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Rates of Foliar Penetration of Chelated Fe(III): Role of Light, Stomata, Species, and Leaf Age

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Rates of foliar penetration of Fe^{III} chelates of imidodisuccinic acid (IDHA), ligninsulfonic acid (Natrel), and citric acid (ammonium ferric citrate) were studied at 20 °C using a leaf disk method. After drying of the donor droplets, the humidity over the donor residues was maintained at 100% because Fe^{III} chelates deliquesce only when humidity is higher than 90%. The wetting agent Glucopon 215 CSUP was added at a concentration of 0.2 g L^{-1} to all donor solutions. With fully expanded stomatous broad bean leaves, penetration of Fe−IDHA followed first-order kinetics and rate constants of penetration were higher in light (0.073 h^{-1}) than in the dark (0.042 h^{-1}). Permeability of broad been leaves to $CaCl_2$ was about 8 times higher than to Fe−IDHA. Doubling the Fe−IDHA concentration in the donor from 2.5 to 5 mmol L^{-1} decreased rate constants of Fe−IDHA penetration by a factor of 2.2. Adding the silicon surfactant Break Thru S240 at 10 g L^{-1} to the donor induced infiltration of open stomata and about 80% of the applied Fe−IDHA penetrated during droplet drying, while with Glucopon 215 CSUP stomatal infiltration was not observed. With broad bean leaves, penetration of Natrel and ammonium ferric citrate also followed first-order kinetics and rate constants were also higher in light than in the dark. Adaxial astomatous surfaces of fully expanded pear, apple, and grapevine leaves were practically impermeable to Fe−IDHA while stomatous abaxial leaf surfaces were permeable, but rate constants of penetration decreased with time and differed greatly among species. Astomatous surfaces of young unfurling grapevine and peach leaves were permeable to Fe−IDHA, but permeability of stomatous surfaces was much higher. The effect of light on permeability of stomatous leaf surfaces is attributed to the presence of aqueous pores in cuticles over guard cells, and it is suggested that permeability of these pores increases as stomata open. Consequences of these results for foliar applications of Fe chelates are discussed.

KEYWORDS: iron chlorosis; foliar nutrition; IDHA; Natrel; ammonium ferric citrate acid

INTRODUCTION

Some economically important plant species may suffer from iron deficiency when grown in calcareous soils. Citrus, grapevines, kiwifruit, peach, and pear are examples of susceptible crops whose leaves turn yellow because chlorophyll synthesis is impaired. Iron chlorosis can cause considerable economic losses due to reduced yields and poor fruit quality (*1*).

Various remedies have been tried to cure iron chlorosis. Soil application of Fe(III) chelates is the most widely used procedure to deal with this problem. Solid Fe sulfate implanted into the stems of pear and peach trees has been found to increase Fe and chlorophyll concentrations in leaves (*2*). Foliar applications of a large variety of iron-containing compounds have also been tried, usually with limited success. These studies have been reviewed recently (*3*−*5*).

Cuticular penetration of Fe chelates has recently been studied using astomatous isolated cuticular membranes (*6*). All chelates tested were not hygroscopic and deliquesced only at 100% humidity. At 90% humidity, rates of penetration were insignificant because Fe chelates did not dissolve on the surfaces of the cuticles. Penetration was a first-order process. Rate constants decreased with increasing concentration, indicating that chelates somehow lowered permeability.

Presence of aqueous pores in cuticles is a prerequisite for penetration of ionic species such as calcium, potassium, and glyphosate salts (*7*−*10*). The astomatous adaxial sides of apple and pear leaves were nearly impermeable to $CaCl_2$, while rates of penetration across their abaxial surfaces were high (*11*). Aqueous pores preferentially occur around guard cells and basal cells of trichomes (*12*−*14*).

