.

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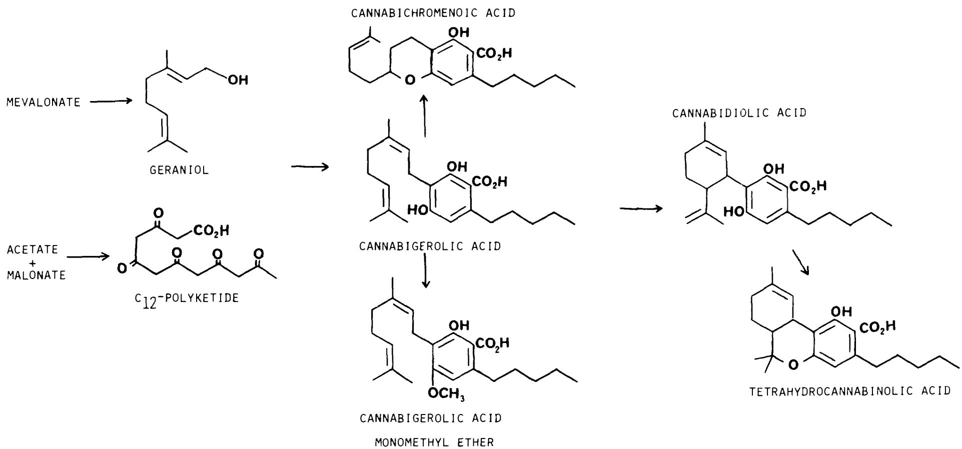

FIG. 1. Biosynthesis of cannabinoids (after Shoyama et al., 1974).

Recent advances in the understanding of cannabinoid biosynthesis have made possible more meaningful experiments with the plant. Shoyama et al. (1974) have elegantly elucidated the biosynthetic pathway to the cannabinoid acids from mevalonate, acetate, and malonate. These are well recognized as the true biosynthetic products of the plant (Fig. 1). Many of the minor cannabinoids isolated have come to be seen as degradation products of the natural cannabinoids on storage, exposure to light, curing, and other processing. Small & Cronquist's work (1976) and the work of Fetterman et al. in Mississippi (1971) have strengthened the view that THC-acid production is more dependent on the genome of the plant than on environmental factors.

Our breeding experiments were designed to take a closer look at the breeding behavior of the plant with respect to cannabinoid production, measured by gas chromatographic techniques, and backed up by mass spectral identification of the gas chromatographic peaks as cannabinoids.

MATERIALS AND METHODS

Seed samples.—JBC-2 was obtained from the Central Siberian Botanical Garden, Novosibirsk, USSR, and labelled *Cannabis ruderalis*. This is possibly identical to the *C. ruderalis* in Small & Beckstead (1973, p. 164, Table 5). It produced small (less than 2 ft) quick-flowering plants with low THC content (less than 0.2%). The seeds were marbled and dropped off at maturity, and the "fleshy caruncle-like growth at the base" was evident, though not obviously so.

JBC-3 was an alledgedly Mexican strain of *C. sativa* which reached 6–7 ft in height when given adequate root space, did not flower until it had reached this height, and contained a relatively high amount of THC (between 1.0 and 2.0%). Seeds were plain and indehiscent at maturity. Despite the lack of an authentic origin for this strain, it conforms to all published criteria for *C. sativa*.

Growing conditions.—Seeds were sown in rows in flats containing two parts of topsoil to one part peat moss. The greenhouse was kept between 70° and 80°F. When several inches tall, the seedlings were transplanted to large plastic tubs 16 inches in diameter and eight inches deep, a size that was found to minimally restrict growth. Smaller pots were not used because this produced smaller plants of *C. sativa*, disguising useful differences in growth and flowering behavior.

Plants were watered twice daily by an automatic capillary watering system, and positioned to receive full sun in the greenhouse.

The time of sowing was not strictly controlled with regard to day-length, however the quickness of flowering was constant within the plants of each strain used in the cross. The Mexican strain (JBC-3) has shown erratic flowering behavior when grown out of step with the normal seasonal variations in day-length. The *Cannabis ruderalis* (JBC-2) flowered promptly when sown at any point from November to May. Convenience dictated that plants of the two strains not be started at the same time, due to the difference in time required for flower production.

