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The Mode of Action of Fatty Alcohols on Leaf Tissue

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Abstract. The mode of action of a mixture of C_8 and C_{10} fatty alcohols, formulated in polyoxyethylene (20) sorbitan mono-oleate (SMO) and used as an emulsion (FAE) to inhibit axillary bud (sucker) growth in tobacco production, was studied using infrared spectroscopy (NIR), photoacoustic spectroscopy (PAS), electrical resistance, and the ability of treated cells to reverse plasmolysis on leaf tissues from *Nicotiana tabacum* L. and other dicotyledonous species. NIR spectra showed that isolated cuticles were affected optically when treated with FAE, but did not dissolve. PAS absorbances in the UV of isolated cuticles and of epidermal peels were similar and showed that cuticles were homogeneous, unilamellar structures. In intact leaf segments, it was possible, over time using PAS absorbances in the visible region, to separate absorbance of the surface components (cuticle) from the absorption of chlorophyll and other subsurface components and to monitor the penetration by FAE into the leaf. Penetration of the FAE to the subcuticular cells took approximately 2 h. Electrical resistance measurements of FAE-treated isolated midveins of tobacco leaves decreased with time, indicating that the plasma membranes of the cells became leaky. The effect of FAE on plasma membranes of cells was confirmed with *Elodea* sp. where leaf cells after treatment with 1 and 5% FAE lost the ability with time to plasmolyze upon exposure to a 10% solution of $Ca(NO_3)_2$. The results of the various studies were interpreted to mean that at the labeled concentration (4–5%) for use in the control of axillary bud growth on decapitated tobacco, FAE passed through the cuticle without disrupting it. However, the plasma membranes of the subtending cells were altered so that, in time, bud tissues desiccated (appeared burned) and growth of the sucker was controlled.

Mixtures of C_8 and C_{10} fatty alcohols, which usually include small amounts of the C_6 and C_{12} alcohols, formulated with polyoxyethylene (20) sorbitan mono-oleate (SMO), are some of the agents used in the control of axillary buds (suckers) in the culture of tobacco. Fatty alcohols as emulsions (FAE) are contact herbicides; they are not translocated and destroy tissue only at the point of contact. In the field, FAE are sprayed over the tops of plants or poured down the stalk so that axillary buds are wetted; they can be applied either when the apical bud appears or later to decapitated plants. FAE penetrate young sucker tissue rapidly, followed by darkening of the tissue, and then desiccation. One suggested mechanism is nuclear membrane damage (Steffens et al. 1967). Field tests (Aycock and McKee 1975, Mylonas and Pangos 1978) revealed that some injury may occur to leaves of tender, fast-growing plants if the spray emulsion accumulates on leaf surfaces or along leaf edges.

To better understand the mode of action of FAE, we examined the effects of the formulation Off-Shoot T on leaves of tobacco and other dicotyle-

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donous species. To measure infrared absorption, we chose infrared reflectance spectroscopy (NIR), which can provide information about translucent or opaque samples, such as leaf surfaces. For UV and visible absorption measurements we used the technique of photoacoustic spectroscopy (PAS) (Rosencwaig 1980). Electrical resistance measurements and plasmolysis were used to measure cell damage.

In PAS, a sample, such as a small leaf segment, is placed in a closed sample chamber with a window transparent to light. When light is directed onto the sample and is absorbed, electrons in the sample are excited to higher energy levels. As the excited electrons decay to ground state, the energy is dissipated primarily through nonradiative emission (heat), which causes expansion of the air in the sample chamber. If the incident light is interrupted (chopped) at a given frequency, the air in the chamber will expand and contract, producing sound waves detectable with a microphone. PAS offered two advantages for this study: first, it could be used on solid samples requiring little or no sample preparation, such as leaves; and second, it provided not only absorbance readings, but also a relative measure of the depth of the absorbing layer within the sample. This depth measurement was possible because there was a lag time between the excitation or absorbance and the generation of sound waves. The deeper within a sample the absorbance occurred, the longer it took for heat to reach the surface of the sample and heat the pocket of air. The separation of components of a spectrum based on these lag times is known as depth profiling.

In the present study, we used PAS depth profiling to demonstrate that a FAE enters a leaf through the cuticle and to measure its rate of penetration. Although the cuticle was not destroyed from FAE treatment, NIR was used to demonstrate that the cuticle may be slightly altered. We also determined from electrical resistance measurements and plasmolysis that after penetration a FAE disrupted the plasma membrane of cells in the tissue below the cuticle, causing desiccation and tissue death.

Materials and Methods

Fatty Alcohols

The fatty alcohol formulation, Off-Shoot T, used in these studies was obtained from Cochran Corporation. The active ingredients (85%) were 0.5% C_6, 42% C_8, 56% C_{10}, and 1.5% C_{12} fatty alcohols. All dilutions were made with respect to the formulation.