On the basis of these previous mechanistic studies, it was decided to study the role of stomata in penetration of Fe chelates. Because isolated cuticles obtained from stomatous leaf surfaces are perforated, such a study cannot be conducted using isolated

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Table 1. Properties of Calcium Chloride and Fe(III) Compounds Used in the Experiments

compound	chemical name of chelator	Fe weight fraction	molecular weight (g mol^{-1})	pH of the applied solution	source
Fe–IDHA	imidodisuccinic acid	0.09	393	5.1	Agrex
Natrel	ligninsulfonic acid	0.11	507[a]	adjusted to 5.0	Agrex
ammonium ferric citrate	citric acid, NH_3	0.20–0.23	254[a]	7.0	Fluka
calcium chloride			111	6.5	Fluka

[a] Average molecular weight calculated from Fe weight fraction and atomic weight of Fe.

cuticles. Leaf disks and some of the chelates used in the previous study (*6*) were employed.

MATERIALS AND METHODS

Plant Material. Experiments were conducted using leaf disks of apple (*Malus domestica* Borkh. cv. Golden Delicious), broad bean (*Vicia faba* L. cv. Hang Down), grapevine (*Vitis vinifera* L. cv. Vroege van der Laan), peach (*Prunus persica* (L.) Batch, cv. Kernechter), pear (*Pyrus communis* L. cv. Conference), and Madagascar jasmine (*Stephanotis floribunda* (R.Br.) Brongn.). Apple, pear, and peach were grown in the orchard of the institute; all others were raised in a greenhouse. Unless mentioned otherwise, leaves that had just fully expanded were used. They will be referred to as mature leaves. With the exception of broad bean, all leaves were hypostomatous. Green healthy leaves were used in experiments. There are no indications in the literature that cuticular permeability of chlorotic leaves differs from that of green leaves.

Chemicals. Donor solutions were prepared by dissolving calcium chloride or Fe chelates in deionized water. Molecular weights and other relevant properties of test compounds are given in **Table 1**. Fe chelates were spiked with $^{59}FeCl_3$ at a concentration of approximately 500 Bq μL^{-1}, which corresponds to a concentration of radioactive ^{59}Fe of 2.55 μmol L^{-1} (Perkin-Elmer, Boston, MA; specific activity 3500 MBq mg^{-1}; radiochemical purity 99%). Incorporation of ^{59}Fe into Fe^{III} chelates occurs very rapidly (*6*). The total Fe concentrations of the donor solutions were either 2 or 5 mmol L^{-1} and are specified in the figure legends. Penetration of $CaCl_2$ was studied using $^{45}CaCl_2$ (NEN, Boston, MA; specific activity 44.7 GBq mmol^{-1}, radiochemical purity 99.9%). Radioactivity (^{45}Ca) in the donor solution was approximately 100 Bq μL^{-1}. The $C_{8/10}$-polyglycoside Glucopon 215 CSUP (Fluka, Neu-Ulm, Germany) was added to all donor solutions at a concentration of 0.2 g L^{-1}. This is a nonionic surfactant that does not complex Ca^{2+} or Fe^{3+} ions. Addition of an effective surfactant is absolutely necessary to establish good contact between aqueous solutions and aqueous pores in cuticles (*7*−*9*). In one experiment the silicon surfactant Break Thru S240 (Goldschmidt, Essen, Germany) was used at 10 g L^{-1} as wetting agent to induce stomatal infiltration. Solutions were freshly prepared for each experiment. As $^{59}FeCl_3$ stock was dissolved in 0.5 mol L^{-1} HCl, an equivalent amount of KOH was added to the donor solutions to neutralize the acid and maintain the pH at the desired level (**Table 1**). For further details and verification of procedures the reader is referred to Schönherr et al. (*6*).

