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RESEARCH ARTICLE

Expansion of female sex organs in response to prolonged virginity in *Cannabis sativa* (marijuana)

Ernest Small · Steve G. U. Naraine

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Abstract Female flowers of *Cannabis sativa* in wild-growing populations and in hemp plantations are almost always well supplied with pollen. The style-stigma portion of the pistils of such plants was found to average only about 3 mm in length and to invariably be two-branched. By contrast, "buds" (congested female inflorescences), the standard form of marijuana now produced in the illicit and medicinal marijuana sectors, are protected against pollen. This report documents that in the absence of pollen, the style-stigma parts of virgin pistils expand notably, average over 8 mm in length, and tend to develop more than two branches and to increase in girth. From an evolutionary viewpoint, this expansion of pollen-receptive tissue is an apparent adaptation for increasing the probability of fertilizing the females when males are extremely scarce. From a practical viewpoint, the expanded presence of stigma tissues may be both advantageous and disadvantageous. The high-THC secretory gland heads of *Cannabis* tend to fall away from marijuana buds, significantly decreasing pharmacological potency, but many gland heads become stuck to the receptive papillae of the stigmas, reducing the loss. Although stigmas constitute a small proportion of marijuana, their distinctive chemistry could have health effects.

Keywords *Cannabis sativa* · Hemp · Marijuana · Pollination · Stigmas · Styles

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Introduction

Wild plants of *Cannabis sativa* L. are among the small minority [4 % according to Yampolsky and Yampolsky (1922), 6 % according to Renner and Ricklefs (1995), or some undetermined higher figure according to Bawa (1980)], of flowering plants with male reproductive organs (stamens) and female reproductive organs (carpels) confined to separate plants (i.e. the populations are dioecious, with unisexual flowers, those on a given plant either entirely male or entirely female). Staminate plants, with male flowers only, are routinely called males, and pistillate (carpellate) plants, with female flowers only, are called females, and this standard terminology is followed here. Numerous monoecious cultivars have been bred, these bearing both male and female flowers. Dioecious plants are thought to be mostly or entirely cross-pollinated, while monoecious plants are selfing to

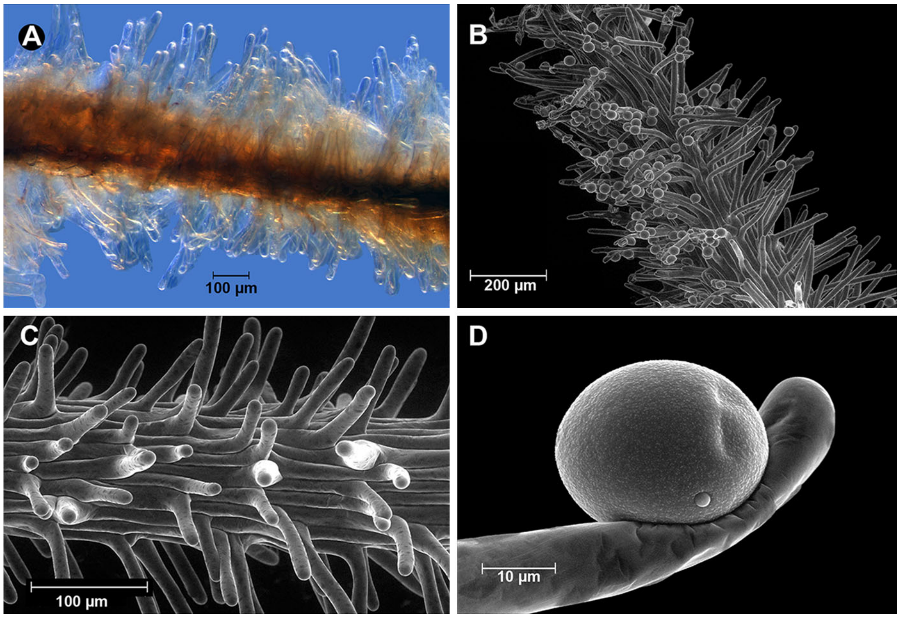

Fig. 1 Close-up photos of stigmas of *Cannabis sativa*. **A** Light photomicrograph of a portion of a stigma. **B–D** Scanning electron photomicrographs. **B** Numerous pollen grains trapped on papillae of stigma. **C** Papillae of stigma. **D** Pollen grain on a stigmatic papilla

some degree [20–25 % according to Finta-Korpel'ová and Berenji (2007)].

