Plant Physiology, December 1999, Vol. 121, pp. 1069–1079, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Apoplastic pH and Fe^{3+} Reduction in Intact Sunflower Leaves[1]

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It has been hypothesized that under NO_3^- nutrition a high apoplastic pH in leaves depresses Fe^{3+} reductase activity and thus the subsequent Fe^{2+} transport across the plasmalemma, inducing Fe chlorosis. The apoplastic pH in young green leaves of sunflower (*Helianthus annuus* L.) was measured by fluorescence ratio after xylem sap infiltration. It was shown that NO_3^- nutrition significantly increased apoplastic pH at distinct interveinal sites (pH ≥ 6.3) and was confined to about 10% of the whole interveinal leaf apoplast. These apoplastic pH increases presumably derive from NO_3^-/proton cotransport and are supposed to be related to growing cells of a young leaf; they were not found in the case of sole NH_4^+ or NH_4NO_3 nutrition. Complementary to pH measurements, the formation of Fe^{2+}-ferrozine from Fe^{3+}-citrate was monitored in the xylem apoplast of intact leaves in the presence of buffers at different xylem apoplastic pH by means of image analysis. This analysis revealed that Fe^{3+} reduction increased with decreasing apoplastic pH, with the highest rates at around pH 5.0. In analogy to the monitoring of Fe^{3+} reduction in the leaf xylem, we suggest that under alkaline nutritional conditions at interveinal microsites of increased apoplastic pH, Fe^{3+} reduction is depressed, inducing leaf chlorosis. The apoplastic pH in the xylem vessels remained low in the still-green veins of leaves with intercostal chlorosis.

Various investigations have shown that leaves may show Fe-deficiency symptoms even with leaf Fe concentrations higher than in green leaves (e.g. Carter, 1980; Mengel and Malissiovas, 1981; Sahu et al., 1987). Aktas and Van Egmond (1979) reported that chlorosis increased with elevated NO_3^- supply. The chlorosis-inducing effect of NO_3^- was also found by Mengel and Geurtzen (1988) and could be reversed by switching from NO_3^- to NH_4^+ without any external supply of Fe. Hoffmann et al. (1992) were the first to report a relationship between leaf apoplastic pH and the form of N nutrition. With NH_4^+ supply the leaf apoplastic pH was low, while NO_3^- resulted in high apoplastic pH. Mengel et al. (1994) and Kosegarten and English (1994) found an inverse relationship between the chlorophyll concentration and leaf apoplastic pH. NO_3^- was thought to be taken up into the cell via a NO_3^-/H^+ cotransport (Ullrich, 1992; Crawford and Glass, 1998), and the perfusion of excised leaves with NO_3^- resulted in microsites with an apoplastic pH of around 7.0 (Hoffmann and Kosegarten, 1995).

These findings suggested that high leaf apoplastic pH restricts cellular Fe acquisition (Mengel, 1995), and this conclusion was corroborated by the observation that spraying leaves with dilute acids resulted in a re-greening of chlorotic leaves (Sahu et al., 1987; Tagliavini et al., 1995). Sahu et al. (1987) found that spraying caused a 2-fold increase in yield; interestingly, the same yield increase was found by treating the plants with Fe-EDDHA. Apoplastic pH has been shown to be related to plasmalemma proton pumps (Petzold and Dahse, 1988; Hoffmann et al., 1992) and spraying chlorotic leaves with fusicoccin resulted in a lowering of leaf apoplastic pH (Hoffmann et al., 1992) and in leaf re-greening (Mengel and Geurtzen, 1988).

Based on these observations, Mengel (1995) hypothesized that high pH in the leaf apoplast hampers the reduction of Fe^{3+}-citrate; reduction of Fe^{3+} is the prerequisite for the transport of Fe^{2+} across the plasmalemma (Chaney et al., 1972; Fox et al., 1996). Recently, a Fe^{2+} transporter has been identified in yeast (Eide et al., 1996). High pH in the root medium depressed the reduction of Fe^{3+} complexes (Romera et al., 1991). The investigations of Römheld and Marschner (1983), Toulon et al. (1992), and Susin et al. (1996) have shown that the reduction of Fe^{3+} in the apoplast of intact roots occurred at low pH. Various researchers (e.g. Brüggemann and Moog, 1989) working with membrane vesicles from barley roots found a pH optimum of Fe^{3+} reduction at pH 6.8; others (e.g. Holden et al., 1991), using vesicles from tomato roots, found an optimum of pH 6.5 for the reduction of Fe^{3+}. These high pH optima, however, presumably relate to the cytosolic side of the plasma membrane-located Fe^{3+} reductase and were also found for vesicles from mesophyll cells (Brüggemann et al., 1993; Rombola et al., 1999). The pH optimum for the apoplastic domain of the Fe^{3+} reductase appeared to be lower (Mengel, 1995). If this apoplastic condition is not met, substantial amounts of Fe remain in the apoplast and are not transported into the symplasm, where it is required for cellular processes.

The main objective of this study was to test the pH dependence of Fe^{3+} reduction in the leaf apoplast. Also, apoplastic pH measurements were carried out with excised leaves fed via the petiole with xylem sap obtained from plants grown on NO_3^-, NO_3^-/HCO_3^-, NH_4^+, or NH_4NO_3 as a control. It was possible to display by use of microscope image analysis apoplastic pH at the cellular level and apo-

[1] This work was supported by the Deutsche Forschungsgemeinschaft.

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plastic Fe^{3+} reduction in intact leaf tissue. Since Fe chlorosis is a symptom of young leaves, measurements were carried out with young green leaves before leaf chlorosis occurred.

MATERIALS AND METHODS

Chemicals

2′,7′-Bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein-dextran and rhodamine were purchased from Molecular Probes (Eugene, OR). All other chemicals were from Sigma Chemical (St. Louis).

