J. Amer. Soc. Hort. Sci. 126(4):486–490. 2001.

Foliar-Applied Surfactants and Urea Temporarily Reduce Carbon Assimilation of Grapefruit Leaves

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Additional index words. *Citrus paradisi*, cuticular penetration, Ortho X-77, scanning electron microscopy, Silwet L-77

Abstract. Although urea can be an effective adjuvant to foliar sprays, we examined effects of additional surfactants on urea penetration through leaf cuticles along with the effect of urea with and without surfactants on net gas exchange of leaves of 'Marsh' grapefruit (*Citrus paradisi* Macf.) trees budded to Carrizo citrange (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.) rootstock. Various combinations of urea, a nonionic surfactant (X-77), and an organosilicone surfactant (L-77), were applied to grapefruit leaves and also to isolated adaxial cuticles. When compared to X-77, L-77 exhibited superior surfactant features with smaller contact angles of droplets deposited on a teflon slide. Both L-77 and X-77 initially increased penetration rate of urea through cuticles, but the effect of X-77 was sustained for a longer period of time. The total amount of urea which penetrated within a 4-day period, however, was similar after addition of either surfactant. Solutions of either urea, urea + L-77, urea + X-77, or L-77 alone decreased net assimilation of CO_2 (A_{CO2}) for 4 to 24 hours after spraying onto grapefruit leaves. A solution of X-77 alone had no effect on A_{CO2} over the 4-day period. Although reductions in A_{CO2} were similar following sprays of urea formulated with two different surfactants, the underlying mechanisms may not have been the same. For the urea + X-77 treatment, X-77 increased the inhibitory effects of urea on A_{CO2} indirectly by increasing penetration of urea into leaves. For the urea + L-77 formulation, effects of L-77 on A_{CO2} were 2-fold, direct by inhibiting A_{CO2} and indirect by increasing urea penetration. One hour after application, scanning electron microscopy (SEM) of leaf surfaces treated with X-77 revealed that they were heavily coated with the residue of the surfactant, whereas leaves treated with L-77 looked similar to nontreated leaves with no apparent residues on their surfaces. The amount of X-77 residue on the leaves was lower 24 hours after application than after 1 hour as observed by SEM.

Foliar application of urea to citrus has become a common practice not only to increase leaf N (Lea-Cox and Syvertsen, 1995), but also to enhance flower induction, fruit set, and fruit yield (Albrigo, 1999; Ali and Lovatt, 1992; Rabe, 1994). Weekly sprays of urea can increase net assimilation of CO_2 within 2 to 3 weeks in 'Duncan' grapefruit (*Citrus paradisi*) leaves (Romero-Aranda and Syvertsen, 1996). On the other hand, urea sprays can be phytotoxic when applied at high rates (Bondada et al., 1995) or when mixed with noncompatible agrochemicals. When urea is sprayed onto leaves, it first must pass through the waxy cuticle which covers all aerial organs of plants (Martin and Juniper, 1970). The mostly lipoidal composition of the cuticle makes it highly impenetrable for water and polar compounds. Urea may diffuse through the citrus cuticles through micropores traversing the cuticular matrix and/or by solubilization in cuticular lipids (Schönherr and Schmidt, 1979). Urea can also act as an adjuvant because it can increase the efficacy of uptake of foliar sprays containing P, Mn, S, Mg, and Fe (Swietlik and Faust, 1984). Penetration of urea through isolated tomato (*Lycopersicon esculentum* Mill.) fruit cuticles increases with ambient temperature (Knoche et al., 1994).

Use of adjuvants to enhance uptake of foliar-applied chemicals is common in modern agriculture (Stock and Holloway, 1993). Adjuvants may act as antifoaming agents, buffers, emulsifiers, spreaders, stickers, and wetting agents. Adjuvants can increase cuticular penetration, diffusion into the apoplast, uptake into leaf cells, and leaf metabolism (Schönherr and Baur, 1996). Action of organosilicone surfactants has been attributed to their ability to increase the spread of droplets by lowering surface tension (Schönherr and Bukovac, 1972). Nonionic surfactants can increase cuticular penetration of an active ingredient (AI) through complex interactions between the AI, surfactants, and leaf surfaces (Sharma et al., 1996; Stock and Holloway, 1993).

