Ultrastructure of Epicuticular Wax in Canola

K. L. Conn and J. P. Tewari

Department of Plant Science, University of Alberta, Edmonton, Alta., Canada T6G 2P5

Z. Naturforsch. **44c**, 705–711 (1989); received December 1, 1988/February 23, 1989

Canola, *Brassica campestris, B. napus,* Wax Ultrastructure

Ultrastructure of the epicuticular wax of four commercially grown Canadian cultivars of canola (*Brassica campestris* cvs. Tobin and Candle, *B. napus* cvs. Altex and Westar) was investigated. Freeze-drying and air-drying methods of specimen preparation for scanning electron microscopy were compared. The freeze-drying method resulted in disruption and possible washing away of wax crystals, whereas there was no visible damage with the air-drying method. The freeze-drying method provided evidence for an amorphous layer of wax beneath the wax crystals. Ultrastructure of wax in the four cultivars was generally similar. All cultivars had an evenly distributed layer of wax crystals superimposed on an amorphous layer of wax. Some trends such as density of wax on leaves and siliques appeared to be species-specific, whereas density of wax on stems did not. There appeared to be at least three types of wax crystals present. These included plate-like, filamentous, and rod-like crystals. The rods were present singly or in blocks.

Introduction

Aerial surfaces of all higher plants are clothed in a layer of amorphous wax upon which may be situated one or more types of crystalline wax deposits [1]. The epicuticular wax (EW) acts as a barrier to loss of water vapor, restricts leaching of inorganic and organic substances, reflects certain wavelengths of solar radiation, and provides disease resistance (or disease escape) [1, 2]. Properties of the EW of rapeseed that confer resistance (or escape) to *Alternaria brassicae* (Berk.) Sacc., causal agent of the black spot disease, have been under investigation in this laboratory for some time. The EW has been shown to reduce deposition of water-borne inoculum by providing a water-repellent surface [3]. The less susceptible *Brassica napus* L. has larger amounts of EW than the more susceptible *B. campestris* L. [3–5]. The larger amounts of wax in *B. napus* reduces the germination of conidia of *A. brassicae,* most likely by restricting movement of leaf exudates [5, 6]. These properties are partly dependent on the physical structure of the EW [1]. There have been studies on ultrastructure of EW in *B. campestris* and B. *napus* [3, 7–10], but none of them have dealt with canola-type [11] cultivars of rapeseed. This paper presents results of a study on ultrastructure of EW of four canola-type cultivars of *B. campestris* and *B. napus* now commercially grown in Canada. Leaves, stems and siliques were investigated because *A. brassicae* causes spotting on all these parts [12]. A preliminary report on this work has been published [13].

Reprint requests to J. P. Tewari.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0700–0705 $ 01.30/0

Materials and Methods

Plant material

Cultivars of canola used were Tobin, Candle *(B. campestris),* Altex and Westar *(B. napus).* Plants were grown in either growth cabinets at day/night temperatures of 18 °C/12 °C and 16 h light, or in a greenhouse where conditions varied throughout the season. Plants in growth stage four [14] were used. The leaf immediately above the cotyledons was designated as the first leaf. Middle leaves from positions 5 to 7 and upper leaves from positions 8 and 9 were used for scanning electron microscopy (SEM). Adaxial leaf surfaces of all four cultivars were prepared for SEM by the two methods described below. Abaxial leaf surfaces, stems and siliques of all four cultivars were prepared for SEM only by the air-drying method. Stem pieces from lower and upper parts and siliques from lower and upper parts of the influorescence were used for SEM.

Scanning electron microscopy

Freeze-drying method

Leaves were placed in glass petri plates containing moist filter paper. Osmium tetroxide (2% solution) was added to the filter paper, and the leaves fixed by osmium tetroxide vapor overnight. Pieces of leaves were frozen by quick immersion in liquid Freon 22, stored in liquid nitrogen and freeze-dried at −80 °C using an Edwards vacuum freeze-drier. They were then mounted on stubs with conductive glue, coated with gold and examined in a Cambridge Stereoscan 150 SEM.

Air-drying method

Leaves were fixed with osmium tetroxide vapor as described above. Pieces of leaves were mounted on stubs and air-dried for 2 days. Pieces of stems and siliques were placed on glass rods in petri plates containing moist filter paper and processed as above. All materials were coated with gold and examined as described above.