Isolation of Cuticles

Cuticles were isolated by taking leaf disks 2 cm in diameter with a cork borer from leaves of *Nicotiana tabacum* L. cv NC 2326 and *Ficus elastica* Roxb. ex Hornam. The disks were vacuum infiltrated with 0.5% pectinase in 0.1 mM potassium phosphate buffer, pH 3.5, with Thimerosal 1:10,000 (vol:vol) (Ammons 1954) added as a bactericide. The disks were then incubated in the buffer–bactericide solution at 40°C for 12 h after which the cuticles were removed.

NIR Measurements

To prepare harvested cuticles from *Nicotiana tabacum* L. for the NIR measurements, they were floated on water over filter paper in petri dishes; then the water was removed from under the cuticles with a pipet. A 10% FAE was added to one dish and water to the other as control. After 2 h the FAE and the water were removed from the respective dishes and the cuticles were rinsed with water and allowed to dry overnight on the filter paper. FAE and water filter paper controls were also prepared. A disk, 3.2 cm in diameter, with or without a mounted cuticle was cut from each filter paper. Infrared reflectance spectra were obtained from isolated cuticles with a spectrometer–computer system described elsewhere (McClure and Hamid 1980).

PAS Measurements

Photoacoustic spectra were obtained from cuticles removed from *Ficus elastica* Roxb. ex Hornam. and *Nicotiana tabacum* L., and from abaxial epidermal peels from *Nicotiana glauca* L., with a Gilford Model R-1500 Photoacoustic Spectrometer interfaced with an Apple II Plus computer at a chopping frequency of 40 Hz. Cuticles and epidermal peels were scanned from 200–400 nm or from 400–800 nm. Cuticles from *Ficus elastica* were soaked overnight in a 5% FAE, washed, and compared with controls over the region 200–400 nm.

For the study of cuticle integrity and FAE penetration using PAS, sections of leaf lamina lacking large veins from *Lamium amplexicaule* L., *Taraxacum officinale* L., *Rosa sp.* L., and *Nicotiana tabacum* L. cv Xanthi were cut to fit the 0.5 × 1.0 cm sample holder with two razor blades fastened together and spaced 0.5 cm apart. There was no further sample preparation. Since the FAE provided no suitable chromaphore between wavelengths of 200 and 800 nm, a dye with an absorption maximum at 550 nm (Sudan III, dark red color) was dissolved in the FAE. A small drop (5 μl) of Sudan III-labeled FAE (5%) was added to the leaf segment in the sample chamber. It was then possible to monitor the absorption peaks of the cuticle (~400 nm), the Sudan III/FAE (~550 nm), and chlorophyll (~680 nm), independently.

Electrical Resistance Measurements

The effect of a FAE on the electrical resistance of tobacco tissue was studied using leaf veins that were impaled across two electrodes made from sewing needles 1 cm apart and connected to a Heathkit Impedance Bridge, Model 1B-2A. The power source was a 600 Hz, 2 V square wave input from a Heathkit Sine-Square Wave Generator, Model EUW-27. A square wave from an external source was used because the generator voltage of the impedance bridge was too high and would have caused a "breakdown" in the tissue analogous to the irreversible break-

down of a capacitor in an electrical circuit. To increase the accuracy of the resistance measurements, a Heathkit Decade Resistance Box, Model 1N-17, was used to place a 100 Ω resistance in parallel with the leaf veins.

Veins were obtained from greenhouse grown *Nicotiana tabacum* L. cv Xanthi plants that supplied a large number (30) of leaves of relatively uniform size just prior to flowering. An equal number of veins (40) were selected for uniformity in size from both sides of the midribs of the leaves and harvested with a 2.0-cm cork borer. The tissue disks were placed in beakers of water to keep them hydrated during collection. The veins were then separated from the lamellar tissue and placed on wet filter paper in covered, deep petri dishes. Veins taken from the left side of the midrib were treated with a 5% FAE, whereas veins from the right side were used as controls. Fresh weight and electrical resistance were determined on each of 10 veins chosen at random from both treated and control groups every 2 h up to 8 h. This procedure was repeated with 40 veins using a 5% solution of 15% SMO in place of the 5% FAE.

In another test, the effect of a FAE on the electrical resistance of veins treated similarly was obtained as the veins were exposed to the laboratory environment on dry paper towels. The weight and corresponding electrical resistance of five different leaf veins were measured every 30 min for 2 h and then every 2 h for a total of 8 h.

Plasmolysis Measurements

The effect of a FAE on the integrity of the plasmalemma was determined using leaves of *Elodea sp.* L. Ten leaves were taken from near the apical meristem and soaked in either 1 or 5% FAE for 1, 5, and 10 min. Each leaf was washed in tap water and mounted on a microscope slide in a 10% (wt:vol) solution of $Ca(NO_3)_2$. The number of plasmolyzed and turgid cells were determined with a microscope. In order to minimize variability, observations were made from the outer edge to the midrib of the leaf approximately 50 cells from the apex measured along the margin. In all cases, the larger cells which plasmolyzed were considered to have intact plasmalemmas, whereas those that did not were considered to be damaged.

