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Light Intensity and Temperature Effects on Epicuticular Wax Morphology and Internal Cuticle Ultrastructure of Carnation and Brussels Sprouts Leaf Cuticles[1]

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Abstract. **At either 440 or 145 $\mu Em^{-2}s^{-1}$, greater amounts of cuticle, cutin matrix, and wax were formed at 15° than 25°C on leaves of Brussels sprouts (*Brassica oleraceae*. L., *Gemmifera* group), but the reverse occurred on leaves of carnation (*Dianthus caryophyllus* L.). At either 15 or 25°, greater amounts of cuticle, cutin matrix, and wax were formed at 440 than 145 $\mu Em^{-2}s^{-1}$ on both species. For Brussels sprouts, at 25°/440 $\mu Em^{-2}s^{-1}$ and 25°/145 $\mu Em^{-2}s^{-1}$, large parallel wax dentrites covered the leaf surface. At 15°/440 $\mu Em^{-2}s^{-1}$, dentrites were smaller and morphologically less elaborate. At 15°/145 $\mu Em^{-2}s^{-1}$, epicuticular wax occurred as scattered rods perpendicular to the leaf surface. Carnation epicuticular waxes consistently occurred as rods, but as temperature and/or light intensity decreased, rod length decreased and density increased. There were no changes in internal cuticle ultrastructure of either species in different environments, but cuticle thickness increased as temperature and light intensity decreased. Epicuticular waxes visualized in surface view by scanning electron microscopy (SEM) were extracted by procedures for transmission electron microscopy (TEM) observation.**

The cuticle is composed of a matrix of cutin impregnated with waxes and polysaccharides and may be covered with epicuticular waxes (13). The morphology and development of epicuticular waxes may be influenced by temperature, light, and humidity (8), thus affecting important cuticular properties such as regulation of gas and water exchange and leaching, protection from environmental contaminants (13), and absorption of foliar-applied compounds (8).

Most studies of environmental effects on cuticles have dealt with epicuticular waxes, characterizing their morphology, composition and quantity (1, 2, 4, 8, 9, 10, 21), while few have studied internal cuticle ultrastructure (8). Yet the internal arrangement of cuticular constituents could have a major influence on penetration. These studies were undertaken to characterize the effect of various temperature/light intensity combinations on morphology of epicuticular waxes and internal cuticle ultrastructure. They are designed to form the bases for further studies correlating differences in epicuticular wax morphology, cuticular wax content, and thickness with permeability of cuticles to inorganic ions (19).

Materials and Methods

'Jade Cross E' Brussels sprouts seedlings and rooted 'White Sim' carnation cuttings were transplanted into 10.2-cm plastic pots containing 1 peat moss:1 perlite:1 loam soil, with 2.4 g/liter each of superphosphate and dolomite lime and 0.8 g/liter of 10–4.4–8.3 (N-P-K) fertilizer.

Plants were grown in controlled environment chambers for 6 weeks at 16 hr photoperiod, and at 25 ± 1.3°C or 15 ± 1.4° in combination with 440 $\mu Em^{-2}s^{-1}$ (400–700 nm) with 22 Wm^{-2} IR or 145 $\mu Em^{-2}s^{-1}$ (400–700 nm) with 8.5 Wm^{-2} IR, measured with a Lambda LI-185A Quantum Sensor. Relative humidity was 58 ± 7.3% for the 25° treatments and 68 ± 6.5% for the 15° treatments as monitored with a dew point hygrometer; this difference was not significant and should not significantly alter results (1). Plants were watered as needed and fertilized weekly with a 200–88–166 ppm (N-P-K) soluble fertilizer. Special precautions were taken to avoid any physical contact with the foliage. Samples were collected after 6 weeks from the most recent fully expanded leaves which had developed entirely under the specified environment.

For SEM observations, a 1-cm leaf disc from each of 5 plants in each treatment was excised, avoiding the midvein and major lateral veins, and was freeze-dried. No wax or tissue damage was observed in freeze-dried specimens. Discs were coated with 200 Å gold in short bursts in a water-cooled sputter coater, which prevented wax damage due to heating. Specimens were viewed with an AMR Model 1000A SEM at 10–20 kV. Wax damage due to beam heating (16) did not occur.

