

Epicuticular Wax Columns in Cultivated *Brassica* Species and in their Close Wild Relatives

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Three different types of epicuticular wax columns were found in *Brassica* species with a chromosome number (n) = 9: long columns (LC), short columns (SC) and netted columns (NC). LC were found in *B. incana* and *B. rupestris*. SC were found in *B. villosa*, *B. macrocarpa*, *B. cretica*, *B. hilarionis* and also in *B. montana*. *B. insularis* columns were intermediate. NC waxes were found in *B. oleracea* and its close allies *B. alboglabra* and *B. bourgeauii*. Samples of *B. rapa* ($n = 10$) and *B. nigra* ($n = 8$) examined did not show any wax columns but their amphidiploids with *B. oleracea* (*B. napus* and *B. carinata*, respectively) seemed to inherit the NC type of wax present in *B. oleracea*.

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Key words: *Brassica*, waxes, wax columns, leaf surface.

INTRODUCTION

Cultivated *Brassica* species include three diploids (*B. rapa* L. with a chromosome number (n) = 10, *B. oleracea* L. with $n = 9$ and *B. nigra* (L.) Koch with $n = 8$) as well as three amphidiploids which combine these three genomes in every possible way (*B. napus* L. with $n = 10 + 9$, *B. juncea* with $n = 10 + 8$, and *B. carinata* with $n = 9 + 8$). While *B. rapa* and *B. nigra* show no apparent close relatives, *B. oleracea* has a few wild related species growing in coastal habitats around the Mediterranean. Much work has been done to elucidate the phylogenetic relationships among these species. However, we are not aware of any comparative studies on the structure of their epicuticular wax. In some *Brassica* species, the epicuticular wax is organized in three layers, i.e. a continuous sheet, flat crystals and upright crystals (Conn and Tewari, 1989*b*). Only the upright crystal layer was studied in the present investigation.

Epicuticular waxes on the surfaces of higher plants are normally associated with conservation of water (Martin and Juniper, 1970). In addition, they confer other unique properties to plant surfaces and may also play other important roles (Tewari and Skoropad, 1976; Bodnaryk, 1992; Knowles, Knowles and Tewari, 1996). By providing a water-repellent surface, wax limits the deposition of water-borne spores of pathogens. In *Brassica*, it has been shown that this is the major mechanism by which the glaucous *B. napus* is less susceptible to *Alternaria brassicae* (Berk.) Sacc. than the non-glaucous *B. rapa* (Tewari and Skoropad, 1976; Conn and Tewari, 1989*a*). Ecologically, waxy plants are mostly associated with dry habitats. In *Brassica*, wax is very often present in coastal species.

MATERIALS AND METHODS

Plant material was mostly obtained from the seed bank of crucifers kept in the Escuela T. S. de Ingenieros Agrónomos,

Universidad Politécnica de Madrid (Gómez-Campo, 1990). The accessions used are listed in Table 1 with their respective chromosome numbers (n), their accession number, species name and authority and seed source or origin.

Seedlings were grown in 25 cm clay pots in a glasshouse. They were watered by dripping water directly onto the soil to avoid any damage to the epidermal wax structure which might occur if sprinklers were used. *Brassica* wax development is affected by environmental conditions (Reed and Tukey, 1982; Shepherd *et al.*, 1995). However, in this study all plants were grown under the same conditions and, therefore, different wax types reflect genetic differences among the various species examined.

Leaves from the sixth to eighth nodes were removed from young plants, taking care not to touch the laminae. Leaves were then transferred into 20 cm Petri dishes on moist filter paper with ten drops of 2% osmium tetroxide solution in water and left overnight in a fume hood. The next day, small pieces of leaves were mounted on metal stubs and allowed to air dry. Both leaf surfaces were mounted. After being coated with platinum they were examined and photographed in a Hitachi-2500 scanning electron microscope.

RESULTS

In taxa which presented upright epicuticular wax crystals, three general types of wax columns were found to exist.