Results and Discussion

Destruction of leaf tissue could be easily explained if the FAE had dissolved the cuticle. However, isolated cuticles did not dissolve in 5% FAE, even after several days, although a drop of the formulation on an isolated cuticle did leave a translucent spot. In addition to this visual change, the FAE-treated and control cuticles also differed in the second derivatives of the infrared reflective spectra (Fig. 1A), with fewer differences between treated and control filter papers (Fig. 1B). The visual and infrared reflectance differences could arise from either of two facts: the FAE could have intercalated into the cuticular matrix (consistent with the translucent spot) or induced some structural change. In either case, although the optical properties were altered, dissolution of the cuticle did not occur.

In the UV-visible range, PAS spectra of detached cuticles showed a maximum absorption around 380–400 nm. Representative traces of the PAS absorption spectrum and the phase lag are shown for the UV region of 200–400 nm (*Nicotiana tabacum* L., Fig. 2, and *Nicotiana glauca* L., Fig. 3), and for the visible region of 400–800 nm (*Ficus elastica*, Fig. 4). Spectra from detached cuticles (e.g., Fig. 2) were similar to those of epidermal peels (e.g., Fig. 3). The phase lag of the spectra remained essentially constant throughout the absorption range, indicating that the samples behaved as homogeneous unilamellar structures over the wavelengths scanned; i.e., the absorbance at each wavelength originated from essentially the same depth within the sample. (At the red end of the spectrum, when absorption approached zero, the phase lag was not well defined and the signal became noisy, e.g., Fig. 4.) Treatment of the isolated cuticles with FAE produced no detectable differences in the PAS spectra.

Because the cuticle behaved as a single, well-defined layer that would contrast with the chlorophyll-bearing leaf interior, it was possible to take PAS spectra of intact leaf sections to determine whether the FAE penetrated the cuticle and entered the interior of the leaf. The FAE did not absorb light at wavelengths from 240–800 nm, so it became necessary to mark the FAE with the red dye Sudan III, which was soluble in fatty alcohols, insoluble in water, and absorbed maximally around 530 nm (Fig. 5).

Absorbance in the visible photoacoustic spectrum (400–800 nm) of an untreated leaf segment of *Rosa sp.* was high around 400 nm, lower in the region of 440–600 nm, and then high in the region of 600–720 nm (Fig. 6). The 400–440 nm band was clearly absorption of the cuticle (see Fig. 2) and the 600–720 nm peak was due to the absorption by chlorophyll. The phase lag was low in the 400–440 nm region, corresponding to absorption by the surface (cuticle), and high from 440–700 nm, corresponding to absorption by subsurface tissues. When the same leaf segment was spotted with the Sudan III/FAE and immediately scanned again from 400–800 nm (Fig. 7), the absorption curve now had three major peaks: 400 nm, 550 nm, and 680 nm corresponding to the cuticle, Sudan III/FAE, and chlorophyll, respectively. The phase lag now had two minima corresponding to the cuticular absorption and the Sudan III/FAE absorption. The phase lag was exaggerated at 680 nm compared to the untreated sample, because the sample increased in thickness when the FAE was placed on the surface; the same effect reduced the amplitudes of both the 400 and 680 nm absorbance peaks.