Tissue preparation for TEM was modified from procedures of Hallam (5) and Karnovsky (12). Freshly excised leaf pieces, 0.5 cm^2, from each of 5 leaves in each treatment were fixed 1 hr in 1.5% paraformaldehyde/3% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.0, plus 5 mg/ml $CaCl_2$, then cut into 1- to 2-mm segments, lightly vacuum infiltrated, and fixed 2 hr. The tissue was buffer washed 1 hr (6 rinses), post-fixed 2 hr in 2% OsO_4 in 0.1 M Na cacodylate, pH 7.0, then buffer washed and dehydrated in a graded ethanol series. The above steps were at 0 to 4°C. Fixed tissue was infiltrated and embedded in a graded propylene oxide: Epon-Araldite series at 22° and polymerized at 60° for 24 to 48 hr (14). Thin sections were stained 30 min in 2% aqueous uranyl acetate then 10 min in lead citrate (20) and viewed and photographed at 80 kV with a Phillips EM 200. Observations by both SEM and TEM revealed

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little difference between the adaxial and abaxial leaf cuticles, so all micrographs are of cuticles of adaxial surfaces.

Cuticles were isolated from 1-cm^2 leaf pieces with several changes over 96 hr of 2% pectinase, 0.2% cellulase, and 0.2% hemicellulase in 0.1 M Na acetate, pH 4.0 at 37°C (15). Remaining cellular debris was removed with a 10 min treatment with 0.589 g $ZnCl_2$/ml concentrated HCl (6). Isolated cuticles were dewaxed by two 10-min changes in acetone (to remove water) then six 20-min changes in chloroform at 22° with constant agitation. Complete wax extraction was assured since longer extraction times did not further decrease cuticle weights (17). Cuticle weights were determined gravimetrically after drying to constant weight over silica gel at 22°.

Results and Discussion

Cuticle Characteristics. At either 15° or 25°C, the cuticular components per unit area (total cuticle, cutin matrix, and cuticular wax) were greater at 440 than 145 $\mu Em^{-2}s^{-1}$ for Brussels sprouts and carnation (Table 1). At either light intensity, the cuticular components per unit area were greater at 25° for carnation, but greater at 15° for Brussels sprouts. The percent wax of Brussels sprouts leaves increased as temperature and/or light intensity decreased; there was little effect on carnation.

Results on total cuticular wax content generally agree with previously reported results for epicuticular waxes (1, 2, 4, 9, 10, 21), determined by differential extraction of epicuticular waxes by brief exposure to chloroform. Yet no report substantiated only epicuticular wax extraction. If cuticles which possess differing quantities and morphologies of epicuticular waxes (compare Fig. 1A to 1D) are subjected to the same dewaxing procedure, variable amounts of epicuticular and internal cuticular waxes could be extracted. Hence, the results concerning epicuticular wax quantities and composition from plants grown in different environments may be due largely to differential wax extraction as opposed to true treatment differences (1, 2, 4, 9, 10, 21).

Epicuticular wax morphology. The epicuticular wax of Brussels sprouts leaves at 25°C/440 $\mu Em^{-2}s^{-1}$ was composed of complex wax dentrites oriented parallel to and completely covering the leaf surface (Fig. 1A). At 25°/145 $\mu Em^{-2}s^{-1}$, the dentrite density decreased slightly, exposing some leaf surface, but morphology was unaltered (Fig. 1B). At 15°/440 $\mu Em^{-2}s^{-1}$, the dentrite density increased but dentrite size was reduced and morphology was less elaborate (Fig. 1C). Temperature changes had a greater effect than light intensity changes (compare Fig. 1C and 1B). At 15°/145 $\mu Em^{-2}s^{-1}$, the epicuticular waxes occurred as wax rods perpendicular to the leaf surface (Fig. 1D), and leaves were not glaucous compared to leaves in the other environments. Similar results have been reported for Brussels sprouts (1), rape (21), and *Clarkia elegans* (9), in which morphologically more complex wax dentrites or plates predominated at higher temperatures and light intensities and morphologically simpler wax rods, tubes, or filaments predominated at lower temperatures and light intensities.