Plants were harvested when mature; i.e., for males, after the pollen had been substantially shed; and for females, after sufficient ripe seed had been collected for further breeding work. All samples analyzed were freeze-dried and stored in the dark in sealed plastic bags at room temperature.

Sampling protocols varied, especially for the Mexican strain, but all parts of the same plant contained identical cannabinoid profiles if they contained any at all. Roots and woody stems did not contain cannabinoids, and leaves contained less than the flowering tops. All *C. ruderalis* plants were analyzed as the whole freeze-dried plant. Where several very small individuals were concerned, several of the same sex and age were combined into the same sample.

Gas chromatography.—The procedure used was based on that of Lerner (1969). One g of plant material was shaken with 40 ml of chloroform at room temperature for one hour in a stoppered Erlenmeyer flask. The solution was filtered, the plant material rinsed with a few ml of chloroform, and the combined solution evaporated under reduced pressure. This residue was taken up first in 2.0 ml of chloroform, and then 2.0 ml of the internal standard solution (5.0 mg/ml androstene-3,17-dione in methanol) was added. This solution was stored in screw-capped vials with teflon inserts in the caps at freezer temperatures. Caps without teflon inserts were leached by both methanol and chloroform. Dioctyl phthalate from the cap leachates was found to have a relative retention time of 0.45, which without mass spectral analysis could be confused with Δ-8-THC.

Sample solutions were injected on a 3% OV-17 on 100/120 Gas Chrom Q column (Supelco, Inc.) 6 ft long, 4 mm i.d., installed in an HCl Scientific gas chromatograph, or Varian model 3700 gas chromatograph, both with flame ionization detection. Injector temperature was 255°C, column temperature isothermal at 220°C, carrier gas flow (He) 120 ml/min. Typically one microliter of sample solution was injected.

Peak areas were determined by multiplying height times half-height width. This was compared to the area of the internal standard peak and corrected for the amount of plant material taken, along with the response factor of each compound.

It should be noted that this procedure does not differentiate between cannabidiol (CBD) and cannabichromene (CBC) (RRT-0.36) (Turner et al., 1975). To ascertain the identity of this peak, gas chromatography at 180°C on a 3 % OV-101 column was performed. The results of this separation were used to get the ratio of CBD to CBC in the combined peak on OV-17.

RESULTS AND DISCUSSION

Chemistry.—The results of glc analysis are presented in Figure 2 as total percent cannabinoids on a dry weight basis versus Δ^9-THC as percent of total cannabinoids (CBD, CBC, Δ^9-THC). This method of expressing cannabinoid content gives a comparison of overall production of cannabinoids, as well as an indication of how much of this cannabinoid production is Δ^9-THC. The percentage of CBC

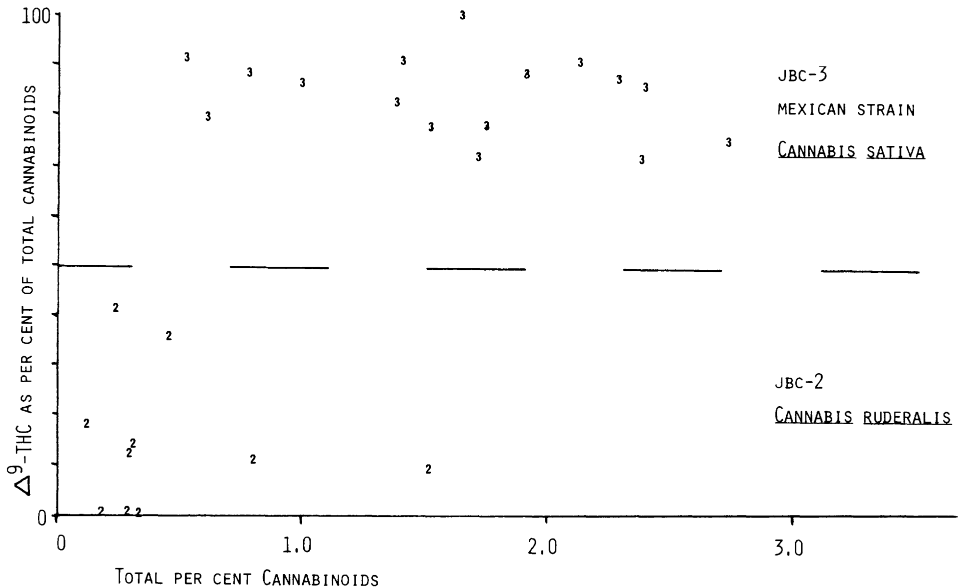

FIG. 2. Cannabinoid profiles for parent strains.