After the FAE was allowed to penetrate the leaf,

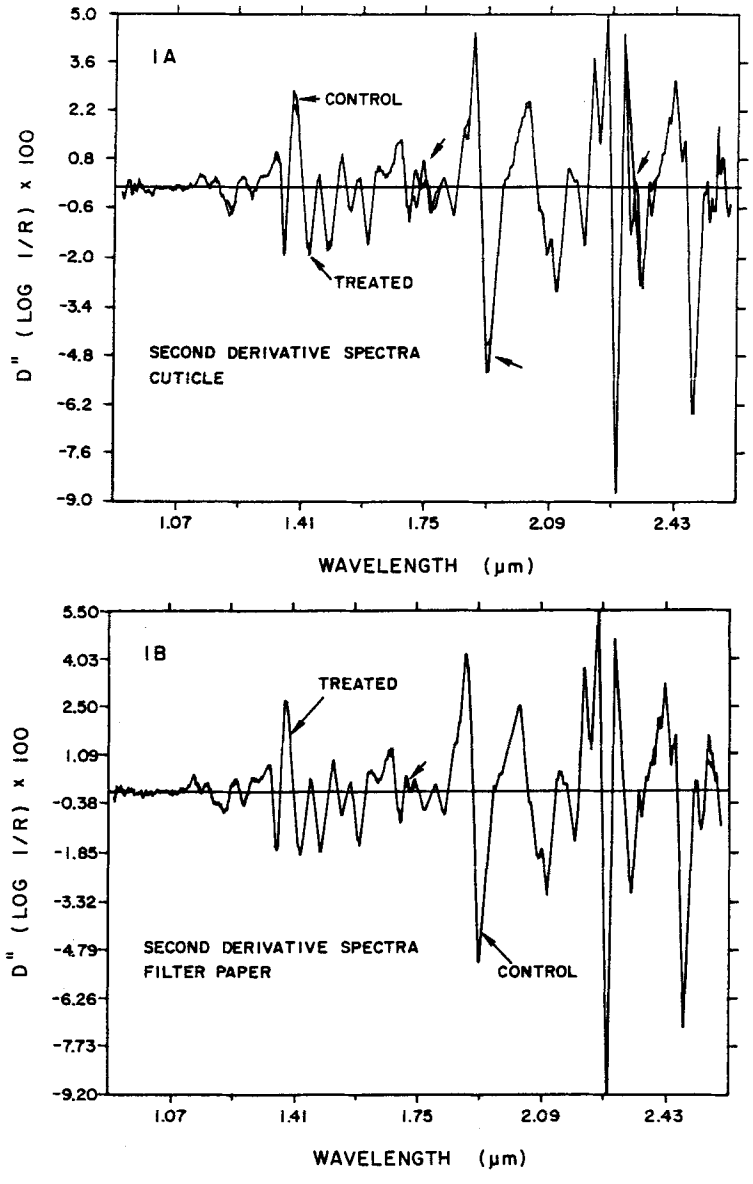

Fig. 1. (**A**) Infrared absorption spectra (2nd derivative) of isolated tobacco leaf cuticles treated with FAE and control. (**B**) Infrared absorption spectra (2nd derivative) of filter paper treated with FAE and control.

the phase lag was more nearly constant over the entire range from 400–800 nm (Fig. 9) because both the cuticle (and probably the intracuticular matrix as well) and the tissue beneath were saturated with the FAE. The FAE changed the thermal diffusivity of the sample making it thermally homogeneous. Further evidence of cuticular saturation was obtained from observing *Nicotiana glauca* L. leaf segments. On treated surfaces, the contact angle of a droplet of water was the same as on untreated surfaces when the FAE was washed off before penetration. However, if the FAE were allowed to penetrate the leaf and the leaf was then washed to remove external FAE, the contact angle was decreased to 0°; i.e., water would not bead on a leaf saturated with FAE.

It was possible to phase separate the surface components from the interior components of a spectrum (Fig. 8). The component with a phase lag of 0° contained the cuticular and FAE (Sudan III) absorption, while the component with a phase lag of 120° contained the chlorophyll (650–750 nm) and other subsurface absorption between 450 and 650 nm (compare to Fig. 6). When the Sudan III/FAE had completely saturated the leaf, as evidenced by a water-soaked appearance, the differences in phase lags were significantly decreased (Fig. 9). The Sudan III absorption peak was not as well defined and was shifted toward shorter wavelengths (compare to Fig. 7). This may have occurred due to the saturation of the cuticle with the FAE, which could change the thermal properties of the cuticle,

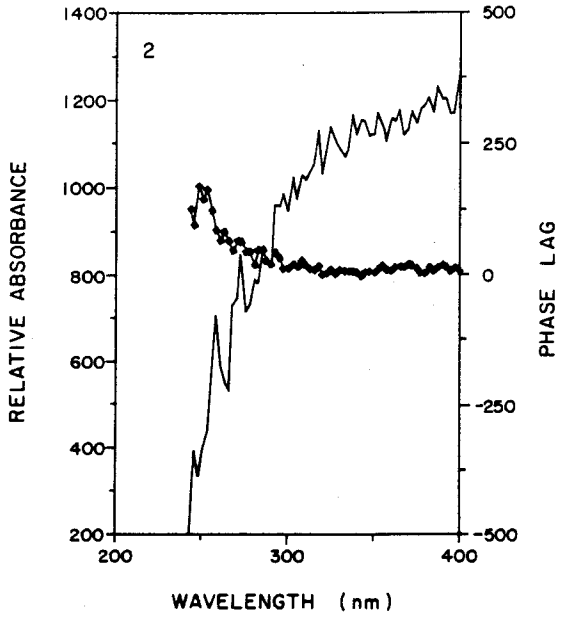

Fig. 2. PAS spectrum of an isolated cuticle from *N. tabacum* L.: —, relative absorbance; ♦, phase lag in degrees. Gain = 1.0; modulation frequency = 37 Hz.