For carnation, the various temperature/light intensity combinations had no effect on epicuticular wax morphology, which always occurred as rods (Fig. 1E–H). At 25°C/440 $\mu Em^{-2}s^{-1}$, the wax rods averaged 2μm in length (Fig. 1E). At lower temperatures and light intensities (Fig. 1F–H), the rod density increased, but length decreased to 1μm at 15°/145 $\mu Em^{-2}s^{-1}$ (Fig. 1H). As with Brussels sprouts, temperature had a greater effect than light intensity on carnation epicuticular waxes (compare Fig. 1F and 1G). Similar results have been reported for *Pisum sativum* (10, 11) and *Eucalyptus* sp. (2, 4), which have simple epicuticular wax forms such as rods, tubes, or small plates that increased in density as temperature decreased or light intensity increased.

Internal cuticle ultrastructure. The outer region of the cuticle on Brussels sprouts (Fig. 2A) and carnation (Fig. 2B) was largely electron-lucent. The inner region contained electron-dense diffuse fibrils in Brussels sprouts, but was impregnated with a fine reticulum of electron-dense fibrils in carnation. There was little effect of the various temperature/light intensity combinations on internal ultrastructure on either plant (Fig. 2) except for differences in cuticle thickness (Table 1). There was a significant increase in cuticle thickness of both plants as temperature and/or light intensity decreased; a decrease in temperature increased cuticle thickness more than a decrease in light intensity (Table 1). These findings are similar to those reported for velvet mesquite *(Prosopis juliflora)* (7, 8).

Epicuticular waxes visualized by SEM in surface view (Fig. 1) were not visualized by TEM (Fig. 2). Observations indicated complete epicuticular wax extraction during the heat polymerization process of the TEM preparation procedure, and no fixation technique preserved epicuticular waxes (18). This might partially explain the lack of observable differences in internal cuticle ultrastructure by environment.

Table 1. Characteristics of the adaxial leaf cuticle of Brussels sprouts and carnation plants grown in different environments.

Environment[y]	Cuticle ($\mu g/cm^2$)	Cutin[z] matrix ($\mu g/cm^2$)	Total cuticular wax ($\mu g/cm^2$)	Total cuticular wax (μg/leaf)	Total cuticular wax (%, w/w)	Cuticle thickness (μm[x])
			Brussels sprouts			
HTHL	56.9	52.3	4.6	181.8	8.1 ± 2.8[w]	0.16a
HTLL	37.2	31.2	6.0	207.0	16.1 ± 1.6	0.19b
LTHL	73.9	57.0	16.9	463.4	22.9 ± 1.6	0.21c
LTLL	20.8	13.2	7.6	267.0	36.7 ± 0.9	0.27d
			Carnation			
HTHL	743.5	577.5	166.0	896.5	22.3 ± 1.1	1.98a
HTLL	316.0	248.8	67.2	356.2	21.3 ± 0.2	2.00a
LTHL	412.3	309.4	102.9	318.8	25.0 ± 0.2	1.94a
LTLL	288.7	222.0	66.7	213.3	23.1 ± 0.9	2.25b

[z]Cutin matrix determined from dewaxed cuticles.
[y]HT = 25°C; LT = 15°; HL = 440 $\mu Em^{-2}s^{-1}$; LL = 145 $\mu Em^{-2}s^{-1}$.
[x]Mean separation within columns by Duncan's multiple range test, 1% level.
[w]± SD