Cannabis is wind-pollinated, male plants producing prodigious amounts of pollen: a single flower can produce about 350,000 pollen grains (Faegri et al. 1989), and there are often hundreds of flowers on larger plants. *Cannabis* pollen can be transported long distances—over 300 km (Clarke 1977). Cabezudo et al. (1997) noted that *C. sativa* pollen, apparently from marijuana cultivated in North Africa, was transported by wind currents to southwestern Europe. Indeed, the pollen is so prevalent that it is a significant allergen for some people (Lindemayr and Jager 1980; Tanaka et al. 1998; Singh and Kumar 2003). Stokes et al. (2000) recorded that in August in the Midwestern United States (where cultivation of hemp is not permitted, but weedy hemp is common) hemp pollen represented up to 36 % of total airborne pollen counts! Because the pollen of *Cannabis* spreads remarkably, an isolation distance of about 5 km is usually recommended for generating pure-bred seed, exceeding the distance for virtually every other crop (Small and Antle 2003). Because of widespread clandestine cultivation, the pollen can be found, at least in small concentrations, over most of the planet.

The stigmas of *Cannabis* are of the so-called "dry" type, a category characterized as having a minimal surface secretion or simply with a thin proteinaceous covering (Heslop-Harrison 1977; Heslop-Harrison and Heslop-Harrison 1985). They are densely covered with receptive papillae to which pollen grains stick (Fig. 1). Physico-chemical events occurring between pollen grains and the papillae of several species with dry stigmas have been examined by Elleman et al. (1992).

Female narcotic plants of *C. sativa* have as much as 20 times the concentration of THC (tetrahydrocannabinol, the chief intoxicant of *Cannabis*) as corresponding males (Clarke and Merlin 2013). Moreover, when females are grown in the absence of males, so that seeds are not produced (seeds contain virtually no THC), they develop higher concentrations of THC in the reproductive parts. Today, most marijuana is made up of so-called "buds," which are seedless ("sinsemilla") compact female inflorescences, as shown in Fig. 2.

The base of the pistil of a female flower of *C. sativa* is enveloped by a protective "perigonal bract" (note Fig. 3A), which grows to cover the achene if the single ovule is fertilized. The widely accepted description of female flowers of *C. sativa* is that they consist of a superior, unilocular (one-celled) ovary and a short apical style with two long filiform (thread-like) stigmas or stigmatic branches. However, as became

Fig. 2 "Buds" (unfertilized, congested pistillate inflorescences) of marijuana. *Left* terminal branch of the strain Purple Erkle, the *whitish appearance* of the stigmas of the clustered female flowers indicating that many are receptive to being fertilized by pollen. *Right* terminal branch of the strain Bullrider, the *brownish appearance* of the stigmas indicating that the stigmas are overmature for pollen reception. The transition from white to brownish stigmas is commonly used as an index of the ideal stage of maturity for harvesting the female inflorescences for use as marijuana buds. Photos by Psychonaught, released into the public domain. (Color figure online)