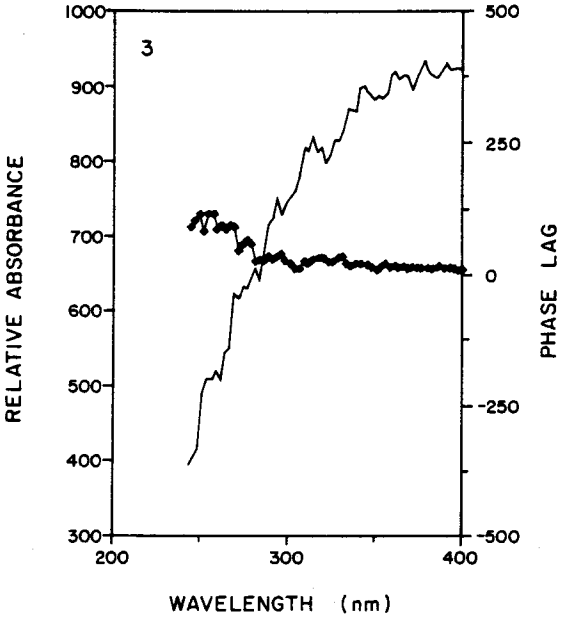

Fig. 3. PAS spectrum of an epidermal peel of *N. glauca* L.: —, relative absorbance; ♦, phase lag in degrees. Gain = 1.8; modulation frequency = 19–21 Hz.

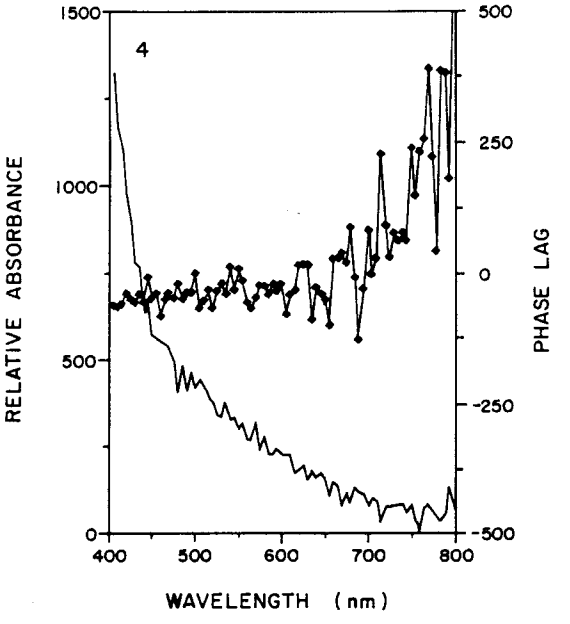

Fig. 4. PAS spectrum of an isolated cuticle from *Ficus elastica* Roxb. ex Horman.: —, relative absorbance; ♦, phase lag in degrees. Gain = 0.027; modulation frequency = 43–47 Hz.

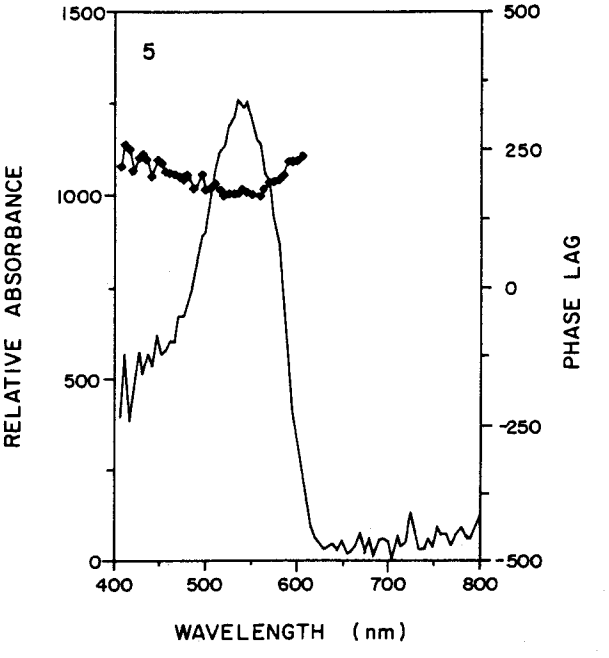

Fig. 5. PAS spectrum of Sudan III dissolved in FAE: —, relative absorbance; ♦, phase lag in degrees. Gain = 0.02; modulation frequency = 40 Hz.

or to some physical change in the interior of the leaf. The same results were obtained using tissue from *Lamium amplexicaule* L., *Taraxacum officinale* L., and *Nicotiana tabacum* L. cv Xanthi (data not shown).