Plant Growth

Sunflower (*Helianthus annuus* L. cv Solostar) seeds were soaked with 0.5 mM $CaSO_4$ (24 h) and then germinated under darkness in a humid chamber at 25°C for 2 d. Plants were cultivated at 25°C during the day (15 h) and at 20°C during the night (9 h) in nutrient solution for 14 d. The control plants were grown for 14 d with NH_4NO_3. The plants of the other treatments were cultivated for 12 d in NH_4NO_3 and then transferred for another 2 d to two different N forms, NH_4Cl and $Ca(NO_3)_2$, and in one treatment to $Ca(NO_3)_2$ and $KHCO_3$. The total N concentration in each treatment was 6 mM, and the HCO_3^- concentration was 10 mM. In a further treatment plants were cultivated for 9 d in 3 mM NH_4NO_3, then transferred for 2 d in solution without N, and then cultivated for a further 3 d with 1 mM NH_4Cl. The Fe concentration in all series was 1 μM. The basic nutrient solution was as described by Kosegarten et al. (1998).

Collection and Analysis of the Xylem Sap

Xylem sap was obtained by sampling the exudation sap as described by Van Beusichem et al. (1988). Collection was carried out 4 h after the onset of the photoperiod, and the xylem sap was sampled for 60 min after plants were decapitated about 3 cm above the root. The sap from the first 5 min was discarded. Analyses of pH and of N compounds (NO_3^-, NH_4^+, and amino acids) were carried out on fresh samples. The pH measurements were conducted with an electrode (U402 M3/S7/60, Ingold, Mettler Toledo, Steinbach, Germany). NO_3^- was analyzed by means of a continuous flow analyzer (Technicon Autoanalyzer II, Bran and Luebbe, Hamburg, Germany). NH_4^+ and amino acids were determined with an amino acid analyzer (Biotronic LC 3000, Eppendorf, Maintal, Germany). The method was modified according to Moore and Stein (1954). Samples were centrifuged at 15,000*g* for 15 min at 4°C, and a 20-μL aliquot of the supernatant was taken and isolated by a cation-exchange column (Eppendorf Biotronic, TS 01044P). Separation was carried out in a buffer system (Eppendorf Biotronic, Typ H1) at increasing pH at a flow rate of 0.2 mL min^{-1}. Each sample comprised the xylem sap from four plants (xylem sap pH) and 24 plants (N compounds), respectively. Xylem sap pH was measured in 20 samples (n = 20).

Apoplastic pH Measurements in Intact Sunflower Leaves

Measurements of leaf apoplastic pH were carried out according to the method of Hoffmann and Kosegarten (1995), working with young leaves with a leaf area of about 800 mm^2. Apoplastic pH was measured after infiltration of fresh xylem sap obtained from the different nutritional N sources into excised leaves. If not noted otherwise, the apoplastic pH was monitored in the interveinal area at the leaf base.

Apoplastic pH measurements were conducted with (a) a fluorescence photometer (LS 50, Perkin-Elmer Applied Biosystems, Foster City, CA) at the tissue level on leaf areas of 9 mm^2 at three different sites per leaf (in each treatment five leaves were analyzed; n = 15), and (b) under a fluorescence microscope (Axiotron/UV-fluorescence microscope, Carl Zeiss, Jena, Germany) at the cellular level. The basic configuration of microscope analysis was as described by Hoffmann and Kosegarten (1995). Excitation light between 450 and 490 nm was specified with a monochromator (bandwidth 15 nm). A measuring diaphragm of 30 × 150 μm was positioned on various cell areas (hair cells, stomata, epidermal cells, and xylem vessels). The illumination field diaphragm was about 20% larger than the measuring diaphragm. Apoplastic pH of xylem vessels (first to fourth order) and hair cells was examined at nine positions per leaf blade; in each treatment three leaves were examined (n = 27). To investigate apoplastic pH distribution in the intercostal leaf area, 20 cell complexes consisting of three to five epidermal and stomatal cells on leaf areas of 50 mm^2 per leaf at the base were examined; in each treatment monitoring of apoplastic pH was conducted with five leaves (n = 100). Apoplastic pH gradients were also measured by microscope image analysis as described by Hoffmann and Kosegarten (1995). A back-illuminated integrating CCD camera (Princeton Applied Research, Trenton, NJ) was used to improve the signal-to-noise ratio.

To monitor the apoplasic pH of green and chlorotic areas of leaves with intercostal chlorosis, leaves were only perfused with 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM KCl. The apoplastic pH was monitored with a fluorescence microscope (Axiotron/UV-fluorescence microscope, Carl Zeiss) in the chlorotic intercostal area (epidermal and stomatal cells) and in the green xylem vessels (mid-rib and first order veins). Green leaves were also examined for comparison. Ten positions per leaf blade were examined and pH measurements were conducted with three leaves (n = 30).

Measurement of Fe^{3+} Reduction in Relation to Apoplastic pH in Intact Sunflower Leaves

Fe^{3+} reduction in relation to apoplastic pH was examined in the xylem vessels (first order according to Canny, 1990) by microscope image analysis. Youngest leaves were excised, ferrozine (1 mM) was preloaded into the leaf for 24 h, and then for a further 6 h, 80 μM $FeCl_3$ and 80 μM citrate were perfused in the presence of various buffers: 100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)/KOH,

pH 4.0 to 6.5, and 100 mM 4-(2-hydroxyethyl)-1-piperazine 2-ethanesulfonic acid (HEPES)/KOH, pH 7.0 to 8.0.