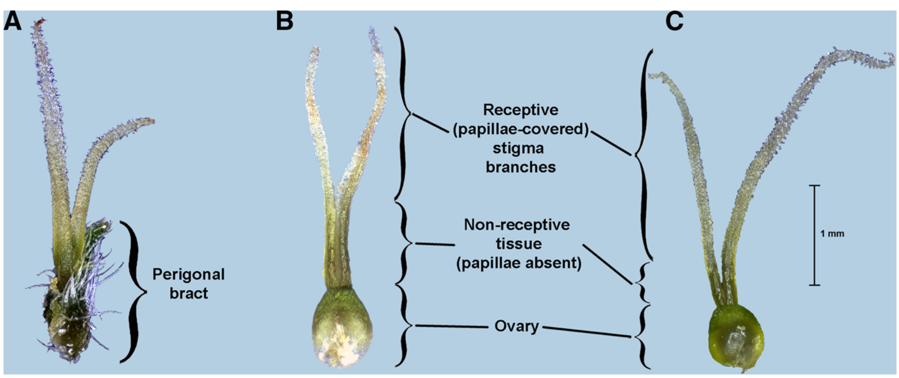

Fig. 3 Young pistils of cultivars of *Cannabis sativa* with two stigma branches. **A** 'Yvonne', showing two stigma branches emerging from a young, protective perigonal bract, which is covered with glandular and cystolithic trichomes. **B** 'Canma', showing a pistil with the two pollen-receptive (covered with papillae) stigma branches emerging from a common, non-receptive base ("style") attached to the ovary. **C** 'Canda', structurally like **B**, but the two style/stigma branches emerge independently from the ovary

evident during the course of this study, the distinction of "style" and "stigma" in *Cannabis* is arbitrary. By definition, a "style" is (non-receptive) tissue joining the ovary to the stigma(s) of a flower. As shown in Fig. 3, whether *Cannabis* has one style or two depends on whether the stigmatic branches emerge in common from the same non-receptive tissue (as in Fig. 3B) or from separated non-receptive tissue (Fig. 3c). Moreover, what appears to be non-receptive basal tissue in young pistils (shown in Fig. 3B, C) with increasing maturity often develops receptive papillae (see Figs. 4, 6, 8), obscuring or obliterating the distinction between stigma and style. Whether the pistil has two stigmas or just one also respectively depends on the presence or absence of material with receptive papillae between the stigmatic branches.

Purpose of this study

This report compares stigma morphology in plants that have been (1) fertilized by pollen with (2) plants that have been protected from fertilization, in order to document and explain the evolutionary and practical significance of the different morphologies developed.

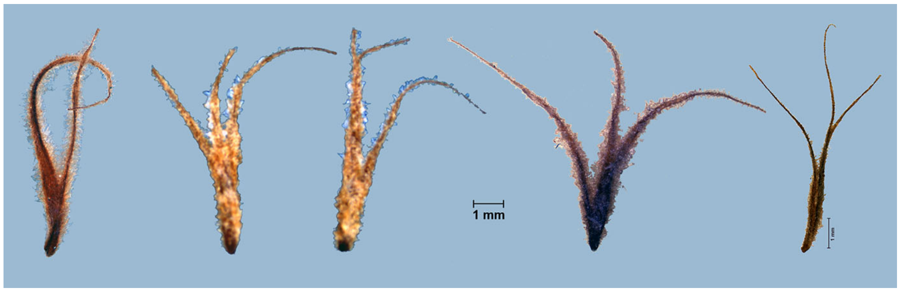

Fig. 4 A two-branched stigma (*far left*) and four three-branched stigmas. Based on a dried "bud" of the medicinal marijuana strain Dinachem

Materials and methods

Material examined is identified in Table 1. Unfertilized plants were represented by samples of marijuana of ten medicinal marijuana strains legally marketed in Canada to licensed patients. Fertilized plants were represented by herbarium (DAO) collections of female, seeded plants which possessed pistils with non-shrivelled style-stigma portions (in *Cannabis*, the pistils eventually become more or less caducous, either falling off the pistils with achene development or remaining as remnants). Ten plants of each of three classes of collections were examined: marijuana land races (un-named selections grown in the middle part of the twentieth century for narcotic purposes), ruderal collections (known to be low in THC), and hemp cultivars (also known to be low in THC).The marijuana strains are referable to *C. sativa* subsp. *indica* (Lam.) E. Small et Cronq. var. *indica*, according to Small and Cronquist (1976). The ruderal strains belong to *C. sativa* subsp. *sativa* var. *spontanea* Vavilov, and the hemp cultivars to *C. sativa* subsp. *sativa* var. *sativa.* In all cases, ten representative pistils were sampled. Total length of all style-stigma branches was measured (i.e., "style" base + each of the two major branches present + any other branches present). Scanning electron photomicrographs were taken with an AMR 1000 SEM; Light photomicrographs were taken using a Leica MZ12.5 dissecting microscope equipped with a Leica DFC420 camera and associated with software to combine multi-level focussed images. Fresh material is illustrated in Figs. 1B–D, 2 and 3, dried or rehydrated material in remaining photos. The weight of ten style-stigmas from each of the ten medicinal marijuana strains was measured on a Metler AG25 balance (d = 0.01 mg).