The fresh, untreated rose leaf segment (see above) behaved as a biphasic or a two-layer structure as opposed to the unilamellar property of the isolated cuticles. A higher modulation frequency

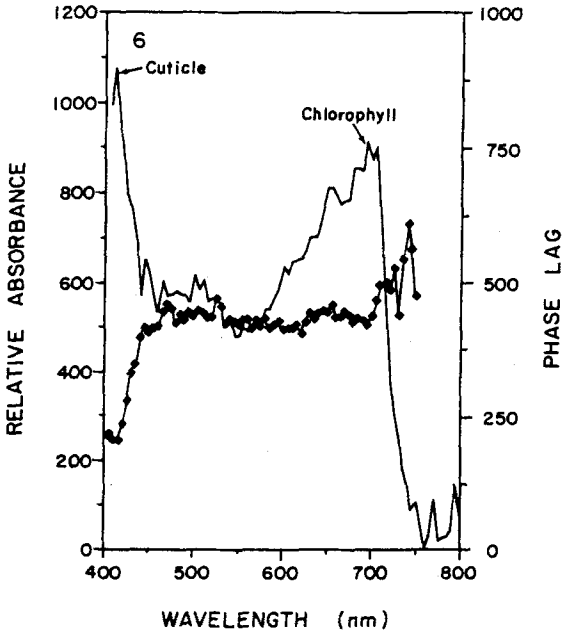

Fig. 6. PAS spectrum of a fresh leaf of *Rosa sp.* L.: —, relative absorbance; ♦, phase lag in degrees. Gain = 0.03; modulation = 40–41 Hz.

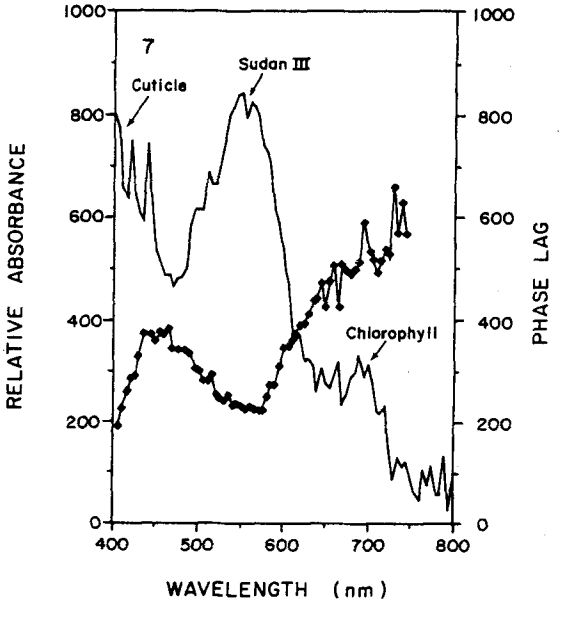

Fig. 7. PAS spectrum of the leaf in Fig. 6 immediately after the application of 5 μl of Sudan-labeled FAE: —, relative absorbance; ♦, phase lag in degrees. Gain 0.03; modulation frequency = 40–42 Hz.

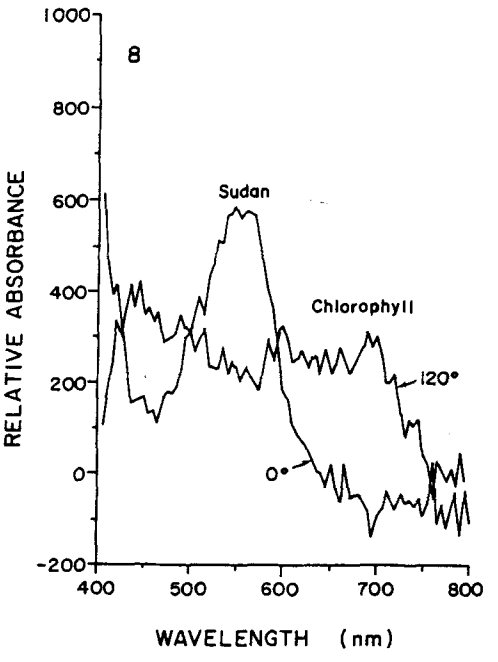

Fig. 8. Separation of the components of the PAS spectra in Fig. 7. Vector projections were taken at 0° and 120°.

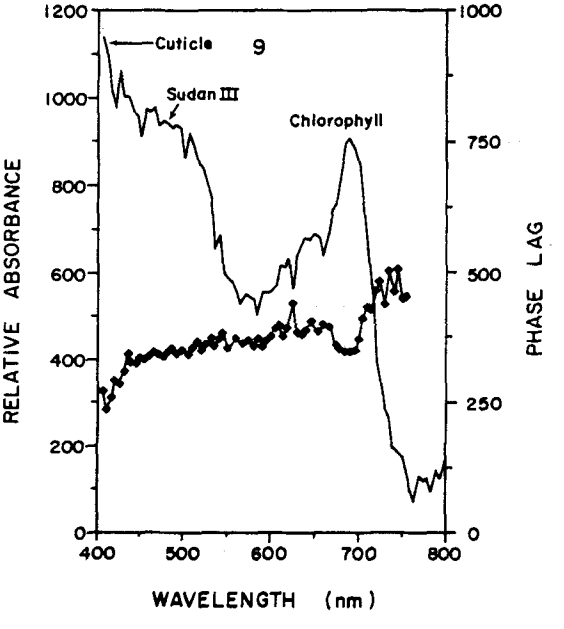

Fig. 9. PAS spectrum of a leaf of *Rosa sp.* L. after Sudan-labeled FAE was allowed to permeate the tissue: —, relative absorbance; ♦, phase lag in degrees. Gain = 0.03; modulation frequency = 30–40 Hz.