was found to be relatively constant within the experiment, from 3 to 9 % of total cannabinoids in the progeny. Parent strains also had CBC as a non-variable cannabinoid at about the same concentration. Thus CBD and THC are the important variables in the cross. The total percent cannabinoids varies rather widely due to variation in sampling technique, but the pattern of cannabinoid production can be delimited into two groups for the cross.

The parent strain JBC-3 (*C. sativa*) is seen to contain at least 70% THC of total cannabinoids. The sex appears to make no difference in cannabinoid total or pattern in this strain.

The other parent, JBC-2 (*C. ruderalis*), contains less than 40% THC of total cannabinoids, with most plants falling below the 20% level. The total percent cannabinoids was lower for *C. ruderalis* ($\bar{x}$ = 0.45%) than for *C. sativa* ($\bar{x}$ = 1.61%), though one "sport" of JBC-2, which was kept for several weeks under high humidity conditions (an inverted paper chromatography tank), showed 1.54% total cannabinoids on a whole plant sample. The sport's cannabinoid pattern was similar to others of its strain, however. The variation in sampling methods makes statistical comparison of the means impossible.

The offspring of the cross were from the female *C. sativa*. The female *C. ruderalis* plants were weak, and though they set seed, the number was small and of poor viability. The seedlings either did not germinate or did not survive much beyond germination. The fact that the reverse cross produced vigorous offspring, and that cannabinoid production does not seem to be linked to sex [in some strains it is linked, according to Small et al. (1975)], means that failure to generate offspring was due to generally weak plants and not to a lack of interfertility.

The progeny of the cross fell into two groups (Fig. 3), separated by a large gap in THC percentage. The total cannabinoid percent of dry weight ranged between 0.36% and 1.30%, with a mean of 0.72% for each group taken separately. All but one value was less than 1.0%. This high value was for the top of a plant selected for a voucher herbarium specimen, not for a whole plant. This underlines the importance of sampling procedure, which will be discussed in detail below.