Results

As shown in Table 1, mean total style-stigma length varied from about 14 to 20 mm in the ten unfertilized collections examined (mean 15.7), and from about 5 to 11 mm in the 30 fertilized collections (mean 6.0). Note that this "total length" (which includes all stigma branches) in the preceding sense should not be confused with the smaller length of the stigma and style from the top of the ovary to the average farthest point of the two stigmatic branches (see Fig. 5). About one in five stigmas of unfertilized samples was found to possess more than one stigma branch—most frequently one of the two major branches branching into two (Fig. 4). Stigmas of fertilized samples always had only two stigma branches. Mean dimensions of fertilized and unfertilized samples are shown in Fig. 5. Virgin stigmas on average became almost three times as long as stigmas of fertilized material.

Although diameter or circumference of stigmas was not recorded, it was obvious that unfertilized stigmas became fatter. Only one herbarium collection of wild-collected material was located which obviously had remained unfertilized for a long period, and the girth of its stigmas was comparatively massive (Fig. 6).

Discussion

Stigma size can be influenced by environmental conditions. For example, Buszard and Schwabe (1995) showed that virgin stigmas in the flowers of domestic apple (*Malus domestica*) are smaller in trees that were stressed the previous year by bearing a heavy crop of fruit. However, the development of most floral parts is quite deterministic in most plants. Close to 90 %

Table 1 Total size of style-stigma segments in dried collections of unfertilized and fertilized *Cannabis sativa*

Group and representative	Mean length (mm)[1]	SD	% Pistils with >2 stigma branches	Mean weight (mg)[2]
Unfertilized, advanced medicinal strains (buds)				
Canadian standard	20.05	1.491	50	0.14
Chocolope	14.90	1.700	50	0.23
Devil Fruit	18.99	2.431	20	0.21
Dinachem	14.63	2.402	30	0.21
G13	14.38	0.827	0	0.16
LA Confidential	12.15	1.379	0	0.14
Nordle	16.25	1.167	10	0.25
Sour Diesel	14.35	1.517	0	0.20
SnickLeFritz	17.59	2.669	20	0.26
Vanilla Kush	14.65	2.343	10	0.15
Grand means	15.695		19	0.195
Fertilized, marijuana land races				
E. Small 289, Mexico	6.63	1.271	0	
E. Small 164, India	4.73	0.492	0	
E. Small 397, South Africa	9.14	1.660	0	
R.E. Schultes et al. 52, Afghanistan	6.27	0.576	0	
R.E. Schultes et al. in 1972, Morocco	6.11	0.500	0	
R.E. Schultes et al. in 1972, Mexico	6.36	1.153	0	
R.E. Schultes et al. in 1972, South Africa	6.01	1.055	0	
R.E. Schultes et al. in 1972, Sierra Leone	5.47	0.450	0	
R.E. Schultes et al. in 1972, Pakistan	5.14	0.961	0	
R.E. Schultes et al. 53, Afghanistan	6.95	1.490	0	
Grand means	6.281		0	
Fertilized, ruderal collections				
G.A. Mulligan 626, Quebec	7.29	1.707	0	
E.G. Anderson 1214, Quebec	7.51	1.274	0	
B. Dawson in 1960, Ontario	10.6	2.265	0	
J.E. Cruise 1018, Ontario	5.55	0.902	0	
E. Vitek in 1990, Austria	7.15	0.647	0	
E. Small 349, Germany	4.89	1.283	0	
E. Small 84, Czechoslovakia	4.68	0.802	0	
E. Small 213, USSR	5.29	0.992	0	
W.H. Minshall in 1938, Ontario	6.08	1.004	0	
R.E. Muir in 1960, Ontario	5.29	0.854	0	
Grand means	6.433		0	
Fertilized, industrial hemp cultivars				
'Uniko BF2', E. Small 134	6.27	0.992	0	
'Biatobrzeski', E. Small 138	5.24	0.824	0	
'Szeged 9', E. Small 87	4.88	1.012	0	
'Pinnatofidofilla', E. Small 108	5.31	1.279	0	
'Fibrimon 24', E. Small 248	4.76	1.311	0	
'Fibrimon 21', E. Small 102	6.96	1.516	0	
'Tiborszallasi', E. Small 88	5.21	0.877	0	