would have given a signal only from the upper layer (cuticle) in the fresh leaf segment. In these experiments a modulation frequency of about 40 Hz was used because this frequency allowed enough time for thermal energy to escape from the chlorophyll-containing regions of most leaves. Even at this fre-

quency, the chlorophyll signal of *Lamium amplexicaule* L. (not shown) was very weak due to the thickness of the trichomes on the leaf surface.

When the Sudan III/FAE was added to the *Rosa sp.* leaf segment, a three-component spectrum was created (cuticle, FAE, and chlorophyll). The triphasic spectrum confirmed that the Sudan III/FAE did in fact penetrate to the interior of the leaf and also allowed the location of the FAE to be tracked, as a function of time, as the FAE penetrated the two leaf layers. However, it was not possible to determine to what extent the stomata were involved. The penetration of the FAE into the leaves was monitored by comparing the phase lag of the cuticle, the FAE, and the chlorophyll components of the absorption spectrum with appropriate phase separation vector projections as in Fig. 8. In *Rosa sp.* penetration took approximately 2 h.

As a further explanation, in a two-layered structure, PAS spectra can be separated into components according to the phase lag. To separate the absorption at a given wavelength into the absorption of the surface layer and the absorption of the interior layer, the absorption vector (magnitude plus phase lag angle) must be projected onto the theoretical phase angle of the surface and onto the theoretical phase angle of the interior. The projected surface and interior absorption magnitudes can be computed using the expression $A\cos(x_a - x_b)$, where A is the absorption amplitude at the wavelength in question, x_a is the experimental phase lag in degrees, and x_b is the theoretical phase lag angle of the surface or of the interior. Whenever $x_a = x_b$, then the original spectrum will be retraced for that wavelength because $A\cos 0° = A$. Either layer may contain minor contributions from components in the other layer unless the two layers are 90° out of phase ($A\cos 90° = 0$). Ideally, one would adjust the modulation frequency such that the interior component of the signal is 90° out of phase with the surface component; the quality of the separation of two components increases as the phase difference between them approaches 90°.

To further explore the question of foliar entry, a leaf segment of *Taraxacum officinale* L. was spotted with approximately 5 μl of Sudan III/FAE and the spectra were obtained from 400–800 nm at 15-min intervals. The phase lag was averaged from 500–600 nm to indicate absorption of Sudan III/FAE and from 650–750 nm to indicate absorption of chlorophyll. In the untreated leaf (time = 0) both phase lags were the same and indicated absorption only in the leaf interior. When the two average phase lags were plotted as a function of time after the addition of the Sudan III/FAE (Fig. 10), the

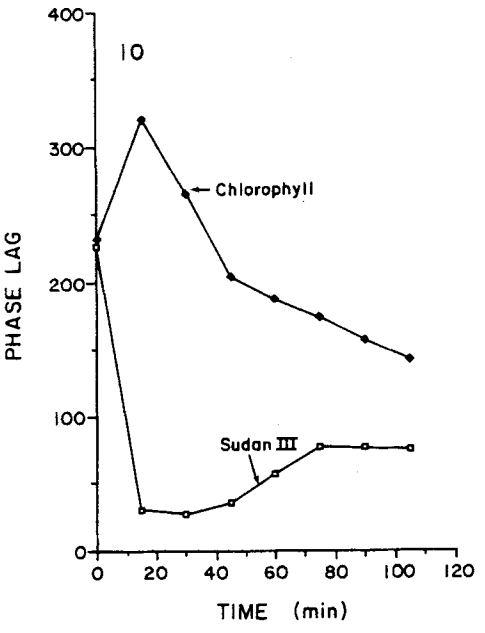

Fig. 10. Average phase lag between 500 and 600 nm (□) and 650 and 750 nm (◆) over time.

Sudan III/FAE absorption appeared at the surface of the leaf (near zero phase lag) and slowly increased in depth (increased phase lag) over time. The phase lag for the chlorophyll absorption increased immediately after addition of Sudan III/FAE because of the extra thickness of the FAE on the surface; as the FAE entered the leaf and altered its thermal conductivity, the chlorophyll phase lag then decreased.