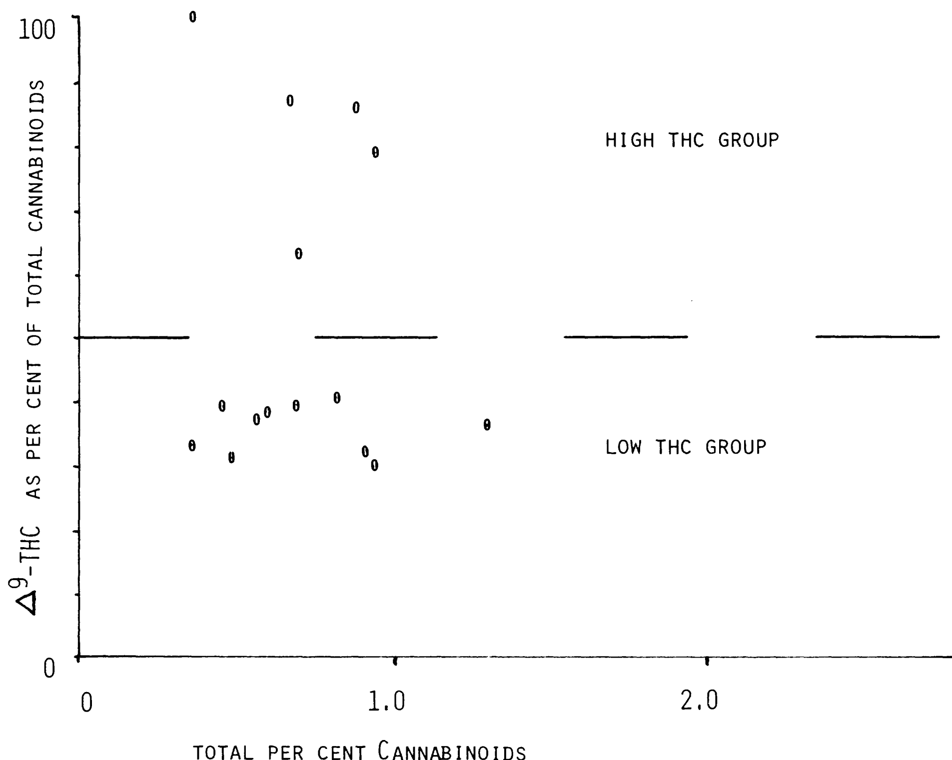

FIG. 3. Cannabinoid profiles for progeny (*Cannabis sativa* × *ruderalis*).

One-third of the progeny (n = 5, 3 females, 2 males) had THC percentages over 60% of total cannabinoids. The other two-thirds of the progeny (n = 10, 6 females, 3 males, one died before flowering) had THC percentages between 30 and 40% of total cannabinoids.

This rather conspicuous chemical dimorphism has no easy genetic explanation. It does not seem to be a case of one enzyme being responsible for the cyclization of cannabidiolic acid to THCA. And if it were that simple, no such "cannabidiolic acid cyclase" has ever been isolated from *Cannabis* of any species.

Morphology.—In habit, the progeny resembled both parents to some extent. They did not show the dimorphism seen in cannabinoid content. Plants were between one and two ft tall, grew vigorously on germination, and flowered after putting out several pairs of true leaves. They continued their growth after initial flowering. The plants were self-crossed to produce an F_2 generation of seed, which was saved for further plantings.

Some of the F_1 seeds were slower to germinate than their *C. sativa* parent. Four seeds remained in the flat for four months before germination. These plants were analyzed when mature, and their cannabinoid profiles fell within either of the two groups of progeny. Delay in germination is characteristic of plants growing outside of cultivation, either in a wild or weedy state. This trait was presumably transmitted from the *C. ruderalis* parent, although it was not observed in the seeds sown.

A voucher specimen of one of the female progeny has been deposited in the Library of Economic Botany of the Harvard Botanical Museum, as specimen no. *35896*.

Such genetic inhomogeneity has not been reported before in strains of *Cannabis*. The nearest case is that of South African *Cannabis* reported by Boucher et al. (1977), in which they found the presence of two chemotypes varying in amounts of THCA and the C_3 THCA homolog tetrahydrocannabivarolic acid.

Small et al.'s breeding experiments (1975) reported only that progeny obtained in crosses were "intermediate" in cannabinoid content. The aim of chemotaxonomy is to demonstrate that the variation in chemistry between two populations has a genetic basis dependent on the presence or absence of enzymes involved with the biosynthesis of the compounds in question. Such information cannot be gotten from studies where populations are pooled or samples are not uniform. In order to do more meaningful work on individual plant cannabinoid content, it is necessary to find sampling methods which are not subject to wide variations, and which are precisely definable and reproducible.

Sampling.—We propose that the unit of sampling for female *Cannabis* plants be the mature bract, at the point where the seed is no longer green and is easily separable from the bract. For the male plant, perhaps the pollen can be used. It has been found in this and other studies (Paris et al., 1975) to contain the same proportions of cannabinoids as the rest of the plant. The male flower is less reproducible for sampling, since it is continuously shedding pollen at maturity, thereby changing the relative proportions of pollen and flower tissue.

This involves working with rather small amounts, since a mature bract when fresh weighs only about 2 mg. Pollen is most easily collected in milligram quantities. But the technique makes possible reproducible multiple samplings from each plant.

As an example of the technique, single fresh bracts were put into a 4 ml screw-capped vial with 0.1 ml of chloroform overnight at room temperaature. In the morning 0.1 ml more of chloroform was added, along with 20 microliters of a methanolic solution of the internal standard, androstene-dione at a concentration of 1 mg/ml. The sample solution was injected into the glc as with the whole plant assay.

Results for this type of assay done with single bracts from the progeny of our cross indicate that the method gives comparable results to the whole plant assay for the percentage of cannabinoids. The percent total of cannabinoids is higher than for the whole plant, as would be expected.