Ferrozine specifically complexes Fe^{2+} and exhibits an absorption maximum at 560 nm (Stookey, 1970). At 720 nm the Fe^{2+}-ferrozine complex shows no absorption (data not shown). The principle of the measurement is based on monitoring the light transmission at 560 nm in the apoplast area of the xylem vessel. To compensate for differences in leaf absorption, light transmission was also measured at 720 nm. By calculating the ratio of light transmission at 720 nm and at 560 nm, a specific measure for the Fe^{2+}-ferrozine complex in the xylem vessel was obtained. The light transmission ratio was calculated on frames of 512 × 512 pixels captured by a standard CCD camera (XC57CE, Sony, Tokyo). The resulting ratio values were displayed in pseudocolor. Figure 1 shows the light transmission at 720 nm (A) and at 560 nm (B) of a control leaf without ferrozine perfusion. The yellow pseudocolor in the xylem vessel corresponds to the maximal light transmission ratio (C). The histogram of Figure 1D shows the distribution of pixel gray values (0–255) with a maximum at a gray level of 101.3 ± 3.9 (n = 9), representing the maximum of the light transmission ratio in the xylem vessel (yellow pseudocolor).

The apoplastic pH of first-order xylem vessels after buffer infiltration was measured in separate experiments using fluorescein isothiocyanate-dextran and 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein-dextran (100 μM each). The different light transmission ratios after the various pH treatments in relation to the formation of Fe^{2+}-ferrozine are shown as different distributions of the pixel intensity in the ratio picture of the xylem vessels (see histograms in Fig. 6). A high pixel value represents a reduction in the light transmission ratio. Changes in gray levels were expressed in pseudocolor. Light transmission at each apoplastic pH value was measured in nine different areas of first-order xylem vessels per leaf (n = 9), and each pH treatment consisted of three leaves (n = 27). In the xylem vessel of the unbuffered leaf (with a xylem apoplastic pH ≤ 5.0; Table V), the light transmission ratio was minimal due to high formation of the Fe^{2+}-ferrozine complex. The minimal light transmission ratio shows a maximum at a gray level of 141.2 ± 4.1 (n = 27; not shown). After subtracting the minimal pixel value of the control leaf (100.5 ± 4.2; n = 27), the maximal rate of Fe^{3+} reduction was obtained and was defined as 100% (Table V). For each pH treatment the corresponding percentage of Fe^{3+} reduction was calculated (Table V). To check for variations in dye loading, the pH-independent fluorescent dye rhodamine (100 μM) was also perfused into leaves. Excitation of rhodamine was conducted at 560 nm and emission was observed at 580 nm, cutting off reflected excitation light by use of a long-pass filter (OG 570, Schott, Mainz, Germany).

Statistical Treatment

Significant differences between the control and the other nutritional treatments were calculated for xylem sap pH and for leaf apoplastic pH by use of the t test (Köhler et al., 1984; Table I).

RESULTS

Effects of N Form and HCO_3^- on Xylem Sap pH and Leaf Apoplastic pH

Table I shows that under alkaline conditions in the nutrient solution (NO_3^- and NO_3^-/HCO_3^-), both xylem sap pH and apoplastic pH significantly increased compared with the NH_4NO_3 treatment (control). Addition of HCO_3^- had no influence on apoplastic pH compared with the NO_3^- treatment. Moreover, when plants were exclusively fed with NH_4^+ (at both 1 and 6 mM), the apoplastic pH decreased. In darkness, the apoplastic pH increased by about 0.1 pH unit in all treatments.

The apoplastic pH values shown in Table I are mean values at the leaf tissue level (9 mm^2) from the intercostal area at the leaf base and were recorded by use of fluorescence photometry. Thus, with this experimental approach,

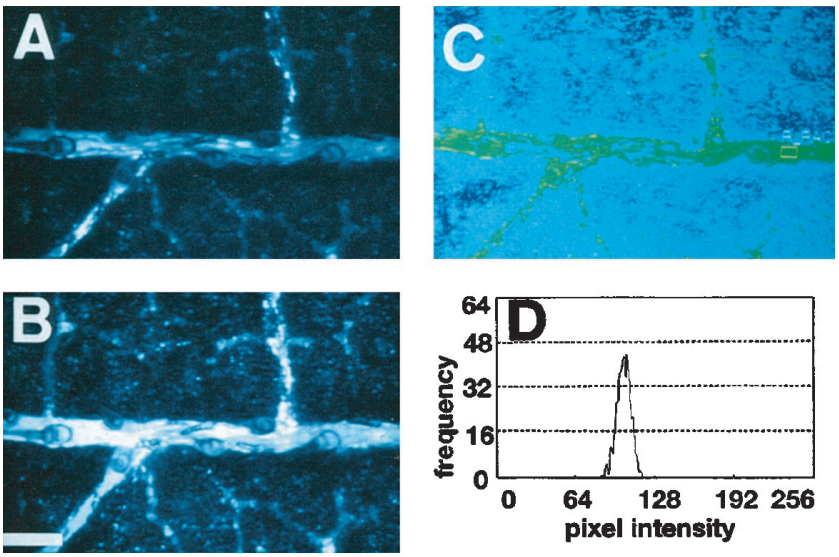

Figure 1. Light transmission at 720 nm (A) and 560 nm (B) and the maximal light transmission ratio (720/560 nm; C) in the xylem vessel of a control leaf (without ferrozine) of sunflower. The maximal light transmission ratio (720/560 nm) in the xylem vessel (without Fe-ferrozine complex) is displayed by yellow pseudocolor (C). Scale, 240 μm. The histogram (D) shows the distribution of the pixel intensity in the ratio picture (C) of the xylem vessel. The maximal light transmission ratio shows a pixel value of 101.3 ± 3.9 (n = 9).

Table I. *Xylem sap pH (n = 20; ±SD) and apoplast pH (n = 15; ±SD) in the intercostal area of young sunflower leaves after infiltration with xylem sap of the different nutritional N sources*

Significant differences between the control and the other treatments at three different levels are denoted by: *, 5% level; **, 1% level; ***, 0.1% level.