Penetration Experiments. Leaf disks (20 mm in diameter) were punched out with a cork borer. Each disk was mounted on a short Teflon tube and covered with a stainless steel cover. Teflon tubes and covers had an outer diameter of 25 mm and an inner bore of 12 mm. In most experiments penetration into the abaxial (lower) leaf surface was studied. In some instances penetration into the adaxial (upper) leaf surface was also measured. The edges of the leaf disks were sealed with silicon grease (Baysilone, Bayer, Leverkusen, Germany) to prevent loss of water from the cut edges. These penetration units were placed upright on wet filter paper in closed Petri dishes. The Teflon tubes were used to prevent direct contact of the leaf disks with the moist filter paper. In this way possible leaching of radioactivity from the leaf disks was avoided. One droplet (5 μL) of aqueous donor solution was placed on the center of the leaf area exposed within the steel cover. Ambient air was blown over the droplets, and solvent water was evaporated within 1 h. The leaf disks with the dry donor residues on cuticles were incubated in light from fluorescent tubes (40 μmol m^{-2} s^{-1} PAR) or in the dark at 20 °C and 100% humidity (Petri dishes covered) in a growth cabinet. Microscopic inspection showed that this relatively low light intensity resulted in stomatal opening. In most instances penetration units were incubated for 1, 2, 4, or 7 h. At the end of the penetration period the radioactive residues of the donor were washed 3 times using 200 μL of deionized water, and radioactivity in the pooled donor solutions was measured after adding scintillation cocktail. These procedures using leaf disks instead of leaves or entire plants provide excellent control of experimental variables, such as light intensity, temperature, and humidity. Because cuticular penetration is a purely physical process, it is highly unlikely that permeability of cuticles of attached leaves might differ from that of leaf disks. When penetration in light and dark or into upper and lower leaf surfaces is compared, leaf disks originating from each leaf were assigned equally to both treatments.

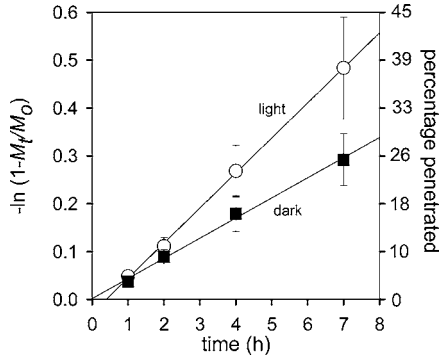

Figure 1. Penetration in light or darkness of Fe–IDHA at 5 mmol L^{-1} into abaxial leaf surfaces of broad bean at 20 °C and 100% humidity. Mean values with 95% confidence intervals of four experiments with 20 leaf disks each are shown.

Radioactivity was determined at constant quench using a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland) set to a 2σ error of 3%. Each treatment consisted of 20 leaf disks, and two to four independent experiments were conducted. To facilitate comparison with data obtained using isolated cuticles, the same data analysis was applied. Results were plotted as $-\ln(1 - M_t/M_o)$ vs time, where M_t is the amount that has penetrated into the leaf disk at time t and M_o is the amount applied. Thus, M_t/M_o is the fraction of Fe chelate that has penetrated and $1 - M_t/M_o$ is the fraction still left on the cuticle. When the natural logarithm of the fraction left on the CM is plotted against time, the slope of the line is the first-order rate constant of penetration (k). Variability among individual leaf disks can be large, and for this reason 20−80 leaf disks originating from two to four experiments were used in calculating the mean values and confidence intervals. Geometric mean values and 95% confidence intervals were calculated from log-transformed data and are shown in the figures.

RESULTS

Penetration of Fe−IDHA into broad bean leaves at 100% humidity followed first-order kinetics, as plots of $-\ln(1 - M_t/M_o)$ vs time resulted in straight lines (**Figure 1**). The slope of the line (rate constant k in h^{-1}) was significantly steeper in light than in the dark, and rate constants amounted to 0.073 and 0.042

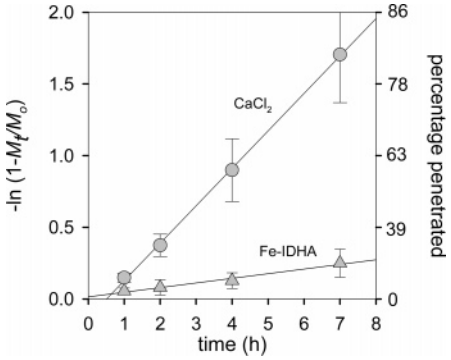

Figure 2. Penetration in light of $CaCl_2$ or Fe–IDHA at 5 mmol L^{-1} into abaxial leaf surfaces of broad bean at 20 °C and 100% humidity.