Table 1 continued

Group and representative	Mean length (mm)[1]	SD	% Pistils with >2 stigma branches	Mean weight (mg)[2]
'Krasnodarskij 35', E. Small 142	4.94	0.612	0	
'Georgina', E. Small on Sept. 13, 2013	4.36	0.436	0	
'Petera', E. Small on Sept. 13, 2013	6.28	0.950	0	
Grand means	5.421		0	

All fertilized samples are from herbarium vouchers at DAO, cited in the first column

[1] As explained in the text, the length indicated here includes the total lengths of the "style" [basal portion(s) above the ovary, whether or not covered by receptive papillae], the main stigmatic branches (usually two) above the "style," and any secondary branches of the main branches

[2] Based on ten style-stigmas

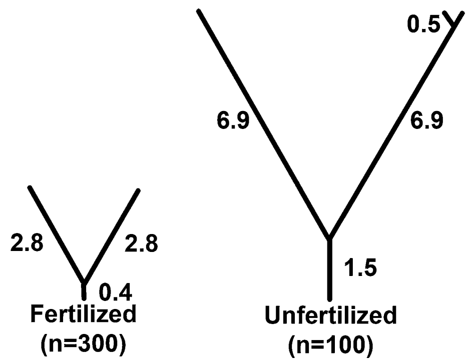

Fig. 5 Mean dimensions (in mm) of stigmas of fertilized plants (*left figure* based on ten stigmas of each of 30 herbarium collections) and of unfertilized plants (*right figure* based on ten stigmas of each of 10 medicinal marijuana samples)

of flowering plants have zoophilous flowers (Ollerton et al. 2011), and the flowers of these animal-pollinated species necessarily have relatively fixed proportions, since their architecture must be compatible with the corresponding dimensions of their pollinators (Miglia and Freeman 1996). By contrast, wind-pollinated species are not so constrained, and the flexibility of stigma size response to lack of fertilization, reported here for *C. sativa*, has been documented for several other anemophilous plants. The phenomenon of unfertilized stigmas continuing to grow in exclusively female flowers has been recorded for spinach (*Spinacia oleracea* L.) and several other species of the Chenopodiaceae (Miglia and Freeman 1996), and for *Juglans mandshurica* Maxim. (Bai et al. 2006). The female flowers of maize (*Zea mays* L.) feature a pair of thread-like styles each terminated by a stigma (in maize, a style + stigma is termed a "silk"). The styles of unfertilized maize flowers continue to elongate for ten