First, there were long needle-like columns (LC) of approx. 7 μm (mean \pm s.d. = 6.86 \pm 1.2, measured in 20 properly oriented columns). This type of wax was dominant in *B. incana* and *B. rupestris* (Fig. 1A). In *B. incana* they were accompanied by abundant epidermal trichomes, while these were very sparse in *B. rupestris*.

Next, there were short thick columns (SC) of approx. 3 μm (mean \pm s.d. = 3.2 \pm 0.6, measured in 20 properly oriented columns). These were dominant in *Brassica villosa*

TABLE 1. Brassica accessions utilised in this research

Chromosome number (n)	Accession number	Species	Seed source/origin
8	0049	<i>Brassica nigra</i> (L.) Koch	Vejer (Spain)
9	6824	<i>Brassica oleracea</i> L.	Granville (France)
	—	<i>Brassica oleracea</i> L.	Cult. commercial sample
	5971	<i>Brassica alboglabra</i> Bailey	Gatersleben (Germany)
	5719	<i>Brassica bourgeau</i> (Christ) Kunze	Tijarafe, La Palma, Canary
	6813	<i>Brassica montana</i> Pourr.	Ste. Marguerite (France)
	6801	<i>Brassica montana</i> Pourr.	Palmaiola (Italy)
	3820	<i>Brassica incana</i> Ten	C. Tindari, Sicily (Italy)
	6558	<i>Brassica incana</i> Ten	Capri (Italy)
	6579	<i>Brassica rupestris</i> Rafin	Cinisi (Sicily, Italy)
	6582	<i>Brassica villosa</i> Biv.	Castelmare di Golfo (Italy)
	3821	<i>Brassica villosa</i> subsp. <i>drepanensis</i> (Caruel) Raim. & Mazz.	Mte. Erice, Sicily (Italy)
	3819	<i>Brassica macrocarpa</i> Guss.	Egadi, Sicily (Italy)
	3814	<i>Brassica insularis</i> Moris	C. Caccia, Sardinia (Italy)
	6020	<i>Brassica cretica</i> Lam. subsp. <i>cretica</i>	Diakoftos (Greece)
	6021	<i>Brassica cretica</i> subsp. <i>aegea</i> (Heldr. & Hal.) Snogerup <i>et al.</i>	Euboea (Greece)
	6344	<i>Brassica cretica</i> subsp. <i>aegea</i> (<i>ibid.</i>)	Limnos (Greece)
	7344	<i>Brassica hilarionis</i> Post	Kyrenia range (Cyprus)
10	8431	<i>Brassica rapa</i> L. (canola)	Prof. J. P. Tewari
17	—	<i>Brassica carinata</i> Braun	Dr. A. de Haro
18	0184	<i>Brassica juncea</i> (L.) Czern	Bot. Garden, Upsala
19	8432	<i>Brassica napus</i> L. (canola)	Prof. J. P. Tewari

(two subspecies). *B. macrocarpa* (Fig. 1B), *B. montana*, *B. cretica* and *B. hilarionis*. Only in *B. villosa* were they accompanied by abundant hairs. *B. insularis* columns were intermediate between LC and SC.

Thirdly, there were netted columns (NC) formed by LC-like elements, apparently interlinked and often topped with transversal columns and wax scraps. They were never accompanied by hairs. Only *B. oleracea*, *B. bourgeai* (Fig. 1C) and *B. alboglabra* showed this type of wax structure. At first sight, the NC pattern appeared as an artefact but the same structure was seen whenever observations on *B. oleracea* were carried out. Transversal columns are often shorter than those shown in Fig. 1C.

No waxes could be found in the leaves of the accessions of *B. nigra*, *B. rapa* and *B. juncea* studied.

DISCUSSION

It has been hypothesized (Gómez-Campo and Gustafsson, 1991) that the two densely haired Sicilian species, *B. villosa* and *B. incana*, are the most primitive within the group, since they also show other primitive characters, such as the presence of two (three) seeds in the pod beak (primitive only for this group). Genetic variation is much higher in *B. villosa* (where four subspecies have been recognized). Other members of the group may be considered more evolved since they have less or no hairs and their beak is further reduced to hold no more than one seed. In this respect, we could consider both SC and LC waxes to be associated with the primitive condition represented by *B. villosa* and *B. incana*. As shown here, the presence of hairs and waxes is not mutually exclusive in the Sicilian brassicas. On the other hand, there is no particular reason why SC and LC waxes should be more primitive.