Some adjuvants can enhance uptake of certain substances but retard the uptake of others (Swietlik and Faust, 1984). There is also evidence of species-specific effects of adjuvants on uptake of some AI that must depend on specific cuticular characteristics (Field et al., 1992; Gaskin and Stevens, 1993). Although the organosilicone surfactant, L-77, facilitated foliar uptake of Fe into chlorotic grapefruit leaves, this surfactant induced disruption of cells in beet (*Beta vulgaris* L. ssp. *vulgaris*) root tissue (Neumann and Prinz, 1974). In addition, L-77 can induce negative effects on citrus fruit color development (Greenberg et al., 1987). Thus, there is the question whether surfactants alone can have physiological effects on plant tissues.

This study was undertaken to determine effects of two types of surfactants, X-77 and L-77, on penetration of urea into grapefruit leaves. We also evaluated net CO_2 assimilation (A_{CO2}) following foliar sprays of urea with and without these surfactants as an indicator of short-term physiological responses of leaves to these spray treatments.

Materials and Methods

Plant material and cuticle isolation. Fifteen-year-old 'Marsh' grapefruit (*Citrus paradisi*) trees budded to 'Carrizo' citrange (*C. sinensis* x *Poncirus trifoliata*) rootstock were used in this study. Trees were part of an unsprayed orchard at the University of Florida, Citrus Research Education Center, Lake Alfred. Fully expanded, 6- to 7-week-old spring flush leaves were harvested (15 Apr. 1999) from these trees for cuticle isolation. All leaves were from sun-exposed, south-facing canopy positions at a height of 1 to 2 m.

Received for publication 15 Apr. 2000. Accepted for publication 29 Mar. 2001. This research was supported by the Florida Agricultural Experiment Station and a grant from the Florida Citrus Production Research Advisory Council and approved for publication as journal series R-7481. We acknowledge assistance of M. Singh and S. Sharma in contact angle measurements and Stephen Sax in statistical analyses. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.
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Cuticles were isolated from leaf disks (1.77 cm^2) cut from the midlamina area of leaves avoiding major veins (Yamada et al., 1964). Leaf disks were digested in a pectinase (4% w/v) + cellulase (0.4% w/v) solution which contained Na–citrate buffer (50 mM) adjusted to pH 4. To prevent growth of microorganisms, sodium azide at 1 $mmol \cdot L^{-1}$ was included in the solution. Leaf disks were incubated for 4 d at room temperature and were exposed intermittently to a vacuum to facilitate infiltration of digestion enzymes into the tissue. Cuticles were gently separated from the tissue under flowing water and any cellular debris remaining on the cuticles was redigested overnight in the enzymatic solution then rinsed for 2 to 4 h in running distilled water. After rinsing, cuticles were floated onto a piece of teflon and left to air dry. Intact adaxial cuticles without stomata were selected by microscopic inspection and stored at room temperature for urea penetration experiments.

Measurement of cuticular penetration. Measurements of cuticular penetration were done as described previously (Petracek et al., 1998). Isolated adaxial cuticles were held with their outer surface up in a 2.8 cm diameter plexiglass ring holder. The ring holder was mounted above a 4 mL reservoir filled with well-stirred, deionized water which was in full contact with the inner cuticle surface. The reservoir had a sidearm for sampling. At time zero, three 1-μL droplets of radioactively labeled urea (concentration = 2.8 $mmol \cdot L^{-1}$; activity of $\approx 60 \times 10^6$ Bq) were deposited onto the cuticle. Urea ^{14}C (Sigma, St. Louis) had a specific activity of 2.05×10^9 $Bq \cdot mmol^{-1}$ and purity of ≥98%. At specific time intervals after urea deposition, 100-μL samples were withdrawn from the reservoir through the side arm, added to 4 mL of scintillation liquid (SX 18-4, Fisher, St. Louis) and placed in a liquid scintillation counter (LS 6000SC; Beckman Coulter, Inc., Fullerton, Calif.) to assess the amount of radioactivity which had diffused through the cuticle. The 100 μL of solution taken from the reservoir was replenished with distilled water after each withdrawal. At the end of each experiment, cuticles were dried, scraped from the cuticle holder, and radio-assayed for the presence of residual ^{14}C urea. All penetration experiments were done at ambient air temperature (28 ± 2 °C) and a relative humidity (RH) of 35% to 50%. Each experiment was conducted three times with two to five replicate cuticles for a total of 10 replicate cuticles tested for each treatment.