Results and Discussion

Comparison of freeze-drying and air-drying methods

Leaves prepared by the air-drying method showed no apparent damage or disruption of the wax layer. Such was not the case when the freeze-drying method was used. Air-dried leaves showed an even distribution of wax crystals (Fig. 1, 2) while freeze-dried leaves showed aggregates of wax crystals interspersed with bare leaf areas (Fig. 3, 4). This indicated redistribution and apparent washing away of wax crystals during the latter preparation process. Rapid immersion in liquid Freon 22 was one of the steps involved in preparing specimens by the freeze-drying method. This resulted in boiling of the coolant as heat was transferred from the specimen to Freon 22, perhaps resulting in physical disturbance of the wax layer. These results indicate that use of the freeze-drying method in the study of ultrastructure of plant surfaces could lead to incorrect interpretations of EW, at least in cases where the wax layer is relatively loose and fluffy.

When studying an undisturbed leaf surface by SEM, one cannot discern whether there is a basal layer of amorphous wax present, although such a layer is generally thought to be present [1]. The specimens prepared by the freeze-drying method provided direct evidence of a basal amorphous wax layer. In Fig. 7, a view of a freeze-dried leaf is shown where a sheet of wax has been dislodged and turned upside down. It can be seen that the wax crystals are supported on a continuous layer of wax.

Ultrastructure of epicuticular wax

Plant surfaces were examined with SEM at many different times during the course of this study. As some plants were grown in a greenhouse, environmental conditions were not the same each time. Therefore, some variation in wax ultrastructure was expected because a complex epicuticular wax like that of *Brassica* is more sensitive to environmental factors than the less complex waxes from some other plants [15, 16]. Density of wax crystals often varied from one sampling time to another. For example, higher temperatures and light intensities during the summer increased the "bloom" on plants. This is consistent with reports by Armstrong and Whitecross [7] and Whitecross and Armstrong [9]. Also, size and proportion of each wax crystal type sometimes varied from one sampling time to another, but the same types of wax crystals were always present on all plant surfaces studied. These variations in ultrastructure were not studied in detail and are not discussed any further.

Leaves, stems and siliques of all four cultivars were covered with a fluffy layer of wax crystals. These wax crystals were evenly distributed over the surface, except for areas immediately surrounding the stomata, where the wax was less dense (Fig. 1, 2). Oblique views revealed that many wax crystals projected away from the plant surface, forming a "forest" of wax crystals (Fig. 5, 6). Density of wax crystals on leaves of *B. napus* cultivars (Fig. 2, 6) was greater than on those of *B. campestris* cultivars (Fig. 1, 5). This agrees with leaves of *B. napus* being glaucous and *B. campestris* being non-glaucous. Adaxial surfaces of upper leaves of all four cultivars were more glaucous than the corresponding surfaces of middle leaves. Trends in the density of wax crystals on abaxial leaf surfaces were similar to the adaxial surfaces. Both upper and lower parts of stems of all four cultivars had high densities of wax crystals (Fig. 8–11). Density of wax crystals on upper and lower siliques of all four cultivars was similar to that on their respective leaves (Fig. 12, 13). Thus, *B. napus* cultivars had a greater density of wax crystals on leaves and siliques than *B. campestris* cultivars, but all the cultivars had high densities of wax crystals on the stems.

The types of wax crystals on all plant surfaces studied were generally similar. The fluffy layer of wax was comprised of at least three types of wax