Nutritional N Source	Xylem Sap pH	Apoplast pH	
		Light	Dark (5 h)
NH_4NO_3 (3 mM)[a]	5.12 ± 0.08	5.36 ± 0.06	5.47 ± 0.07
NH_4^+ (6 mM)	5.14 ± 0.07	5.36 ± 0.06	5.43 ± 0.07
NH_4^+ (1 mM)	5.31 ± 0.02**	5.41 ± 0.09	Not determined
NO_3^- (6 mM)	5.45 ± 0.05***	5.50 ± 0.05**	5.62 ± 0.07*
NO_3^-/HCO_3^- (6 mM)	5.53 ± 0.04***	5.51 ± 0.07*	5.60 ± 0.08*

[a] Control.

the mean pH response in the apoplast of several hundred cells and also of various cell types, i.e. the apoplastic pH of leaf epidermal, stomatal, and hair cells, was measured. Since such apoplastic pH values at the tissue level may average out more pronounced pH changes at the cellular level, the local apoplastic pH of the various cell types was recorded by fluorescence microscopy (Fig. 2; Table II) combined with digital image processing (Fig. 3). These approaches revealed distinctly different apoplastic pH values at various microsites at the cellular level in the leaf.

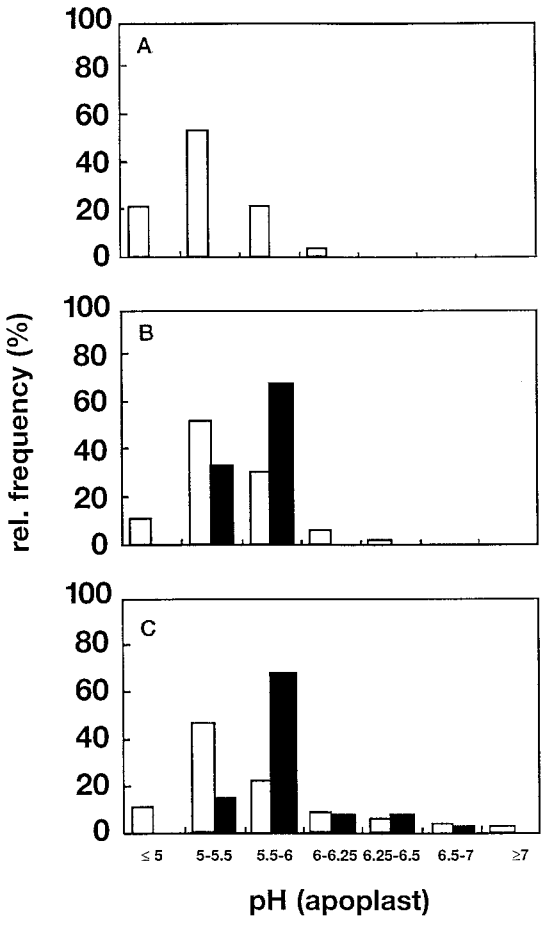

Figure 2. Relative frequency of apoplast pH ($n = 100$) of epidermal and stomatal cells in the intercostal area at the leaf base in relation to different N nutrition and light/dark changes. A, 1 mM NH_4^+; B, 3 mM NH_4NO_3; C, 6 mM NO_3^-/10 mM HCO_3^-. Dark period, 5 h. White bars, Light; black bars, dark.

Figure 2 shows the frequency distribution of apoplastic pH in complexes comprising about three to five leaf epidermal and stomatal cells in the interveinal leaf area. Independent of N form and the addition of HCO_3^-, about 50% of the apoplastic pH values in these complexes was between pH 5.0 and 5.5; about 20% to 30% was between pH 5.5 and 6.0; and 10% to 20% of the apoplastic pH was ≤5.0. In darkness, the frequency distribution of apoplastic pH shifted to values between 5.5 and 6.0 (70%). Only under alkaline nutritional conditions was about 10% of the apoplastic pH ≥ 6.3 (Fig. 2C), which was not different between light and dark. Such leaf cell complexes with high apoplastic pH levels are indicated by green and yellow color (arrows) in Figure 3A. The light-blue color represents a mean pH of about 5.7. The restriction of high apoplastic alkalization to small complexes of epidermal and stomatal cells (about 10% of the leaf apoplast) under alkaline nutritional conditions explains the small overall pH increase (0.15 pH unit) at the tissue level (hundreds of leaf cells) compared with the NH_4NO_3 treatment (Table I).

Figure 3B shows the distribution of the dye fluorescence intensity at 490 nm with highest intensities around the stomatal apoplast and in the xylem area. High fluorescence intensities are caused by the high optical pathlength of the xylem vessels and by dye enrichment around the stomatal apoplast at high transpiration rates, and can be eliminated by the use of the fluorescence ratio technique (see Hoffmann and Kosegarten, 1995).

The apoplastic pH of the hair cells and of the xylem vessels is shown in Table II. Apoplastic pH at these microsites was affected by neither the N form nor HCO_3^-. Apoplastic pH of the hair cells was considerably higher (0.5 pH unit) than that of the xylem.

Table III shows the contribution of various N compounds in the xylem sap being infiltrated into the leaf. The concentration of NO_3^- was higher in treatments of alkaline nutrition (NO_3^- and NO_3^-/HCO_3^-) than in the NH_4NO_3 treatment (control); it was lowest when plants were fed exclusively with NH_4^+. The reverse was true for the concentration of NH_4^+, Gln, and Asn in the xylem sap under the applied nutritional conditions. At 1 mM NH_4^+ in the nutrient solution, the NH_4^+ concentration in the xylem sap was not much different from that found in alkaline nutritional

Table II. *Effect of different N forms (NH_4NO_3, NO_3^- in the presence of HCO_3^-) on apoplast pH in xylem vessels (first to fourth order according to Canny, 1990) and of hair cells (n = 27; ±SD) of young sunflower leaves after infiltration with xylem sap of the different nutritional N sources*

Apoplast pH	Light		Dark	
	NH_4NO_3	NO_3^-/HCO_3^-	NH_4NO_3	NO_3^-/HCO_3^-
Xylem vessel	5.14 ± 0.24	5.16 ± 0.24	5.21 ± 0.10	5.22 ± 0.14
Hair cell	5.61 ± 0.23	5.72 ± 0.22	5.80 ± 0.15	5.82 ± 0.23

treatments. However, the Gln concentration in the xylem sap increased 7-fold over that under alkaline conditions.