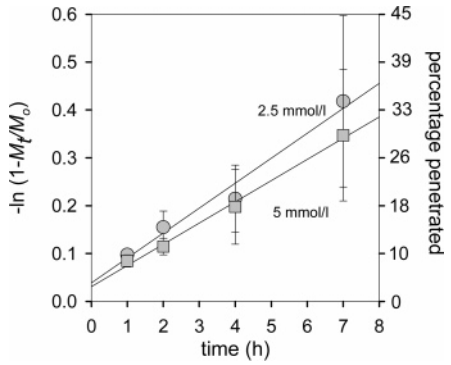

Figure 3. Penetration in light of Fe–IDHA at 2.5 or 5 mmol L^{-1} into abaxial leaf surfaces of broad bean at 20 °C and 100% humidity.

h^{-1} in light and dark, respectively. At the beginning of each experiment Fe−IDHA dissolved in water precipitated on the leaf surface when the water of the donor evaporated. For penetration to occur, solutes must be in solution. The 1 h data point represents penetration during droplet drying, which implies penetration from a donor of increasing chelate concentration. When the covers of the Petri dishes were closed after 1 h, apparently 100% humidity was reached quickly, the residues redissolved, and penetration resumed. If dissolution had taken a long time, penetration plots would not have been linear.

Penetration of $CaCl_2$ in light also followed first-order kinetics (**Figure 2**), but the rate constant was much higher (0.26 h^{-1}) than that measured with Fe−IDHA (0.032 h^{-1}). Rate constants of Fe−IDHA depended on Fe−IDHA concentration (**Figure 3**). Rate constants were 0.097 and 0.044 h^{-1} at donor concentrations of 2.5 and 5 mmol L^{-1}, respectively. With Glucopon (0.2 g L^{-1}) as wetting agent, penetration of Fe−IDHA in light followed first-order kinetics and the rate constant amounted to 0.038 h^{-1} (**Figure 4**). When Break Thru S240 (10 g L^{-1}) was added to the donor, about 80% of the Fe−IDHA penetrated during droplet drying, the leaf tissue under the droplet turned dark-green and developed a glassy appearance and penetration was no longer a first-order process.

Natrel is a Fe^{III} chelate based on ligninsulfonic acid (**Table 1**). In light and at 100% humidity it penetrated into bean leaves at a rate constant of 0.074 h^{-1}, while in the dark *k* was only 0.051 h^{-1} (**Figure 5**). With ammonium ferric citrate, rate constants were smaller and amounted to 0.042 and 0.031 h^{-1} in light and dark, respectively. In all cases penetration followed first-order kinetics.

Penetration in light of Fe−IDHA into stomatous abaxial leaf surfaces of pear, apple, and grape vine was not first-order, and slopes of the plots decreased with time (**Figure 6**), while with *Stephanotis*, penetration was first-order and *k* amounted to 0.2

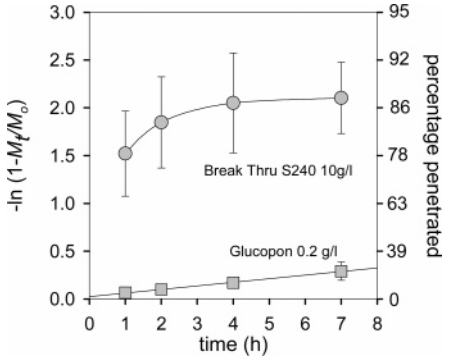

Figure 4. Penetration in light of Fe–IDHA at 5 mmol L^{-1} into abaxial leaf surfaces of broad bean at 20 °C and 100% humidity. Donor solutions contained either 0.2 g L^{-1} Glucopon 215 CSUP or 0.2 g L^{-1} Glucopon 215 CSUP plus 10 g L^{-1} Break Thru S240 as wetting agents.