Measurement of contact angles. Aqueous solutions of surfactants were freshly prepared at concentrations of 0.15% (v/v) for L-77 and 0.3% (v/v) for X-77. These concentrations were chosen because they are just above the critical micelle concentration for each surfactant when tested on at least five different weedy plant species (Sharma and Singh, 1999). Nonionic Ortho X-77 is a mixture of aklylaryl-polyoxyethylene glycols, free fatty acids, and isopropanol (Loveland Industries, Greenley, Colo.). The organosilicone Silwet L-77 is a polyalkene-oxide copolymer (Loveland Industries). Two to three 2-μL droplets were deposited onto the surface of a teflon slide and contact angles on two sides of the droplets were measured with a NRL contact angle goniometer (Rame-hart, Inc., Mountain Lakes, N.J.). Although solutions with surfactants interact with surfaces of both teflon and waxy citrus leaves similarly (Sharma et al., 1989), insufficient rigidity of the isolated cuticles caused the droplets to disperse such that we were not able to measure contact angles of surfactant solutions on cuticles. Thus, we used the teflon slide as a model to measure contact angles of solutions on leaf surfaces.

Gas exchange measurements. A_{CO2} was measured using 2-month-old, fully expanded leaves selected from the same trees that supplied leaf cuticles. All leaves were from a south-facing canopy position at a height of 1 to 2 m. Leaves were tagged and sprayed to runoff with a hand-sprayer using one of six solutions: urea alone, urea combined with X-77 or L-77, X-77, or L-77 alone and deionized water as a control. The urea concentration of 0.28 $mol \cdot L^{-1}$ (1.65% w/v) was high enough to increase leaf N and A_{CO2} (Romero-Aranda and Syvertsen, 1996) but well below concentrations that are phytotoxic (Lea-Cox and Syvertsen, 1995). Surfactant concentrations were 0.15% (v/v) for L-77 and 0.3% (v/v) for X-77. Spray treatments began at 0900 HR on selected, clear days. At designated time intervals from 1 h to 4 d after spraying, light-saturated (photosynthethic photon flux >1000 $mmol \cdot m^{-2} \cdot s^{-1}$) A_{CO2}, stomatal conductance, and substomatal CO_2 concentrations were determined using a closed, portable photosynthesis system (LI-6200; LI-COR, Inc., Lincoln, Nebr.) with a 0.25-L leaf chamber. Although each experiment was conducted four to eight times at weekly intervals, not all treatments were included in all experiments. Three to five different leaves were used in each treatment so that averaged A_{CO2} measurements represent 20 to 33 replicate leaves per treatment. This pooling of replicate leaves was valid since, during all measurements of A_{CO2}, leaf temperature only varied from 30 to 35 °C and RH ranged from 30% to 50%. Each leaf was sprayed only once. When surfactant solutions alone were sprayed, A_{CO2} measurements were conducted twice using four different leaves in each treatment so there were eight replicate leaves per treatment.

Scanning electron microscopy. Small (5 × 5 mm) tissue samples were cut from the midlamina area of mature 2-month-old leaves similar to those used in gas exchange measurements. A droplet of surfactant solution was placed on the upper (adaxial) surface, and leaf samples were attached to metal stub holders using double-sided sticky copper tape. Stubs were placed in small baskets, plunged into liquid nitrogen, then freeze dried using a modified version of the method described by Katoh and Matsumoto (1980) but omitting the acetonitrile step. After drying, the samples were remounted on microscope stubs, coated lightly with gold–palladium (Katoh and Matsumoto, 1980), and their surfaces examined with a Hitachi S530 scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

Statistical analyses. All experiments were analyzed as a completely randomized design. Treatment means were compared at $P \leq 0.05$ using Duncan's multiple range test.

Results

Contact angles of 2-μL droplets of the urea solution alone on the teflon slide averaged 89.8 ± 0.31%. In contrast, contact angles of urea droplets with L-77 decreased to 16.3 ± 0.78% and 34.3 ± 0.58% for urea + X-77.

Addition of X-77 and L-77 to urea solutions greatly augmented the initial penetration rates of urea, which reached a maximum at 20 min (Fig. 1). Thereafter, the rate of urea penetration declined gradually for solutions containing surfactants. Initial penetration rate for urea alone increased to a maximum at 40 min after deposition. After 1 h, urea penetration rate for the urea + L-77 solution declined to the same level as that of urea alone. The penetration rate for urea + X-77 was higher initially than the penetration rate of urea alone, but after 2 h, rates did not differ (Fig. 1).