Assuming that the bracts are relatively uniform within the same plant, how does this sampling method compare with other methods, such as the whole plant, or the manicured plant? The whole plant analysis involves parts (seeds, roots, and woody stems) which have little if any cannabinoid content. The relative proportion of these parts to leaf and bract varies widely with cultural conditions; e.g., if the plant is grown under dry conditions, the root will become a very short, stocky structure, with few branchings; but if it is grown with adequate moisture it will be longer, thinner, and branched with many fine rootlets that are hard to collect completely. The amount of stem and vegetative growth varies also with plant nutrition.

These factors induce error in the measurement of the plant's actual genetic capacity to produce cannabinoids, while having nothing to do with cannabinoid production. Cannabinoids and their precursors are formed entirely in the leafy portions of the plant (Crombie & Crombie, 1975).

The bract, assuming the flower is pollinated, is the most stable part of the plant both for morphology and for sampling.

The number of flowers produced on a plant will influence the relative levels between the bract sampling method and the whole plant assay. We have observed within the progeny of the cross a variation in the number of flowers produced

per plant. This is reasonable because *Cannabis* has been bred for higher seed and flower production. The Mexican parent produces seed quite heavily, while the presumably wild *C. ruderalis* produces relatively few seeds, concentrating its energies into the production of fewer, more viable seeds. This difference in characters shows its genetic base by its mixed transmission into the offspring of the cross.

An aim of taxonomy is to find independent, definable characters, based where possible on a single gene system. The two traits of cannabinoid production and number of flowers per plant are mixed using a whole or manicured plant assay, but not in a bract assay, because the proportion of dry weight due to high cannabinoid bracts is greater for many-seeded varieties of *Cannabis* than it is for those producing relatively few seeds.

The cellular locus of cannabinoid biosynthesis is not known at present. It has been found that it does not occur exclusively in the glandular hairs, that at least the number of glandular hairs on the bract surface does not correlate positively with cannabinoid content (Turner et al., 1977). Thus, it is likely that the site of cannabinoid biosynthesis is spread throughout the cells of the epidermis of the bracts and leaves, as well as in the cells of the glandular hairs.

If the site of cannabinoid biosynthesis were the glandular hairs, the most reproducible assay for cannabinoid production would involve the isolation of the hairs. This has been done for a member of the Lamiaceae (Croteau, 1977). Number of glandular hairs would then be a second character not controlled by cannabinoid biosynthetic ability. Since the hairs are not the specific locus of biosynthesis, the most specific assay should choose a definable unit of epidermis, and this unit of epidermis is the bract. The leaves vary in size and would be hard to define precisely for sampling. The bract is less variable, if the age on sampling (maturity of the seed) is specified.

This technique is useful for herbarium specimens, with the advantage of disturbing valuable voucher specimens less than other methods.

We would suggest that this method be adopted in further studies of *Cannabis* breeding and cannabinoid biosynthesis. We intend to use it in the extension of the present experiment using botanically validated stocks of *Cannabis sativa, C. indica,* and *C. ruderalis* under more tightly controlled breeding conditions and using standard plant breeding formats, with more detailed attention to individual crosses. The F_2 generation of the present cross will be followed to see if its cannabinoid content is different from the F_1.

Other compounds (terpenes, flavonoids) will be examined for their utility as biochemical "markers."

SUMMARY

The results of this experiment strengthen the evidence that the cannabinoid content of *Cannabis* species is genetically controlled, and would suggest a search for the enzyme or enzyme system responsible for "cannabidiolic acid cyclase" activity. Feeding experiments with radiolabelled CBDA and tissue homogenate fractions could result in the isolation of such an entity. This might in turn lead to the elucidation of a specific enzyme inhibitor which would block high potency strains from producing THC.

The experiments also indicate that *C. sativa* and *C. ruderalis* are interfertile under greenhouse conditions, and that the cannabinoid profiles, while differing in the amount of cannabinoid predominating, show no novel cannabinoids. This is one piece of evidence supporting the view that *Cannabis* is a monotypic genus. More studies are indicated to explore this further, particularly to note if other

specific biochemically significant chemotaxonomic markers (e.g., essential oils, flavonoids) can be located which might lend validity to the polytypic concept.

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