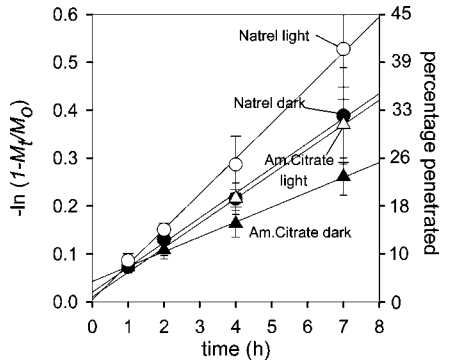

Figure 5. Penetration in light or darkness of Natrel and ammonium ferric citrate at 5 mmol L^{-1} into abaxial leaf surfaces of broad bean at 20 °C and 100% humidity.

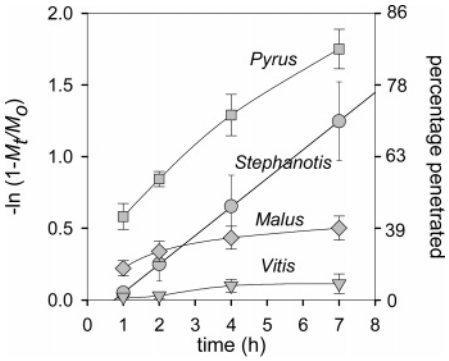

Figure 6. Penetration in light of Fe–IDHA at 5 mmol L^{-1} into abaxial leaf surfaces of pear, *Stephanotis*, apple, and grapevine leaves at 20 °C and 100% humidity.

h^{-1}. Permeability of stomatous leaf surfaces among these four species differed greatly. Permeability of mature astomatous adaxial leaf surfaces of these species in light was so small that it could not be measured accurately (data not shown). However, Fe−IDHA penetrated across astomatous surfaces of very young leaves of grapevine and peach that were just unfurling (**Figure 7**), but rates differed greatly. With peach leaves about 40% penetrated in 24 h (**Figure 7B**), while with grapevine, penetration amounted to less than 5% (**Figure 7A**). Rates of penetration into stomatous lower leaf surfaces were also higher with young unfolding leaves.

DISCUSSION

Rates of foliar penetration of Fe chelates were measured at 100% humidity. At this humidity cuticles are fully swollen and

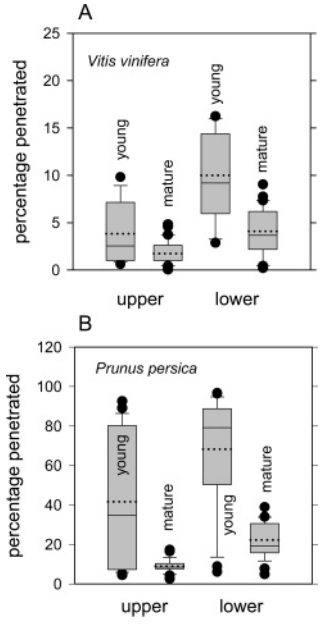

Figure 7. Penetration in light of Fe–IDHA at 5 mmol L^{-1} into adaxial (upper) or abaxial (lower) leaf surfaces of grapevine (A) and peach (B) at 20 °C and 100% humidity. Mature leaves were just fully expanded, and young unfolding leaves had reached about half-maximum size or less. Data are represented as box plots showing all outliers, arithmetic mean (dotted lines) and median (solid lines) values, respectively.

Fe chelates deliquesce. In a previous study it was shown that penetration ceases if humidity is 90% or lower because Fe chelates solidify on the leaf surfaces (*6*). This is a severe limitation in Fe foliar nutrition, and it explains why foliar Fe nutrition often failed (*3*−*5*). In humid climates dew often occurs during the night, chelates deliquesce, and penetration can take place. In arid areas lacking regular dew, penetration would be limited to the period of spray droplet drying. This problem might be solved by searching for adjuvants in which Fe chelates remain in solution even if humidity is lower than 100%. As long as this problem has not been solved, application of Fe chelates to the soil or into the stem (*2*) might be the only alternatives.