Addition of either L-77 or X-77 increased the amount of urea penetration through cuticles within the first hour following deposition of droplets (Fig. 2). The penetration-promoting effect of L-77 decreased within 4 h, whereas enhancement of penetration by X-77 lasted for 24 h. Urea penetration was almost complete within the first 24 h after deposition, regardless of formulation. Ninety-six hours after urea application, the amount of urea that penetrated the cuticles

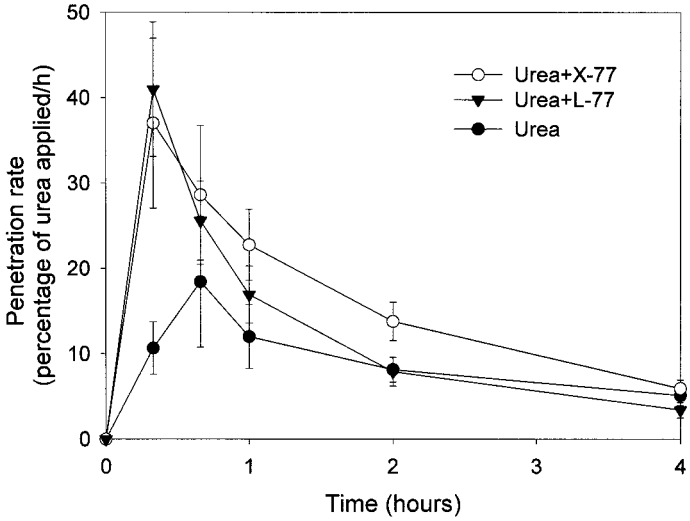

Fig. 1. Effect of surfactants on the penetration rate of urea through isolated 'Marsh' grapefruit leaf cuticles at 28 ± 2 °C and RH of 35% to 50%. Symbols represent means ± SE of 10 replicate cuticles.

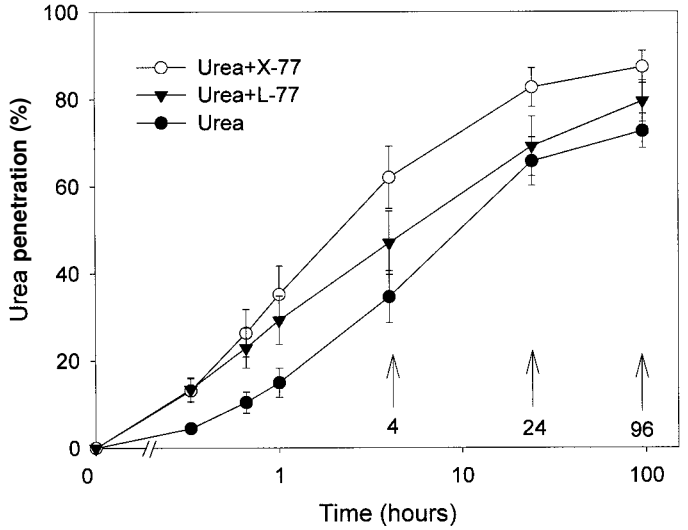

Fig. 2. Effect of surfactants on the percentage of the total amount of urea penetrated through isolated 'Marsh' grapefruit leaf cuticles at 28 ± 2 °C and RH of 35% to 50%. Symbols represent means ± SE of 10 replicate cuticles. Note log scale on X-axis.

was similar whether urea was applied alone or applied with L-77 but was slightly higher when urea was applied with X-77. When ^{14}C-urea was applied alone, with L-77 or X-77, residual radioactivity associated with the cuticles after 96 h was similar (1.5% to 4.5% of the initial amount applied).

Electron micrographs of the surface of nontreated (control) leaves revealed platelets of epicuticular waxes scattered over the area (Fig. 3A). Leaves treated with L-77 appeared similar to control leaves 1 h and 24 h after droplet application (Fig. 3A, C, and E). Thus, there were no visible traces of L-77 left on leaves. One hour after application of X-77, however, leaf surfaces were obscured by a heavy coating (Fig. 3B). Wax platelets were not visible since they were covered by the deposit of X-77. The leaf surface was less obscured after 24 h than after 1 h following application of X-77 droplets (Fig. 3D).