Henceforth, at least five lines of partial or total hair loss and parallel development of waxes can be distinguished (Fig. 2). (a) *B. rupestris* is geographically confined to the Sicilian area of Palermo, between the distribution areas of *B. villosa* and *B. incana*. *B. rupestris* has not completely lost its hairs and presents no particular difference in the structure of its LC waxes compared with the last two species. (b) *B. insularis* from Corsica, Sardinia and Tunisia has no hairs at all, while it exhibits waxes of intermediate size between those found in the Sicilian species. (c) *B. macrocarpa*, isolated in distribution in the Egadi Archipelago (NW Sicily), has no hairs, while its waxes are SC as in the neighbouring *B. villosa*. (d) *B. cretica* and *B. hilarionis*, from Greece, Turkey and Cyprus, have undergone parallel evolution (waxes of SC type and no hairs). The derivation of *B. cretica* from Sicilian material is probably indirect and could be related either to Tunisian *B. insularis* or to Adriatic *B. incana*. (e) The last (fifth) line apparently derives from *B. incana* (on geographic and morphological grounds). It persists with *B. montana*, growing on the coasts of Italy, France and NE Spain. However, it has no hairs and its wax columns are more like the SC type. Further to the west, the Atlantic kale, *B. oleracea*, is also glabrous and shows NC waxes.

NC waxes are also present in *B. bourgeai* and *B. alboglabra*. Although these taxa have been listed as species in Table 1, the opinion that both should be included within the intraspecific variability of *B. oleracea* is widespread (Bothmer, Gustafsson and Snogerup, 1995; Lannér, 1998). Our results strongly support this opinion. *B. bourgeai* is a rare taxon from the Canary Islands, of uncertain origin and might merely represent an ancient escaped cultivar of *B. oleracea*. The same can be said for *B. alboglabra*, a cultivated form which is supposed to have reached China in ancient times.