Apoplastic pH in Green and Intercostal Chlorotic Leaves

To investigate the influence of chlorosis on leaf apoplastic pH, the intercostal area and xylem vessels of leaves with intercostal chlorosis (arrows; Fig. 4) and of green control leaves were analyzed. No pH differences were found in the xylem vessels of the mid-rib and the first-order veins for green and intercostal chlorotic leaves. The apoplastic pH in the intercostal region of the chlorotic leaf was remarkably higher (about 0.5 pH unit) than that monitored in the green vessels and that in the intercostal area of the green leaf (Table IV).

Fe^{3+} Reduction in Relation to Leaf Apoplastic pH

Various pH-buffered solutions were infiltrated into excised leaves, and both xylem apoplastic pH and Fe^{3+} reduction were measured; the latter by the formation of the Fe^{2+}-ferrozine complex in the xylem vessels (first order veins; Table V). In Figure 5 the light transmission of different leaves in the region of the xylem vessel after infiltration of Fe^{3+}-citrate and ferrozine at a low (pH 5.4; Fig. 5, A and B) and a high (pH 7.7; Fig. 5, C and D) xylem apoplastic pH is shown. After both pH treatments the light transmission at 720 nm in the xylem vessels (Fig. 5, A and C) was similar and comparable to the control leaf without ferrozine (Fig. 1A). Small differences were due to differences in leaf absorption. At 560 nm and high apoplastic pH (pH 7.7; Fig. 5D), light transmission in the xylem vessels was high and was similar to that in the control leaf (Fig. 1B). This finding shows that Fe^{2+}-ferrozine formation at pH 7.7 was negligible. In contrast, at low pH levels (pH 5.4), light transmission at 560 nm was much reduced, as shown by fewer whitish strands in this picture (Fig. 5B). Therefore, at low pH, Fe^{3+} reduction took place and the Fe^{2+}-ferrozine complex was formed.

The images in Figure 6 show the light transmission ratio (720/560 nm) after infiltration of Fe^{3+}-citrate and ferrozine at high and low xylem apoplastic pH. At pH 7.7 (Fig. 6A) the light transmission ratio in the xylem vessel was high, as indicated by yellow pseudocolor. The blue pseudocolor of the ratio picture in the xylem vessel of Figure 6B indicates a low light transmission ratio at low apoplastic pH (pH 5.4). The degree of light transmission ratio at various apoplastic pH levels corresponded to the degree of Fe^{2+}-ferrozine formation and therefore to the capacity of Fe^{3+} reduction. Table V summarizes the percentage data of the mean light transmission ratio of the Fe^{2+}-ferrozine complex in the xylem vessels under various pH conditions. The capacity of Fe^{3+} reduction decreased with increasing apoplastic pH. Formation of the Fe^{2+}-ferrozine complex, and therefore Fe^{3+} reduction, was the same at an apoplastic pH ≤ 5.0 (leaf without buffer) and at pH 5.4 (leaf with 100 mM MES, pH 5.0; Table V); therefore, the effect of buffer infiltration appeared negligible. To check for variations in dye loading, the fluorescence intensity of the pH-independent dye rhodamine was monitored as a direct measure of dye concentration inside the xylem vessels.

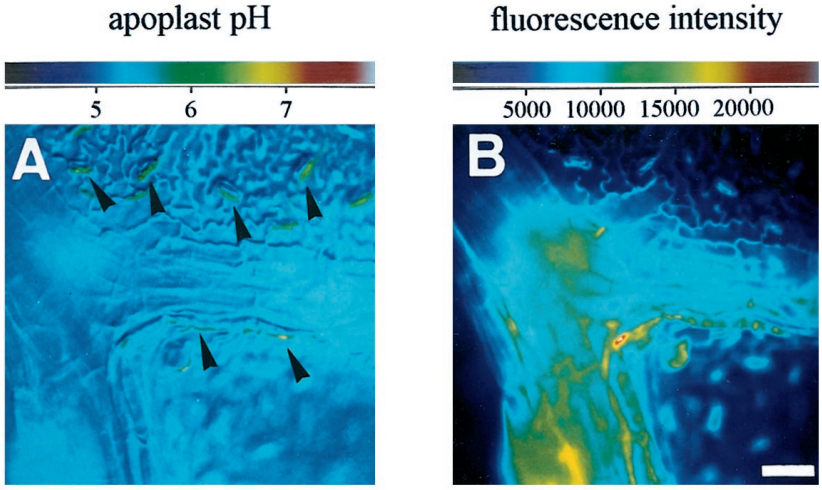

Figure 3. Apoplast pH (A) and fluorescence intensity after excitation at 490 nm (B) of the upper cell layer of a sunflower leaf after 5 h of darkness as examined by microscope image analysis. Plants were cultivated with 6 mM NO_3^-/10 mM HCO_3^-. The light-blue pseudocolor in the left picture corresponds to a pH of around 5.7, the green pseudocolor to a pH of around 6.5, and the yellow pseudocolor to a pH of around 7.0. The fluorescence intensity is high in the xylem vessels and in the stomatal region, as shown by the red, yellow, and light-blue pseudocolors (B). Scale, 80 μm.