There was a transient reduction of net A_{CO2} following sprays with urea alone, L-77 alone, and urea formulated with either surfactant (Fig. 4). This reduction was most apparent during the first hour following treatment applications with reductions of 12% for urea alone, 27% for urea + L-77, and 33% for urea + X-77. There was a gradual recovery of net A_{CO2} to the level of water-treated, control leaves during the course of the experiment from 4 to 96 h. Four hours after treatment, A_{CO2} of leaves treated with L-77 alone, urea + L-77, and urea + X-77 were still significantly lower than that of control leaves. The A_{CO2} of all, except the leaves treated with L-77 alone, had recovered to the control level at 24 h. By 96 h after spraying, all treatment effects on A_{CO2} disappeared (Fig. 4).

Time courses of effects of spray treatments on stomatal conductance (data not presented) were similar to those on A_{CO2}. The average ratio of internal to external CO_2 concentration (Ci:Ca) was 0.78 ± 0.01 (SE) and did not differ significantly between treatments.

Discussion

Organosilicone surfactants (such as L-77) have a high surface activity which greatly reduces surface tension of solutions, enhances spreading over leaf surfaces, and in some cases, facilitates direct penetration through open stomata (Field et al., 1992). Consistent with known properties of organosilicone surfactants (Singh and Singh, 1995), droplets of urea + L-77 solution had the smallest contact angles. Cuticular penetration of urea within 24 h following application, however, was enhanced more by addition of X-77 (nonionic surfactant) than by L-77 (Fig. 2). Although these data were not consistent with their relative effects on contact angle, similar results have been obtained for uptake of glyphosate by six different species of grasses (Field et al., 1992; Gaskin and Stevens, 1993) and also by field bean (*Vicia faba* L) (Gaskin, 1995). In the first 8 h following application of glyphosate, leaf uptake of herbicide solution plus L-77 was greater than uptake of herbicide alone (Gaskin and Stevens, 1993). However, in the first 24 h, less glyphosate penetrated into the leaves when it was applied with L-77 than when glyphosate was applied alone. This suggested the antagonism may have been due to a rapid initial absorption into the cuticle of L-77 which could have affected pathways for cuticular penetration (Gaskin and Stevens, 1993). Our observations support this idea of rapid initial absorption. Within 1 h, scanning electron micrographs revealed no visible residue of L-77, suggesting it had already penetrated into the leaf (Fig. 3C). Penetration of urea through cuticles can also be affected negatively by volatilization of urea which is influenced by air-temperature and RH (Orbović et al., 2001).

Spraying leaves with urea alone, or with urea formulated with either surfactant, resulted in a transient inhibition of photosynthesis for only 1 h (Fig. 4). Although previous studies have described short-term reductions in A_{CO2} following foliar sprays of N–P–K–S solutions (Harder et al., 1982), our data are the first to describe inhibitory effects on A_{CO2} by urea or adjuvants alone and their combinations. Previous studies using comparable rates of urea resulted in increased A_{CO2} within 2 to 3 weeks after application but short-term effects of urea on leaves were not measured (Romero-Aranda and Syvertsen, 1996). Bondada et al. (1995) reported long-term phytotoxicity following high concentrations (6%) of urea sprays. Krogmeier et al. (1989) addressed the question whether urea itself or ammonia, as its breakdown product, is toxic to plant tissue. When combined with urease inhibitor, urea was more toxic to soybean [*Glycine max* (L.) Merrill] leaves than when it was applied alone and it could have been broken down to ammonia and CO_2. The calculated Ci:Ca ratio did not differ between treatments and was generally ≈0.78, which is similar to the ratio that has been reported widely for healthy C_3 leaves (Drake et al., 1997). This indicates that reductions in A_{CO2} were probably due to factors in the mesophyll rather than to