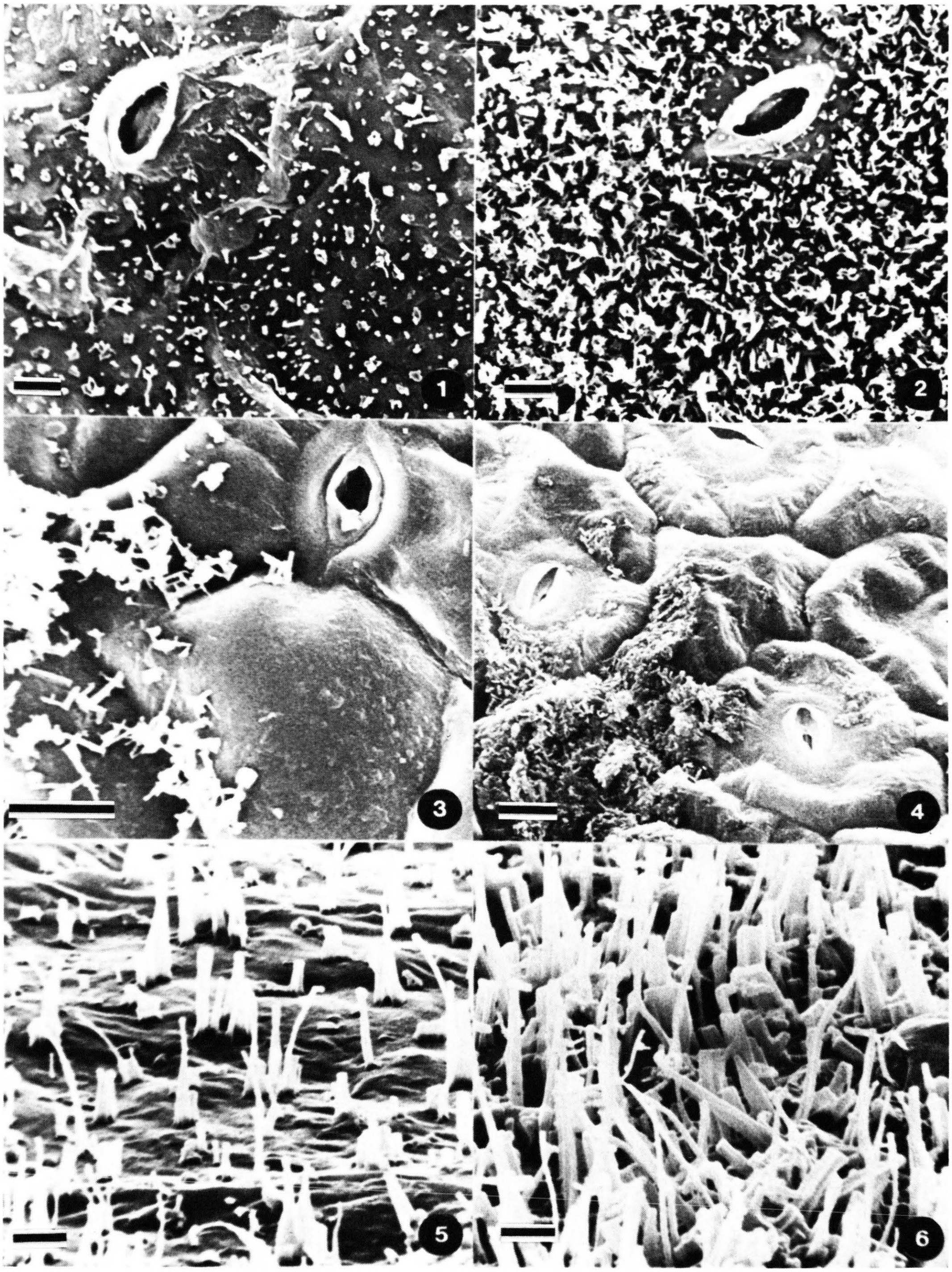

Fig. 1, 2. Top view of adaxial surfaces of middle leaves of Candle and Westar, respectively, prepared for SEM by the air-drying method. Note the even distribution of wax crystals (bars = 5 μm). Fig. 3, 4. Top view of adaxial surfaces of middle leaves of Candle and Westar, respectively, prepared for SEM by the freeze-drying method. Note the displacement of wax crystals, leaving bare areas (bars = 10 μm). Fig. 5, 6. Oblique view of adaxial surfaces of middle leaves of Candle and Westar, respectively, prepared for SEM by the air-drying method. Note that most wax crystals project away from the surface (bars = 2 μm).