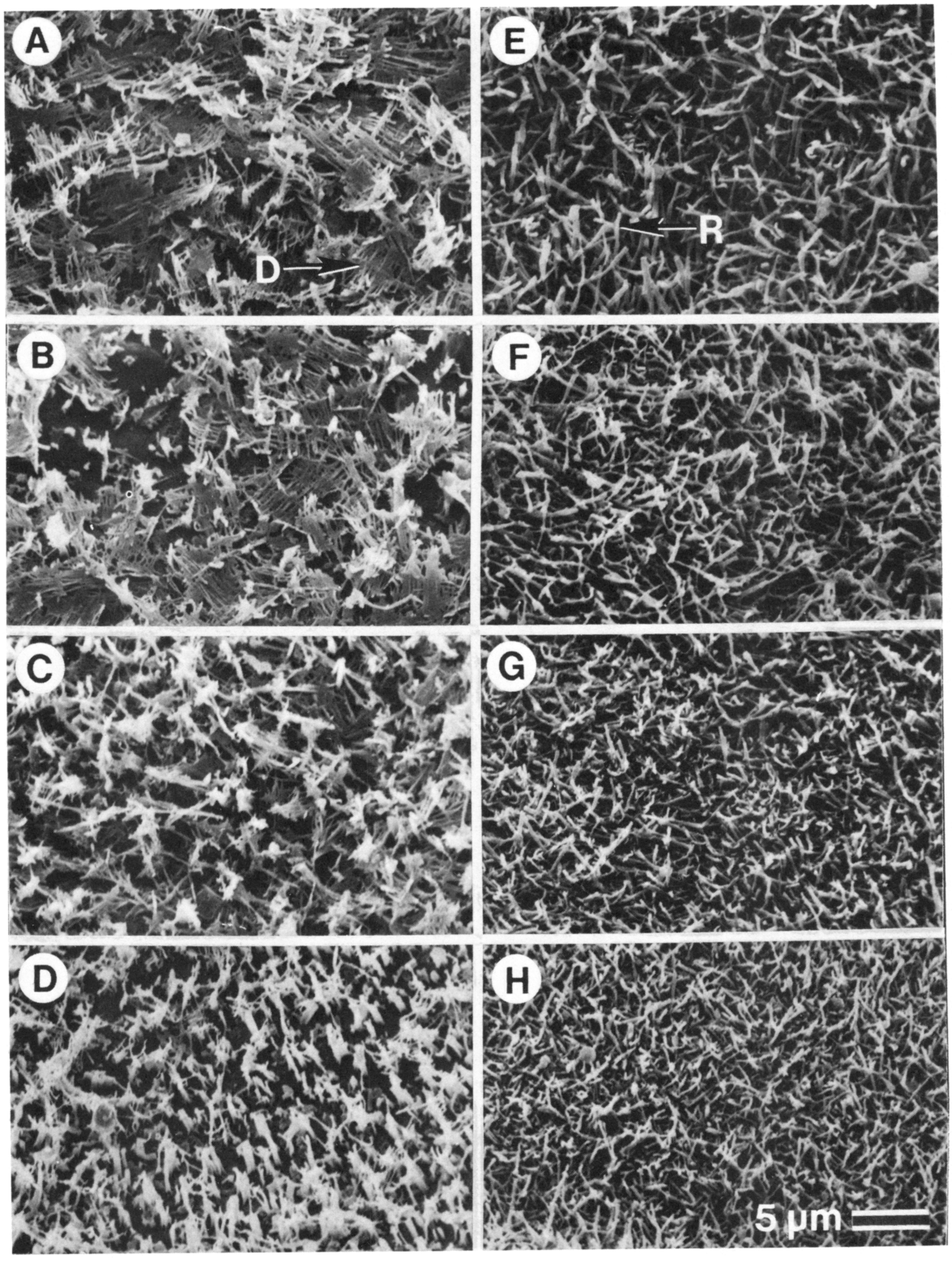

Fig. 1. Epicuticular waxes on the adaxial surface of Brussels sprouts (A–D) and carnation (E–H) leaves which developed in different temperature/light intensity combinations showing wax dentrites (D) and rods (R); all × 2,500.
A & E) 25°C/440 $\mu Em^{-2}s^{-1}$;
B & F) 25°/145 $\mu Em^{-2}s^{-1}$;
C & G) 15°/440 $\mu Em^{-2}s^{-1}$;
D & H) 15°/145 $\mu Em^{-2}s^{-1}$