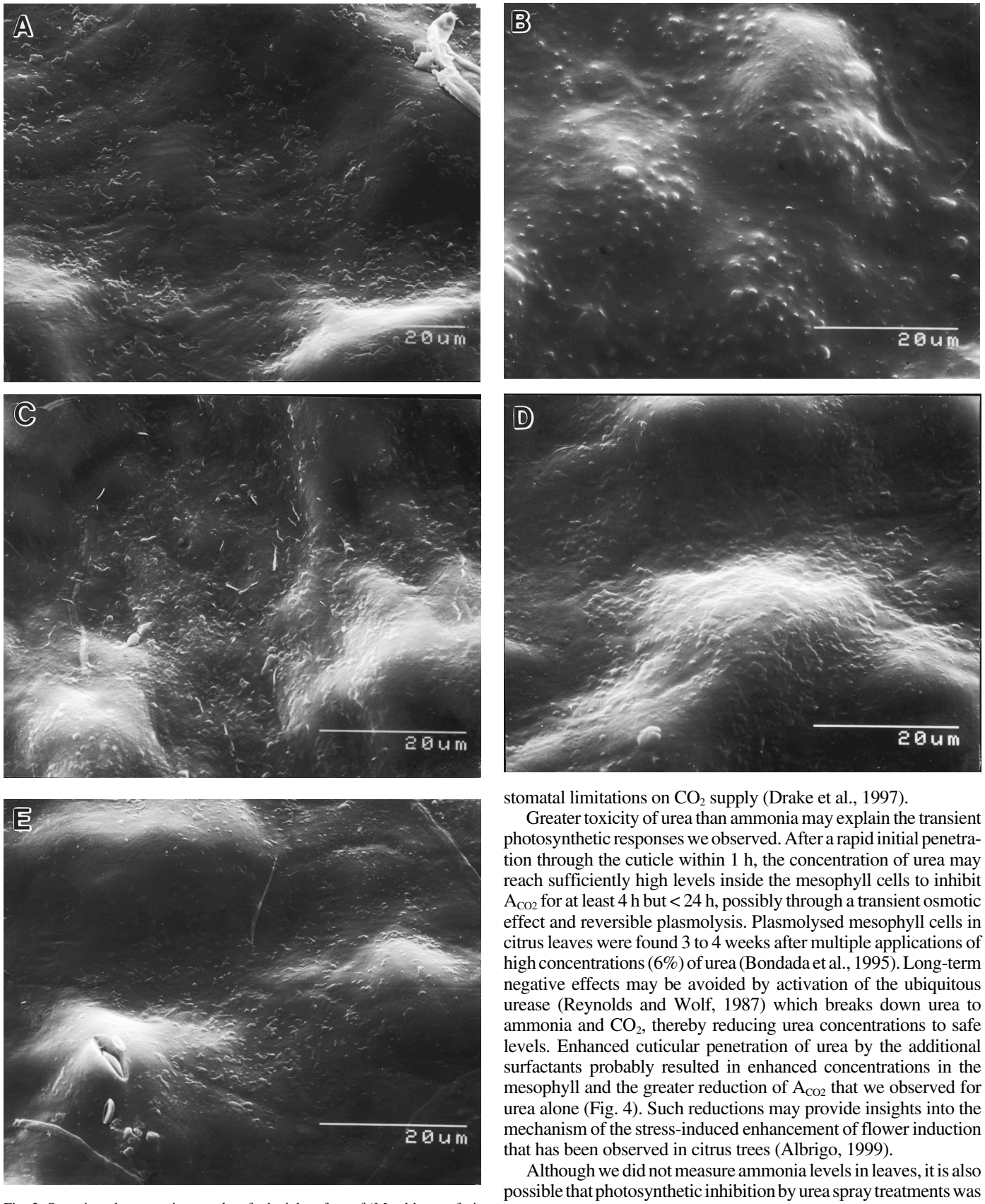

Fig. 3. Scanning electron micrographs of adaxial surface of 'Marsh' grapefruit leaves: (**A**) control–no deposit; (**B**) X-77, 1 h after deposition; (**C**) L-77, 1 h after deposition; (**D**) X-77, 24 h after deposition; and (**E**) L-77, 24 h after deposition.

stomatal limitations on CO_2 supply (Drake et al., 1997).

Greater toxicity of urea than ammonia may explain the transient photosynthetic responses we observed. After a rapid initial penetration through the cuticle within 1 h, the concentration of urea may reach sufficiently high levels inside the mesophyll cells to inhibit A_{CO2} for at least 4 h but < 24 h, possibly through a transient osmotic effect and reversible plasmolysis. Plasmolysed mesophyll cells in citrus leaves were found 3 to 4 weeks after multiple applications of high concentrations (6%) of urea (Bondada et al., 1995). Long-term negative effects may be avoided by activation of the ubiquitous urease (Reynolds and Wolf, 1987) which breaks down urea to ammonia and CO_2, thereby reducing urea concentrations to safe levels. Enhanced cuticular penetration of urea by the additional surfactants probably resulted in enhanced concentrations in the mesophyll and the greater reduction of A_{CO2} that we observed for urea alone (Fig. 4). Such reductions may provide insights into the mechanism of the stress-induced enhancement of flower induction that has been observed in citrus trees (Albrigo, 1999).