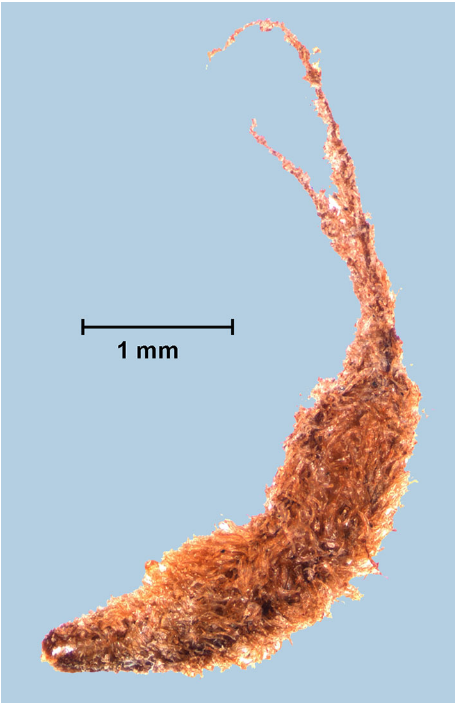

Fig. 6 Stigma of an unfertilized plant (herbarium collection, G.E. Woodley in 1961 from Upper Canada Village, Ontario). Note the massively developed basal portion

or more days, and indeed excessively elongated silks are widely employed by corn growers as a sign that the crop has been inadequately fertilized. Whether the stigmas or the styles or both (as in *C. sativa*) enlarge, the effect is the same: making it more likely that the ovules will encounter pollen and be fertilized.

Miglia and Freeman (1996) observed that several wind-pollinated dioecious species of the Chenopodiaceae which have the ability to develop larger stigmas when pollen is very limited, occur in arid or semiarid regions. They explained this correlation on the basis that plant distribution tends to be quite heterogeneous in such environments, hence pollen is quite variably available, so stigmas with flexible growth response can be especially adaptive. As noted in the introduction, today *Cannabis* pollen is extensively present in the atmosphere, but this is the result of humans distributing the species extremely widely. *Cannabis sativa* originated in semi-arid regions of central Asia (Clarke and Merlin 2013), and it seems probable that the ability of its stigmas to expand in pollen-poor environments served it well in past times when pollen was much less available.

Until recently, the genus *Cannabis* and the genus *Humulus* (best known for *H. lupulus* L., the hop plant) were considered to constitute the Cannabaceae (Small 1978). Recent phylogenetic studies have considerably expanded the family (Sytsma et al. 2002; Yang et al. 2013), but it is clear that *Cannabis* and *Humulus* are well separated from the eight or so other genera that are now included in the Cannabaceae, and constitute a coherent phylad. Commercial hop inflorescences (i.e., the cones, strobili or "hops," used as a brewing ingredient) are mostly produced on virgin female plants in the absence of pollen, and so are quite homologous with marijuana "buds." About 200 vouchers of cones of hop cultivars (most listed in Small (1981)) are preserved at DAO; none of these possessed stigmas, indicating that the stigmas are much less persistent than in *Cannabis*. Examination of an unfertilized female clone revealed that in the absence of pollen the stigmas grow to about 8 mm in length (very comparable to the data reported here for unfertilized stigmas of *Cannabis*), and are then caducous. Photos of a young hop stigma and a young strobilus with well-grown, long stigmas are shown in Fig. 7. It appears, therefore, that *H. lupulus* is, like *C. sativa*, another example of a dioecious, wind-pollinated plant in which the stigmas expand considerably in pollen-poor circumstances. Unlike *Cannabis*, *H. lupulus* is a perennial which spreads aggressively by rhizomes. Isolated female clones are quite common (both in the wild and in cultivation), and so the species can particularly benefit from an adaptation like expanding stigmas which increase the probability of generating seeds.

We have not found a parallel example to our discovery that unfertilized stigma branches in *C. sativa* themselves tend to branch. Mutants with more than the normal number of stigma branches have been recorded in several species, including *Pennisetum glaucum* (L.) R. Br. (Ratnaswamy 1954), *Crocus sativus* L. (Khan 2007) and *Oryza sativa* L. (Singh et al. 2012), but mutation is not the cause of greater branching in *C. sativa*. In *C. sativa* the development of extra stigma branches occurs routinely and only when the stigmas remain unfertilized and grow large. For the most part, when branching occurs it does so by splitting of one of the two normal stigma branches. Pistils of *C. sativa* usually develop two stigmatic branches by a dichotomy of the basal "style," and it appears that this tendency to dichotomize is carried over when a given branch becomes abnormally large. The effect of stigma branching parallels the effects of stigma girth increase and stigma length increase: it increases the likelihood that the ovules will encounter pollen and be fertilized.