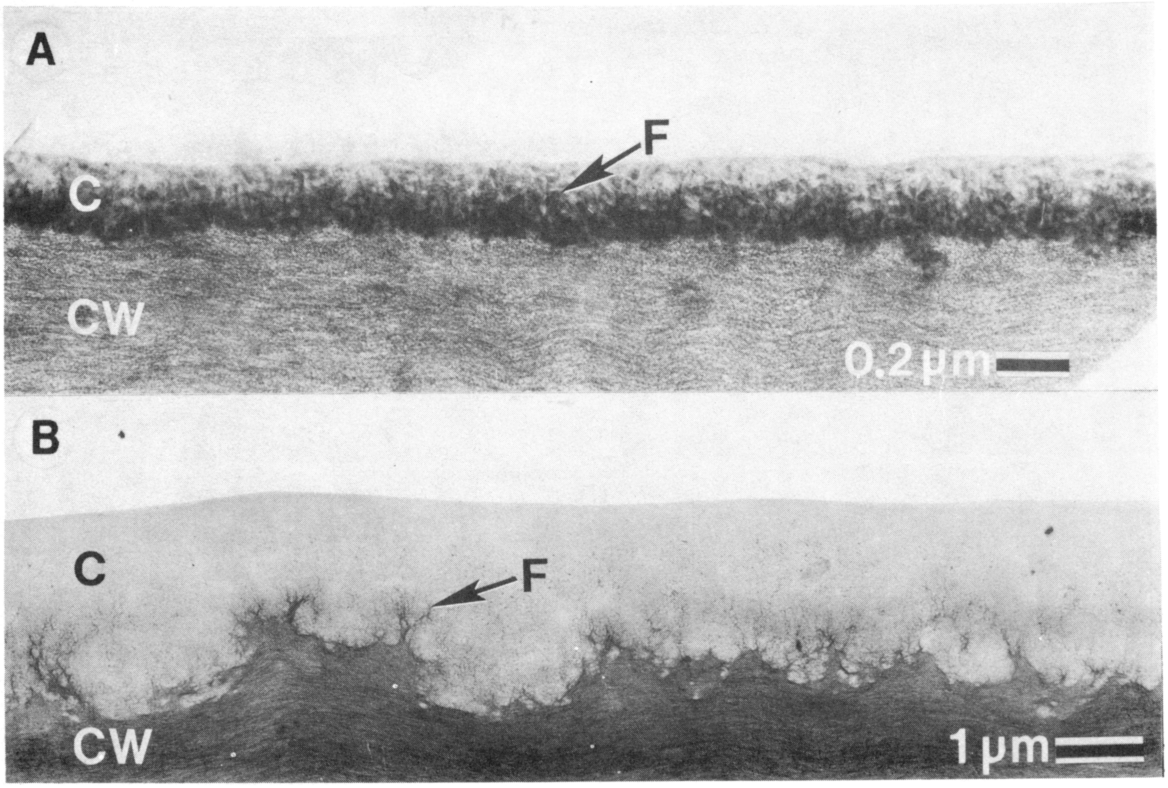

Fig. 2. Internal cuticle ultrastructure of the adaxial cuticle which developed at 15°C/145 µEm $^{-2}s^{-1}$ showing cell wall (CW) and fibrils (F) in the inner region of the cuticle (C). A) Brussels sprouts, × 47,000; B) Carnation, × 11,600

These studies demonstrate that environment may drastically affect the morphology, thickness, and wax content of cuticles. Further studies indicated that the permeability of isolated Brussels sprouts and carnation cuticles to Rb phosphate decreases when grown at lower temperatures and light intensities and that the decrease is correlated with increased cuticle thickness and wax content (19). This suggests that environment may alter important cuticular functions such as foliar absorption of beneficial and harmful compounds, leaching, and water loss.

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