With broad bean and Madagascar jasmine leaves, penetration was a first-order process that could be characterized using rate constants (k) or half-times of penetration ($-\ln(0.5/k)$). First-order kinetics implies that solutes on the cuticle disappeared exponentially with time and accumulation of Fe chelates in the outer epidermal walls did not occur. Hence, translocation into the leaf tissue was not rate-limiting.

In separate experiments, the mean rate constants of penetration in light measured with abaxial surfaces of broad bean and Fe−IDHA ranged from 0.032 to 0.073 h^{-1} (**Figures 1**−**4**), corresponding to half-times ranging from 22.7 to 9.5 h. This variability in permeability of leaves from different lots of plants cannot be explained. Because the same age of plants and leaves and the same variety were used, it may be related to growing conditions in the greenhouse. Plants were raised in a greenhouse, and humidity, light intensity, and temperature varied considerably during the seasons.

A comparison of the rate constants measured with $CaCl_2$ (k = 0.26 h^{-1}) and Fe−IDHA (k = 0.032 h^{-1}) shows that permeability to Fe−IDHA was much lower (**Figure 2**). Aqueous pores in broad bean leaf cuticles are size-selective, and rate constants of calcium salts decrease in a predictable way with increasing molecular weight (*13*). Allowing for the difference in molecular weight between $CaCl_2$ and Fe−IDHA (**Table 1**), the value of k for a hypothetical calcium salt with the molecular weight of Fe−IDHA (393 g mol^{-1}) can be estimated as 0.12 h^{-1}. This is still higher by a factor of 3.8 than that measured for Fe−IDHA. It was shown previously that adding Fe−IDHA or Fe−EDTA to the donor decreased $CaCl_2$ permeability of poplar (*Populus canescens*) cuticular membranes by a factor of 7 (*6*). Furthermore, Fe−IDHA permeability of poplar leaf cuticular membranes decreased with increasing concentration of Fe−IDHA, and it was suggested that Fe chelates somehow reduced permeability of aqueous pores. A similar effect was observed in the present study. Rate constants of penetration of Fe−IDHA decreased by a factor of 2.2 when Fe−IDHA concentration was increased from 2.5 to 5 mmol L^{-1} (**Figure 3**). With poplar cuticular membranes, rate constants also decreased by a factor of 2.2 when Fe−IDHA concentration was raised from 10 to 20 mmol L^{-1} (*6*). Mixing Fe chelates with other nutrients should be avoided, and frequent foliar applications of Fe chelates might interfere with foliar nutrition with other macronutrients or micronutrients when applied separately.

From the rate constants and the dose applied, the amount penetrated at any time interval can be estimated. A 5 μL droplet having a concentration of 5 mmol L^{-1} results in a dose of 25 nmol. The equation for an exponential process, $-\ln(1 - M_t/M_o) = kt$, which was used to plot the data, can be solved for the fraction that has penetrated (M_t/M_o), which is $1 - e^{-kt}$. For a 5 h penetration period the fraction penetrated would amount to 0.2% or 20% of the dose or 5 nmol. With the lower concentration of 2.5 mmol L^{-1}, 38% of the dose would have penetrated, which is 4.75 nmol. In this particular example the difference in amount penetrated is not large, and if the price of the chelate is high, half the cost could be saved by using the lower concentration.

The correlation between rate constants of penetration and anhydrous molecular weights of Ca salts (*12*, *13*) is surprising because these salts are hydrated. In contrast, rate constants of penetration of Fe chelates across poplar CM were independent of anhydrous molecular weight in the range 254−507 g mol^{-1} (*6*). Ca^{2+} ions carry hydration shells, while anions are not or only weakly hydrated (Israelachvili, 1991). Residence times of water molecules in the primary hydration shell of Ca^{2+} are of the order of 10^{-8} s^{-1}, and it has been argued that hydration water oscillates between calcium ions and dipoles lining the pore walls (*14*). With trivalent cations such as Fe^{3+} rotational correlation times are much longer and range from seconds to minutes. In narrow hydrated pores this may lead to a competition for water between Fe^{3+} and ionized carboxyl groups leading to partial dehydration of the pore. Trivalent cations may bind water very strongly, and ion−water complexes of fixed stoichiometry can be formed. If this happens in the narrow aqueous pores, it could explain the lack of correlation between rate constants and molecular weights observed with Fe^{III} chelates. Furthermore, hydrogen bonds might be formed between the $Fe^{III}(HOH)_n$ complexes and dipoles of the pore wall. This could slow diffusion of Fe chelates and Ca salts in these pores, as was in fact observed (*14*). Whatever the reason for the low permeability of cuticles to Fe chelates, relatively long time intervals are needed for significant penetration.