Upon reaching stigmas, pollen sends gametes through the stigma and style by means of a pollen tube, to fertilize ovules some distance away. The longer this distance is, the more expenditure of resources is required, and so the gamete delivery distance is a critical adaptation (Cruden and Lyon 1985; Cruden 2009). While a shorter distance saves resources, a longer distance may serve to filter out weaker gametes (Travers and Shea 2001; Ramesha et al. 2011). In *C. sativa*, only one pollen grain is indispensable to effect fecundation of the single-ovuled pistil, reflective of the general tendency of wind-pollinated flowers to have few ovules (Friedman and Barrett 2009). A pollen grain could land at any point of the elongated stigma—either very close to the ovule or quite distant from it. Unfertilized pistils of *C. sativa* can develop inflated stigma bases (Fig. 6), which would increase the probability of a pollen grain landing close to the ovule, or they can develop extra-long, branched stigmas (Fig. 4) which would increase the probability of a pollen grain landing distant from the ovule, and presumably both morphologies are adaptive.

Female plants of dioecious species have three potential strategies for dealing with pollen limitation: (1) increase stigmatic surface area; (2) extend their flowering period (or the longevity of the flowers); (3) reverse sex and produce pollen (Quinn et al. 2000). *Cannabis sativa* primarily employs the first two strategies, but sex reversal in response to various stresses has been documented, some strains

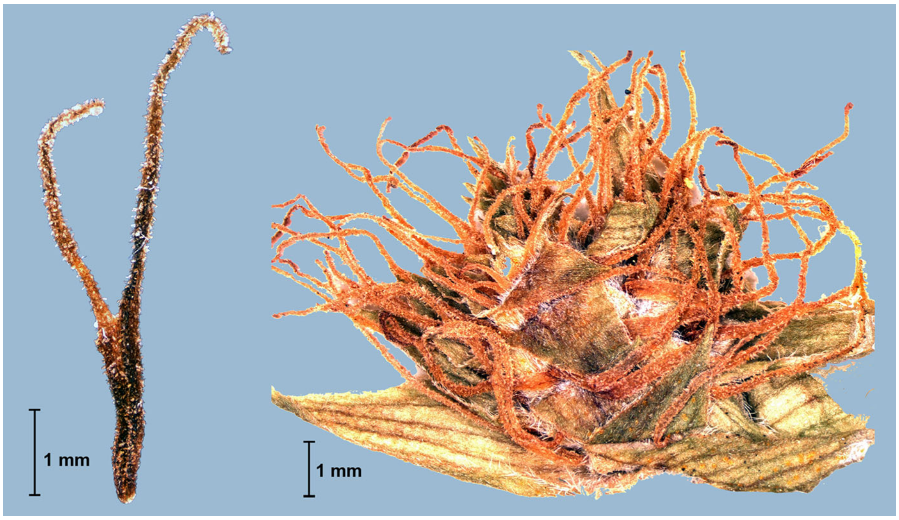

Fig. 7 Stigmas of hop (*Humulus lupulus*), a close relative of *Cannabis* (based on herbarium collections). *Left* a relatively short stigma from a young pistil (from V. Harms 3915, DAO). *Right* a young unfertilized strobilus with pistils that have developed very long stigmas (from E.H. Moss 6569, DAO)

particularly susceptible to this (Cristiana Moliterni et al. 2004). This is rare, however, as seeds are almost never set in plants protected from pollination. Apomixis has not been reliably demonstrated to occur in *Cannabis*, and seed set in isolated female plants is likely the result of contaminant pollen or self-pollination from sex reversal.