Table III. *Concentration (mM) of various N compounds in the xylem sap of sunflower, as depending on the nutritional N source*

Nutritional N Source	NO_3^-	NH_4^+	Glu	Asp	Other Amino Acids
NH_4NO_3 (3 mM)[a]	20.08	0.64	3.42	0.76	0.47
NH_4^+ (6 mM)	1.49	0.92	7.49	1.17	1.10
NH_4^+ (1 mM)	0.69	0.30	11.88	0.74	1.61
NO_3^- (6 mM)	30.45	0.32	1.39	0.38	0.24
NO_3^-/HCO_3^- (6 mM)	26.92	0.16	1.54	0.34	0.42

[a] Control.

Table V shows no difference in the fluorescence intensity of rhodamine between the pH treatments; therefore, for ferrozine infiltration variations in dye loading could be excluded as well.

DISCUSSION

Apoplastic pH of Young Green Leaves under Alkaline Conditions

Fe chlorosis occurs mainly on calcareous soils, where NO_3^- is the exclusive N form in the soil solution due to increased nitrification (Darrah et al., 1986) and NH_3 volatilization (Paramasivam and Alva, 1997). As shown in Table I, the N form clearly influenced xylem sap pH; the highest pH values were observed with NO_3^- nutrition in solution culture. Interestingly, the presence of HCO_3^- (as in the soil solution of calcareous soils) did not influence xylem sap pH (Table I). Presumably, proton pumps adjacent to the xylem (Canny, 1987; Wilson et al., 1988) are efficient enough to neutralize HCO_3^-. In addition, the low partial pressure in the xylem (Zimmermann et al., 1993) should favor the formation of CO_2 from HCO_3^-. Feeding young excised leaves with the xylem sap obtained from various N treatments resulted in a substantial apoplastic pH increase at microsites (pH $\geq$ 6.3) in the intercostal leaf area only in plants that had received exclusively NO_3^- from the nutrient solution (Figs. 2C and 3A).

Leaf apoplastic alkalization upon NO_3^- nutrition was not homogenous in the intercostal leaf area, when inspected at the cellular level. About 10% of the leaf apoplast showed elevated pH levels $\geq$ 6.3 (Fig. 2C) at distinct apoplastic microsites on complexes of stomatal and epidermal cells. The section shown in Figure 3 with an area of 500 $\times$ 500 μm^2 comprises about 100 cells. With microscope imaging only the upper cell layer could be analyzed and showed a number of epidermal and stomatal cells, as indicated by the green and yellow color (arrows), with pH levels $\geq$ 6.3 (Fig. 3A). The apoplast of underlying mesophyll cells may also show these increased pH levels, but this has to be proven by use of confocal microscopy. Such microsites of high apoplastic pH were not found in the case of NH_4^+ and NH_4NO_3 supply (Fig. 2, A and B).

The apoplastic pH values shown in Table I are mean data at the tissue level of several hundred leaf cells and show a significantly higher apoplastic pH of only 0.15 pH units under alkaline nutritional conditions (Table I) compared with the control (NH_4NO_3 treatment). From this observation it is clear that at the tissue level apoplastic pH measurements average out more pronounced apoplastic pH increases at the cellular level (Figs. 2C and 3A). Therefore, the small pH increases at the tissue level (Table I) do not reflect the real physiological, site-specific apoplastic pH response of young leaves exposed to alkaline nutritional conditions. Such microsites of high apoplastic pH are dis-

Figure 4. Intercostal chlorosis in young sunflower leaves. The apoplast pH was measured with a fluorescence microscope in the yellow intercostal area and the green xylem vessels (arrows).

Table IV. *Apoplast pH in the intercostal area and of xylem vessels (mid-rib and first order) in the tip of green leaves and of leaves with intercostal chlorosis (n = 30; ±SD) of H. annuus*

In the leaf with intercostal chlorosis the apoplast pH was measured in the area of green leaf veins and in the chlorotic intercostal area. Apoplast pH was monitored in the light after perfusion of 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM KCl.

Apoplast pH	Green Leaf	Leaf with Intercostal Chlorosis
Mid-Rib	4.78 ± 0.10	4.68 ± 0.24
Xylem vessel (first order)	4.51 ± 0.22	4.72 ± 0.21
Intercostal area	4.46 ± 0.18	5.30 ± 0.14

tributed throughout the leaf blade (not shown), and we speculate that they are related to growing sites of a young leaf where high NO_3^- uptake rates occur. This means that these sites need N for protein synthesis, as well as NO_3^- for osmotic reasons in expanding cells (McIntyre, 1997). According to the composition of N compounds in the xylem sap (Table III), when NO_3^- is the sole N source, it may also provide N for protein synthesis. Like N demand, Fe demand in the growing cells is high, in particular for the synthesis of ribonucleotide reductase (Reichard, 1993) and for chlorophyll synthesis (Terry and Abadia, 1986). This assumption is in line with the observation of Kosegarten et al. (1998) that in sunflowers fed with NO_3^- the development of leaf primordia was inhibited in contrast to the treatment with NH_4NO_3 nutrition.

It is well known from the work of Maksymowych (1973) that the entire blade of a dicotyledonous leaf is involved in growth. Accordingly, we have conducted a frequency study at the leaf base related to a leaf area of 50 mm^2 (Fig. 2). Interestingly, in older leaves apoplastic alkalization induced by NO_3^- nutrition was not observed (not shown), and this may be the reason why mature leaves are not sensitive to Fe chlorosis. In mature leaves, growth processes have been completed and, unlike young leaves, have a low demand for NO_3^- (Van Egmond and Breteler, 1972). In addition, mature leaves show high net photosynthetic rates (Turgeon and Webb, 1975), presumably providing enough energy for the plasmalemma H^+ pump and therefore may efficiently regulate leaf apoplastic pH.