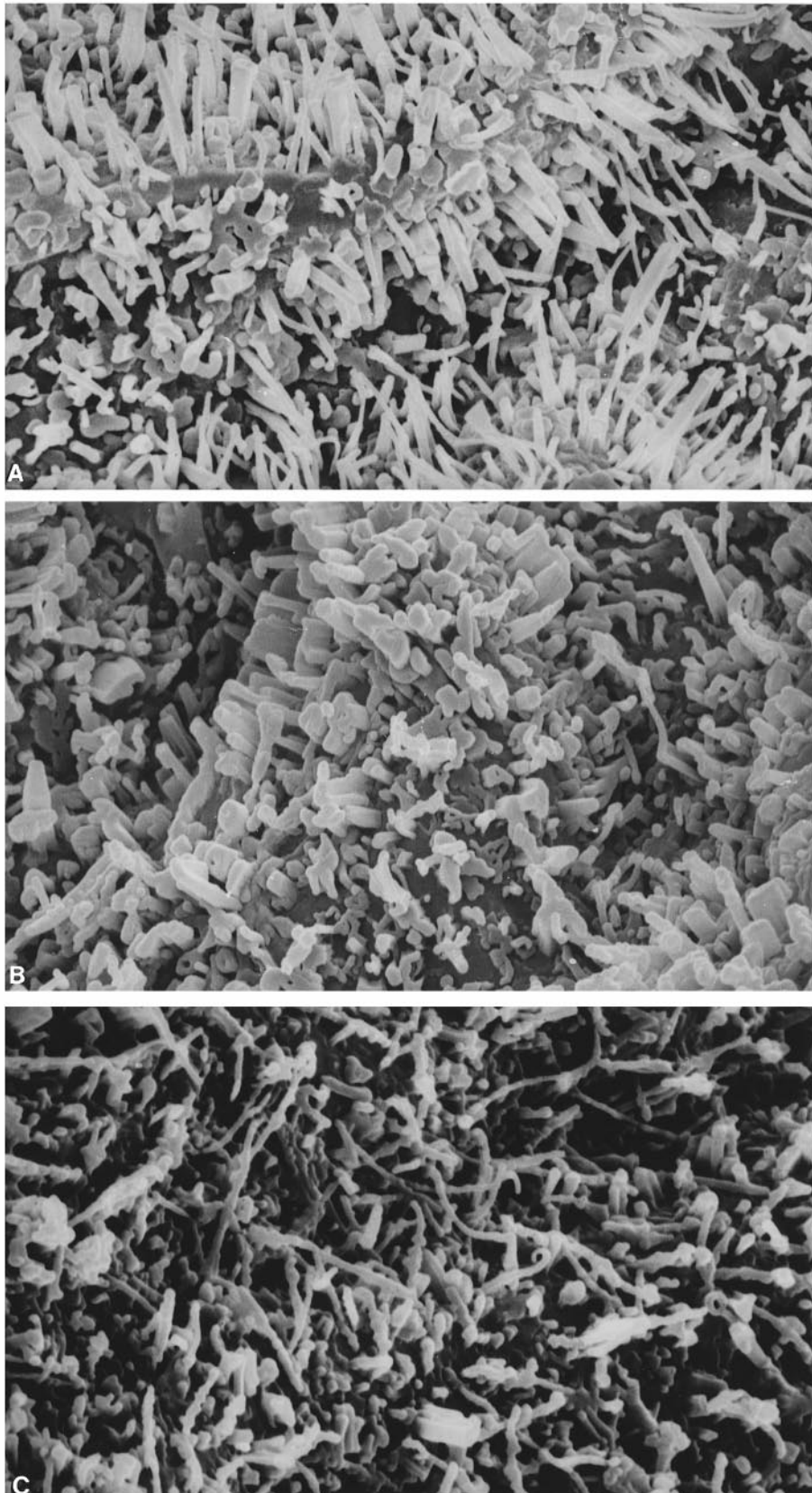


FIG. 1. Long (A), short (B) and netted (C) epicuticular wax columns exemplified by *Brassica rupestris*, *B. macrocarpa* and *B. oleracea*, respectively. $\times 3300$.