In practice, greatly expanded stigmas will be encountered almost exclusively in marijuana in the black or medicinal markets, and indeed very conspicuous stigmas are invariably present in high-quality herbal material (Fig. 2). Most THC of *C. sativa* occurs in the minute secretory heads of the stalked glandular trichomes of the plant, and these heads are extremely difficult to dislodge in fresh material (merely touching causes them to burst). In dried material (such as marijuana), however, the gland heads are easily detached with handling, and are often lost, significantly decreasing pharmacological potency. We have observed that many gland heads become stuck to the receptive papillae of the stigmas (Fig. 8), reducing the loss of potency.

Aside from their indispensable importance in reproduction, stigmas are not usually thought to be significant. Saffron is composed of the stigmas of *Crocus sativus*. Because it is the world's most expensive spice, its chemistry and pharmacology, although not yet well understood, have been more thoroughly examined than that of any other species' stigmas. Saffron stigmas have pronounced medicinal and toxic properties (Moghaddasi 2010). By contrast, the chemistry and pharmacology of the stigmas of *Cannabis* do not seem to have been examined at all. Stigmas have distinctive chemical components in order to reject or accept pollen biotypes and to regulate pollen tube growth. Mean weight of the stigmas of the ten marijuana samples examined was only 0.2 mg (Table 1). Based on a careful dissection, we found that our sample of the strain LA Confidential was made up of about 0.5 % stigmas (dry weight basis). Although stigmas constitute a small proportion of marijuana, their distinctive chemistry could have health effects, and are therefore in need of study.

Conclusions

Female flowers of *C. sativa* normally are well supplied with pollen. The mature style-stigma portion of their pistils on average grow to only about 3 mm in length (when dry), and are invariably two-branched. By contrast, illicit and medicinal marijuana "buds" (dried female inflorescences) are produced in the absence of pollen. The style-stigma parts of virgin pistils expand notably in girth and length (averaging over 8 mm), and occasionally develop more than the normal two branches. From an evolutionary viewpoint, this expansion of pollen-receptive tissue is an apparent

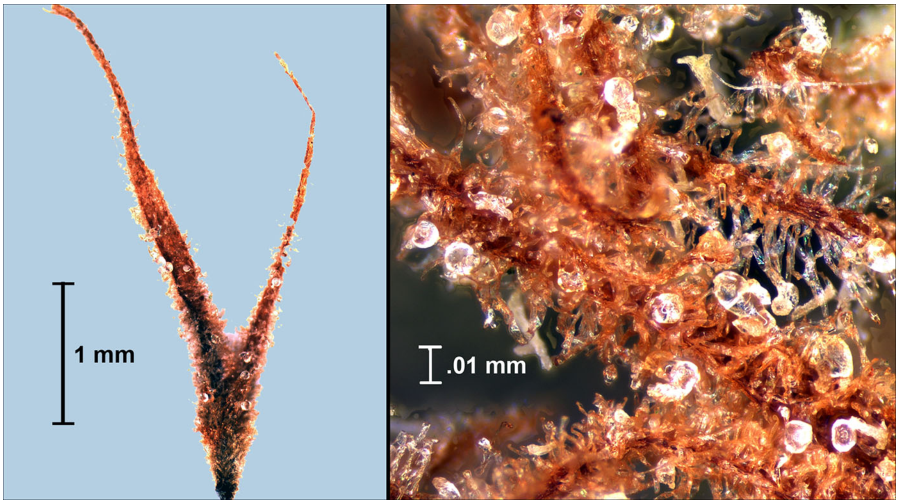

Fig. 8 Stigmas from a "bud" of the medicinal marijuana strain Dinachem, showing how THC-rich secretory gland heads accumulate on the papillae. *Left* an entire pistil. *Right* close-up of stigma with numerous adherent gland heads

adaptation for increasing the probability of fertilizing the females when males are extremely scarce. The high-THC secretory gland heads of *Cannabis*, where most THC is located, tend to fall away from marijuana, significantly decreasing pharmacological potency, but many gland heads become stuck to the receptive papillae of the stigmas, reducing the loss. Although stigmas constitute a small proportion of marijuana, their distinctive chemistry could have health effects.

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