The process of apoplastic alkalization supposedly resulted from the removal of protons from the apoplast upon proton cotransport of NO_3^- (Ullrich, 1992; Crawford and Glass, 1998) into the adjacent cells. NO_3^- typically is the main inorganic N form transported to the leaf (Pate, 1973; Van Beusichem et al., 1988) and, presumably, at microsites of the meristematic and rapidly expanding leaf cells, high NO_3^- uptake rates necessary for the growth of a young leaf occur. As evident from Table III, the NO_3^- concentration in the xylem sap was high in all treatments with NO_3^- in the nutrient solution.

In the treatment with NH_4NO_3 and NH_4^+, however, the NH_4^+ concentration in the xylem sap was relatively high (Table III). Therefore, in these treatments NH_4^+ also may play an important role in N nutrition of leaf cells. Even at a concentration of 1 mM NH_4^+ in the nutrient solution (Table III), reflecting the concentration of most agricultural soil solutions (Wolt, 1994), a concentration of 0.3 mM NH_4^+ and a low NO_3^- concentration (0.69 mM) were found in the xylem sap. In the leaf apoplast of *Brassica napus* grown on sandy soil, Husted and Schjoerring (1995) found NH_4^+ concentrations up to 0.8 mM and reported high uptake rates for NH_4^+, which according to Nielsen and Schjoerring (1998), may be related to a transporter with channel-like properties. NH_4^+ uptake depolarizes the membrane potential (Herrmann and Felle, 1995) and stimulates the H^+-ATPase, which results in a low apoplastic pH (Kosegarten et al., 1999).

Until now, very little information has been available concerning NH_4^+ transport from the leaf apoplast into the symplasm. Ninnemann et al. (1994) isolated and characterized the AMT1 gene for a high-affinity NH_4^+ transporter in leaves of Arabidopsis. In addition to NH_4^+, Gln was a major N compound in the xylem sap upon treatment with NH_4NO_3; with exclusive NH_4^+ supply, Gln was even the dominating N compound in the xylem sap (Table III). Uptake systems for amino acids in leaves have been identified by Van Bel et al. (1986) and uptake found to occur presumably via proton cotransport (Li and Bush, 1990; Williams et al., 1990). Amino acids are protonated at the apoplastic pH level, and therefore uptake into the mesophyll cell may remove fewer protons from the apoplast than with NO_3^-. If NH_4^+ and/or amino acids contribute to

Table V. *Fe^{3+} reduction in intact leaves of sunflower in relation to apoplast pH of xylem vessels (first order)*

Fe^{3+} reduction and apoplast pH was monitored after infiltration of various buffer solutions to the xylem vessels ($n = 27$; ±SD). The pH-insensitive dye rhodamine was infiltrated into leaves and used as an internal standard to check for variations in dye loading.

Buffer (pH)	Xylem Apoplast pH	Fe^{3+} Reduction	Rhodamine Fluorescence Intensity
		%	
Leaf without buffer	≤5.0 (Light)	100	2,690.1 ± 202.2
100 mM MES (pH 5.0)	5.44 ± 0.07	98 ± 5	2,651.8 ± 208.4
100 mM MES (pH 6.0)	5.92 ± 0.43	78 ± 15	n.d.[a]
100 mM HEPES (pH 7.25)	6.86 ± 0.62	54 ± 20	n.d.
100 mM HEPES (pH 8.0)	7.71 ± 0.41	22 ± 11	2,821.3 ± 177.5

[a] n.d., Not determined.

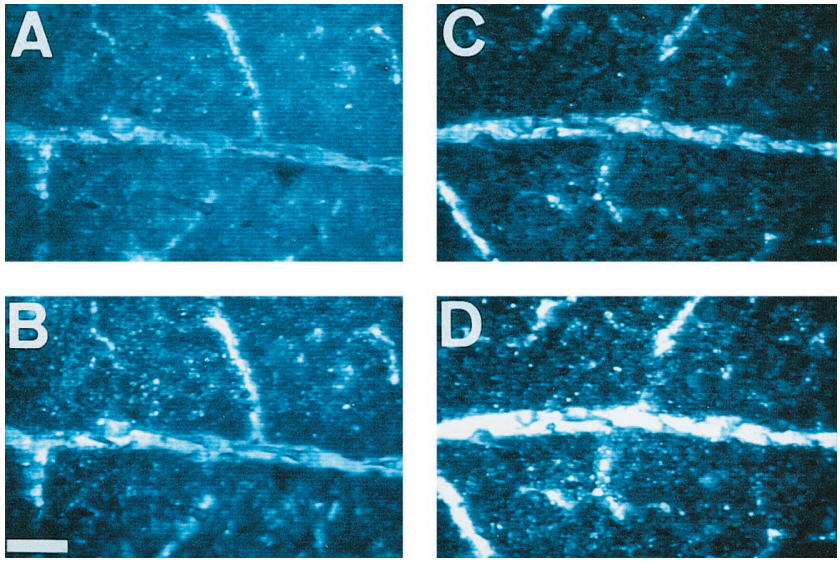

Figure 5. Light transmission in the xylem vessels at pH 5.4 (A, 720 nm; B, 560 nm) and at pH 7.7 (C, 720 nm; D, 560 nm) after infiltration of Fe^{3+}-citrate and ferrozine at different apoplastic pH levels. Scale, 240 μm.

the N nutrition of leaf mesophyll cells from the apoplast, the apoplastic pH may be lowered and high apoplastic pH levels at microsites would not prevail (Fig. 2, A and B).

An increase of apoplastic pH here was observed between different cell types according to the following sequence: xylem vessel (Table II) < the main portion of epidermal and stomatal cells (Fig. 2) < hair cells (Table II). The observed cell-specific differences in apoplastic pH may result from a differential abundance of H^+ pumps in the different leaf cells (Bouche-Pillon et al., 1994; Michelet and Boutry, 1995) and/or from differential removal of protons from the respective apoplast space because of differential N uptake of the various cells. In all nutritional treatments, the pH in the apoplast of the mesophyll, the xylem, and the hair cells was higher in darkness than in light (Tables I and II; Fig. 2, B and C). This finding indicates that apoplastic pH is influenced by the prevailing metabolic condition and is in line with previous results of Hoffmann and Kosegarten (1995). Mengel and Malissiovas (1982) have shown that net proton excretion of roots of intact vine trees was higher during the day than at night.