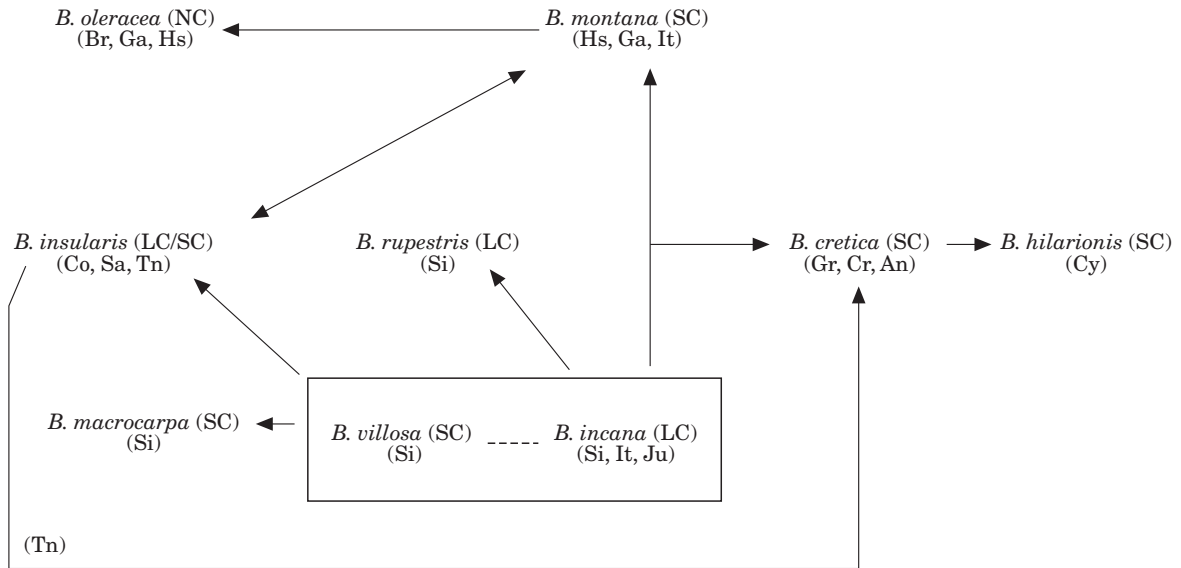


FIG. 2. Scheme showing the geographic distribution of $n = 9$ *Brassica* species studied with indication of their respective wax types. Sicilian densely hairy species are boxed. Arrows express hypothetical derivations. Abbreviations for countries and islands are as in Med-Check List (Greuter *et al.*, 1986): An, Anatolia; Br, British Isles; Co, Corsica; Cr, Crete; Cy, Cyprus; Ga, France (continental); Gr, Greece (cont.); Hs, Spain (cont.); It, Italy (cont.); Sa, Sardinia; Si, Sicily; Tn, Tunisia; Ju, former Yugoslavia.

Within each type of wax column, some minor differences have been observed, for instance between Turkish and Greek *B. cretica* or between the two populations of *B. incana* studied.

Thus, lines *c* and *d* seem to be related to *B. villosa* while line *b* (*B. insularis*) might have originally been of either LC or SC type and later introgressed by an opposite stock. Line *a* (*B. rupestris*) seems to be related to *B. incana* which, in turn, is situated in the origin of line *e*. The phenetic and phylogenetic reality of this line (*e*) across the coasts of Italy, France, Spain and Great Britain seems reasonably well established. Therefore, perhaps we should admit that SC waxes of *B. montana* are also the result of introgression, probably from an SC ancestor of *B. insularis* which also provided glabrousness. The disruption of this line along the east, south and west coasts of the Iberian Peninsula and the new Atlantic habitat conditions—temperature, salinity, etc.—may have somehow favoured the development of NC waxes.

According to our results with other cultivated *Brassica* species outside the $n = 9$ group, *B. nigra* ($n = 8$) has no waxes while *B. carinata* (an amphidiploid between *B. nigra* and *B. oleracea*) inherits the NC waxes of *B. oleracea*. In turn, *B. napus*, an amphidiploid between *B. rapa* (syn. *B. campestris*) and *B. oleracea* also has waxes of the NC type, like its parent *B. oleracea*. The other parent, *B. rapa*, did not present any type of wax, at least in the material we studied. The same occurred with *B. juncea*.

Conn and Tewari (1989*b*) reported the presence of waxes in *B. rapa*. However, given the wide genetic diversity of this cultivated species (where it is not uncommon to find glaucousness in the upper leaves), we consider that this apparent contradiction is just a reflection of variability. Also, waxy (NC type) and waxless varieties can be found in *B. carinata* (de Haro, pers. comm.). *B. napus* may show both

LC (Tewari and Skoropad, 1976; Conn and Tewari, 1989*b*) and NC waxes (our results). Although further confirmation is required, we should mention here molecular studies by Song and Osborn (1992) based on the RFLP pattern of cp and mt DNAs provide evidence for multiple origins of *B. napus*, suggesting that an ancestor similar to *B. montana* was the cytoplasmic donor of many *B. napus* accessions.

At first sight, netted columns (NC) may appear to result from branching, but after closer observation it is more reasonable to interpret them as a consequence of a massive production of feeble wax columns where newly formed columns push, lift, tilt and sometimes bend the older ones, giving rise to the clumps and scraps, and finally producing the appearance shown in Fig. 1C. Secondary welding may also be present, but this may also occur in other wax types. Thus differences observed in different varieties of *B. napus* and *B. carinata* might just be the result of different quantitative balances between inheritance from *B. oleracea* and from their second parent.

Future studies with additional populations, especially those from Sicily, Corsica and South France, may provide further details for the scheme described above.

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