Permeability of stomatous leaf surfaces was higher in light than in the dark. With broad bean and Fe−IDHA the factor was 1.74 (**Figure 1**), while rate constants for Natrel and ammonium ferric citrate were higher by factors of 1.45 and 1.68, respectively (**Figure 5**). Permeability of broad bean leaves to calcium salts was 1.82 times higher in light than in the dark (*13*). The phenomenon was observed with other solutes and

species as well (cf. *14*) and has been attributed to an increase in permeability of the cuticle around guard cells when stomata open (*13*, *14*, *16*). The presence of stomata and their degree of opening are of paramount importance for foliar penetration. This argument is supported by the fact that permeability of astomatous leaf surfaces to Fe chelates was practically zero as soon as they were fully expanded (**Figure 7**). When spraying crops with hypostomatous leaves, the lower stomatous leaf surfaces are the major targets for spray droplets.

Mass flow of donor solution in open stomata while the droplets are drying may contribute to the light effect. With various calcium salts penetration during droplet drying amounted to a few percent when donor solutions contained Glucopon 215CSUP (*13*). This surfactant lowers surface tension to 29−30 mN m^{-1}, which is not quite sufficient for spontaneous flooding of open stomata (*17*). With Glucopon as the sole surfactant, linear penetration plots were obtained that intersect the origin (**Figure 4**). Adding the silicon surfactant Break Thru S240 resulted in flooding of intercellular spaces, as surface tension fell to around 22 mN m^{-1}. This could be seen with the naked eye as the leaf under the droplet turned translucent and dark-green. About 80% of the radioactivity entered the leaf during droplet drying. Rate constants decreased thereafter, and they were higher during the intervals 1−2 and 2−4 h than those observed with Glucopon alone (**Figure 4**). This might indicate liquid continuity through open stomata between a liquid film on the cuticle and the intercellular space. The data show that massive and rapid loading of intercellular spaces with Fe chelates via open stomata can be accomplished with Break Thru S240. Rather high concentrations are required to obtain infiltration of open stomata, and it remains to be seen if the surfactant damages cell membranes and interferes with photosynthesis.

Penetration in light of Fe−IDHA into stomatous leaf surfaces differed among plant species. The decrease in slopes with time was most conspicuous with apple and grapevine leaves (**Figure 6**). It is not known why penetration plots were linear with broad bean (**Figures 1**−**4**) and Madagascar jasmine (**Figure 6**) but not with the other species tested. If Fe chelates accumulate under the cuticle and are not translocated rapidly, rate constants would decrease with time. Furthermore, microscopic inspection indicated that stomata of these species partially closed following treatment with Fe chelates. However, this was not studied in detail but it is clear that rates of penetration obtained in this study with a very narrow selection of plant species should not be extrapolated to other plants.

With grapevine and peach leaves the effect of leaf age on Fe−IDHA penetration was studied. Permeability of stomatous abaxial surfaces was higher than that of astomatous adaxial surfaces (**Figure 7**). Possible reasons have been discussed above. Significant rates of penetration into astomatous leaf surfaces were observed with young unfolding leaves. This indicates the presence of aqueous pores in cuticles of very young leaves and their disappearance during leaf expansion. From these data it is concluded that frequent applications of Fe chelates are necessary to fertilize newly formed unfurling leaves and the chances for regreening of old and mature leaves are limited with many crops.

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