Once it was determined that FAE had indeed entered the leaf tissue, it was logical to ask whether the plasma membrane was being adversely affected by the FAE. The cell membrane can be described as a dielectric bounded by the conducting intra- and extracellular media (Schanne and Ceretti 1978); i.e., the cell acts as a capacitor. It is well accepted that membrane damage will allow ions to be released into the apoplastic regions causing a large local increase in ion concentration and thereby decreasing the electrical resistance of the tissue (Tattat and Blanchard 1976). The electrical resistance of excised veins of *Nicotiana tabacum* L. cv Xanthi decreased significantly after treatment with FAE compared to the control (Fig. 11). There was no significant decrease in the SMO-treated veins compared to the control (not plotted).

The veins that were exposed to the laboratory environment and were weighed prior to the resistance measurements showed that no significant change in weight occurred over time among the un-

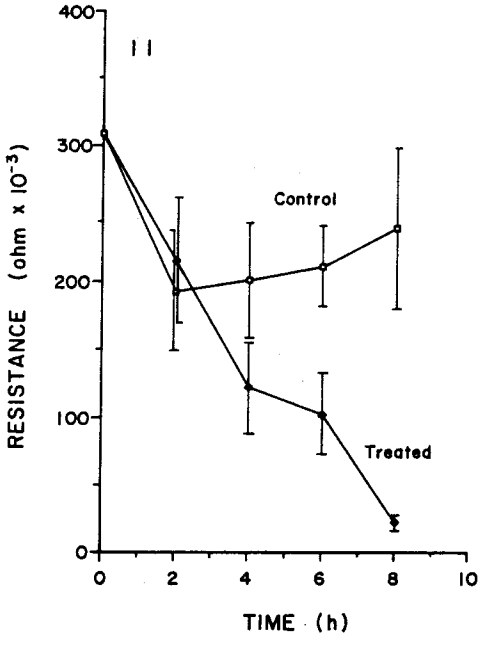

Fig. 11. Electrical resistance of the leaf veins over time: ♦, FAE treated; □, control.

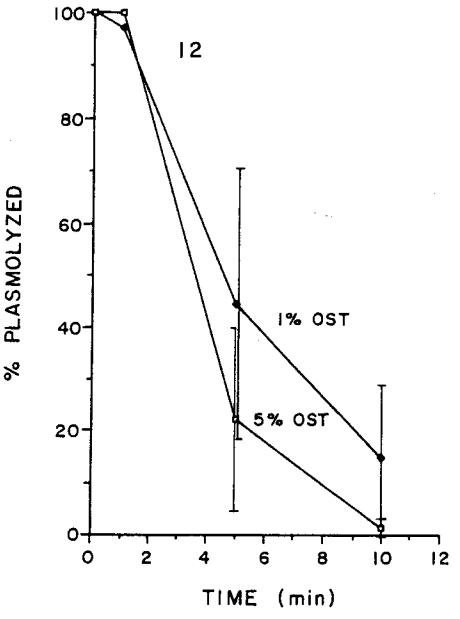

Fig. 12. Percentage of leaf cells of *Elodea sp.* that plasmolyzed in the presence of 10% $Ca(NO_3)_2$ after being immersed up to 10 min in 1% (♦) and 5% (□) FAE (OST).

treated, the SMO-treated, and the FAE-treated veins, but resistance measurements decreased in those that were treated with FAE. These results suggested that damage to the plasma membrane occurred prior to loss of weight; i.e., desiccation. Thus, the initial pathic event that determined the control of suckers by FAE was not desiccation, as has been reported earlier (Tobacco Information 1983), but rather was the effect of the FAE on the plasma membranes with the result being damaged tissue that lost water with time and appeared desiccated (burned).

The plasmalemma integrity measurements on *Elodea sp.* leaves immersed in water, 1% FAE, and 5% FAE for 1, 5, and 10 min, respectively, showed that the cells lost the ability to plasmolyze when exposed to the $Ca(NO_3)_2$ solution with increased exposure time and increased concentrations of FAE (Fig. 12). In leaves immersed in water all cells plasmolyzed. Assuming that the integrity of the plasma membrane of a cell is necessary for the cell to plasmolyze, these results showed that the plasma membrane was altered directly by the FAE concentration and that this alteration increased with exposure time.

Conclusions

It was concluded from NIR that the leaf cuticle was altered optically but not dissolved by the application of the FAE. The PAS of leaves treated with Sudan III/FAE showed that the FAE did penetrate to the interior of the leaf because the absorption of the dye could be observed strongly on the surface initially, and then more weakly in the interior after incubation. PAS depth profiling showed that the FAE entered the leaf through the cuticle, but the role of the stomata was not determined. Following penetration of the cuticle, tissues below the cuticle were injured by treatment with the FAE as determined by a decrease in electrical resistance and the failure of leaf cells to plasmolyze. Thus, it is the loss of integrity of the plasma membrane which results in tissue desiccation, intepreted as control, when a FAE is used to control suckers in the production of tobacco.

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