Although we did not measure ammonia levels in leaves, it is also possible that photosynthetic inhibition by urea spray treatments was due to an increased presence of ammonia in the mesophyll cells. Ammonia is a potent uncoupler of photophosphorylation and can inhibit photosynthesis by depleting reducing power accumulated in ATP (Devlin and Witham, 1983).

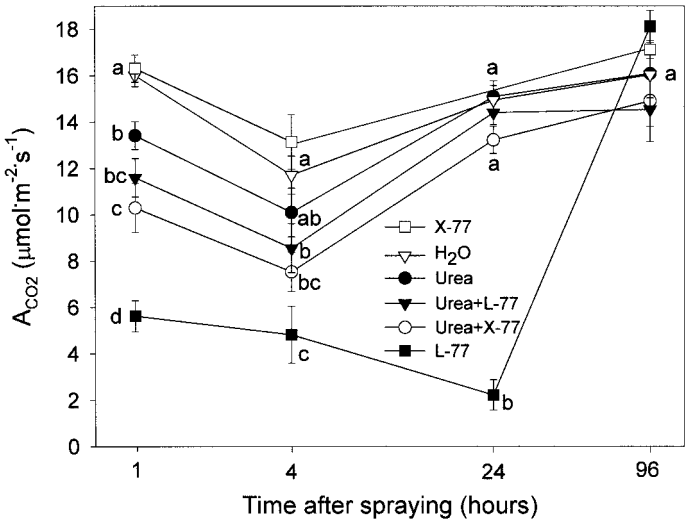

Fig. 4. Effect of urea and surfactants sprayed on 'Marsh' grapefruit leaves on the rate of CO_2 assimilation (A_{CO2}) measured in the field at 30 to 35 °C and RH of 30% to 50%. Symbols represent means of 8 to 33 replicate leaves. Within a time point, mean separation by Duncan's multiple range test, $P \leq 0.05$. Note log scale on X-axis.

Decreases in A_{CO2} were similar for 1 to 4 h after sprays of urea formulated with the two surfactants (Fig. 4), but the underlying mechanisms may not have been the same. Greater inhibition of A_{CO2} by urea + X-77 than by urea alone (at 1 h) may be related to either the initially increased urea penetration or increased retention of urea solutions on leaf surfaces when urea was mixed with X-77 (Figs. 2 and 4). Inhibition resulting from application of urea + L-77 was probably due to the effect of both urea and the surfactant, since L-77 alone also reduced A_{CO2} (Fig. 4). Greenberg et al. (1987) noted only minor negative effects on citrus fruit physiology when L-77 was applied alone, whereas Silwet-408, another organosilicone surfactant, had detrimental effect on flowers of nectarines [*Prunus persica* (L.) Batsch (Nectarine Group)] and peaches [*Prunus persica* (L.) Batsch (Peach Group)] and is being used as a thinning agent in Israel (Klein and Cohen, 2000). It is possible that L-77 alone had a larger effect than urea + L-77 because urea slowed down penetration of L-77 into the leaf. After evaporation of water, urea solutions leave a thin deposit visible on the leaf surface (Orbović et al., 2001). When urea + L-77 is applied to leaf surface, the surfactant may be competing with urea for available penetration pathways and could also be bound by the residual film of urea on the leaf surface. It is also possible that L-77 could have enhanced the amount of urea solution retained on leaf surfaces and subsequent penetration. If either surfactant resulted in increased retention of urea solution, then this effect only lasted for 1 h because there was no difference between the effect of urea alone and urea formulated with either surfactant on A_{CO2} at 4 h after spraying (Fig. 4).

In summary, two types of surfactants (X-77 and L-77) enhanced penetration of urea through isolated grapefruit leaf cuticles. A high urea penetration rate was sustained for a longer period with X-77 than with L-77 in spite of the greater wetting capacity of L-77. A transient reduction of A_{CO2} was associated with foliar urea sprays and also with L-77 alone. The degree of photosynthetic inhibition was greater for urea combined with surfactants than by urea alone.

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