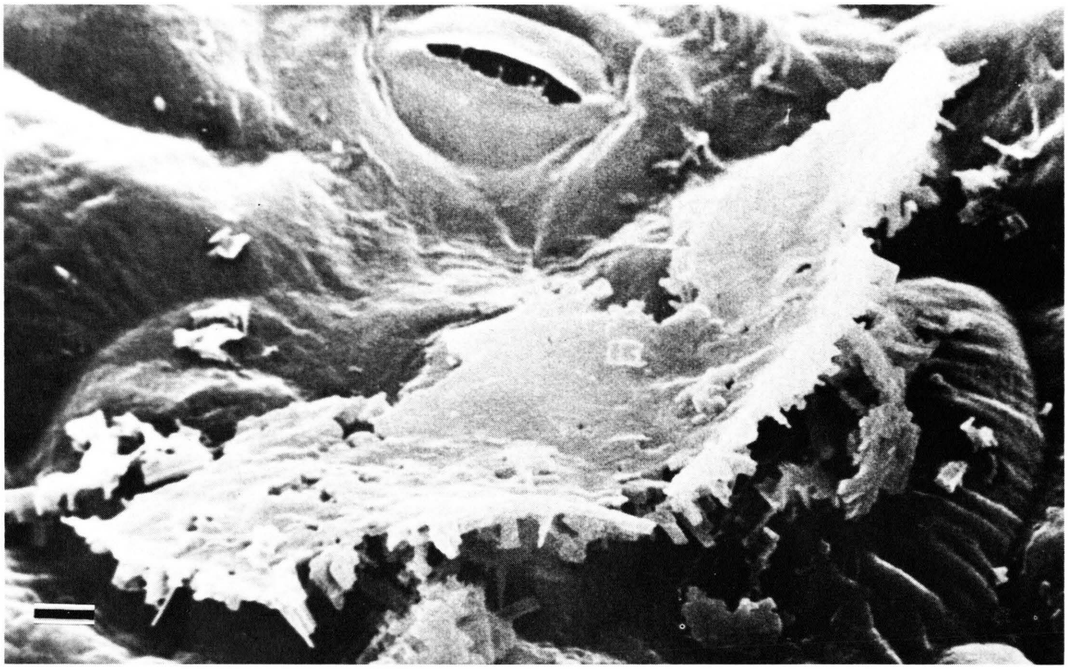

Fig. 7. Adaxial surface of an upper leaf of Westar prepared for SEM by the freeze-drying method showing a sheet of wax that has been turned upside down (bar = 4 μm).

crystals. These were plate-like, rod-like, and filamentous crystals. Plate-like crystals were oriented flat on the surface, while rod-like and filamentous crystals projected away from the surface. Plate-like crystals were of variable shapes and sizes, and some contained holes (Fig. 14). Margins of some of them were barely discernable while others had well defined edges and were slightly raised. Rod-like crystals were the most plentiful type of crystal. They appeared singly or in blocks. Individual rods were stocky and relatively straight (Fig. 15). Wax crystals described here as being blocks, appeared to be groups of rods fused together. Their sides were undulating with contours resembling the individual rods (Fig. 16, 17). The blocks had flat tops and most of them contained one or more holes which were lacking in the individual rods (Fig. 16, 17). There appeared to be growth rings in some blocks indicating periodic growth (Fig. 17). Filamentous wax crystals were thinner and usually longer than the rods (Fig. 18). They were also often curved and sometimes branched (Fig. 19). Wax crystals similar to the plates, individual rods, and filaments described here have been reported in rapeseed before [5, 7–9]. However, this appears to be the first report of the occurrence of wax crystals described here as blocks of fused rods. Many blocks when viewed from the top appeared similar to some of the wax crystals described as plates (Fig. 14). The height of blocks varied considerably. They ranged from tall ones to those that were just higher than some of the crystals described as plates (Fig. 14). It is, therefore, possible that some of the plate-like crystals are actually young blocks. Wax crystals in the form of tubes reported in some other types of rapeseed [3, 8] were not present in the cultivars investigated during the present study. The blocks described here did contain holes, but their morphology was very different from that of tubes, and thus, are not likely related.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and Alberta Crop Research and Development Programme to J. P. T. The help received from Mr. George Braybrook in SEM work is gratefully acknowledged.

Fig. 8, 9. Top view of stem surfaces of Candle and Westar, respectively, prepared for SEM by the air-drying method (bars = 10 µm). Fig. 10, 11. Oblique view of stem surfaces of Candle and Westar, respectively, prepared for SEM by the air-drying method (bars = 2 µm). Fig. 12, 13. Oblique view of silique surfaces of Candle and Westar, respectively, prepared for SEM by the air-drying method (bars = 2 µm).

Fig. 14. Adaxial surface of an upper leaf of Altex showing plate-like wax crystals (arrows). Fig. 15. Stem surface of Altex from the middle of a plant showing rods (arrows). Fig. 16. Abaxial surface of an upper leaf of Tobin showing a fused rod (arrow). Fig. 17. Adaxial surface of an upper leaf of Westar showing fused rods (arrows). Note the growth rings in these crystals. Fig. 18. Adaxial surface of an upper leaf of Tobin showing filamentous wax crystals (arrows). Fig. 19. Adaxial surface of a middle leaf of Tobin showing a branched filamentous wax crystal (arrow). The plant surfaces depicted in Fig. 14–19 were prepared for SEM by the air-drying method (bars = 1 μm).

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