Fe^{3+} Reduction in Relation to Apoplastic pH in Young Green Leaves

Growing tissues need a continuous Fe supply (Brown, 1978), and the anatomy of the growing leaf tissue is complex (Taylor, 1997). It is of interest whether growing tissues receive Fe from the xylem and/or from the phloem. According to U.W. Stephan (personal communication) and in contrast to their earlier findings (Stephan and Scholz, 1993), Fe in the phloem sap is mainly transported in the form of a Fe^{3+} complex, presumably bound to a small peptide. In our study we used young leaves of about 800 mm^2. At that developmental stage, showing high transpiration rates (not shown), the supply of Fe to the expanding leaf should proceed mainly via the xylem. Here, Fe is translocated in form of Fe^{3+}-citrate (e.g. Tiffin, 1966; Clark et al., 1973).

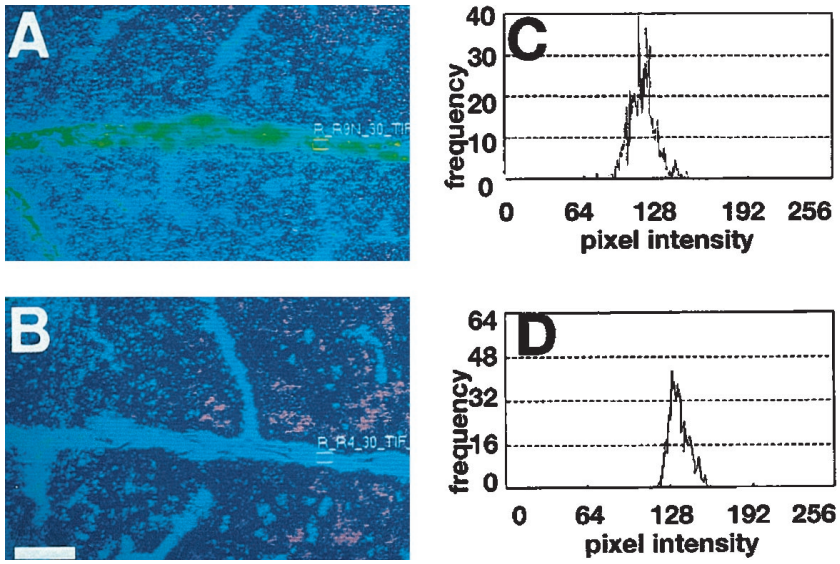

Figure 6. Light transmission ratio (720/560 nm) in the xylem vessels (A and B) after infiltration of Fe^{3+}-citrate and ferrozine at different apoplastic pH levels. A, pH 7.7; yellow pseudocolor represents high light transmission ratio. B, pH 5.4; light-blue pseudocolor shows low light transmission ratio. Scale, 240 μm. The distribution of the pixel intensity in the histogram is shown at pH 7.7 (C) and at pH 5.4 (D), with a maximum at 106.6 ± 3.4 (n = 9) and at 139.7 ± 5.8 (n = 9), respectively. A shift to higher pixel values indicates a reduction in the light transmission ratio.

This Fe^{3+} complex presumably needs to be reduced before passing the plasmalemma (Chaney et al., 1972; Fox et al., 1996). Fe^{3+} reductase activity in the leaf has been evidenced (Brüggemann et al., 1993; De la Guardia and Alcantara, 1996) and has been suggested as the prerequisite for Fe uptake into the growing leaf cell (Crowley et al., 1991; Mengel, 1995).

In this context it is of interest that the mesophyll tissue of young green leaves showed minute areas with a high apoplastic pH exclusively with NO_3^- supply (Figs. 2C and 3A). As mentioned previously, we suggest that NO_3^- is taken up with high rates at these microsites of high apoplastic pH, and that these microsites comprise meristematic and rapidly expanding cells, where NO_3^- is used for protein synthesis and as a major osmoticum (McIntyre, 1997). Such cells also require Fe for the synthesis of ribonucleotide reductase (Reichard, 1993) and for chlorophyll synthesis (Terry and Abadia, 1986). If at such sites the activity of the Fe^{3+} reductase is restricted because of a high pH at the apoplastic domain of the reductase, intracellular Fe deficiency will occur, with a concurrent reduction in leaf growth (Mengel and Malissiovas, 1981; Kosegarten et al., 1998) and hampered chlorophyll synthesis (Terry and Abadia, 1986).

In our experiments, Fe^{3+} reduction was measured by the formation of a Fe^{2+}-ferrozine complex in the leaf xylem after infiltration of Fe^{3+}-citrate in the presence of various buffers and was determined by means of assessing the light transmission in the xylem vessel (Figs. 1, 5, and 6). As shown in Table V, Fe^{3+} reduction rates clearly declined upon increase of apoplastic pH in the xylem. Our measure for Fe^{3+} reduction is a relative one and the most important conclusion that can be drawn from our data is that the xylem of intact leaves shows a pH-dependent Fe^{3+} reduction, with maximal rates at apoplastic pH 5.0 and lower. With increasing xylem apoplastic pH, Fe^{3+} reduction concomitantly decreased; e.g. at pH 7.7 the reducing power was only 22% of that found at apoplastic pH 5.0 (Table V). To our knowledge, until now no relationship between Fe^{3+} reduction power and apoplastic pH in intact leaves has been described. The maximal rates of Fe^{3+} reduction in the leaf apoplast at apoplastic pH 5.0 and lower compare well with that in intact roots of *B. napus* (Toulon et al., 1992) and of *Beta vulgaris* (Susin et al., 1996). Toulon et al. (1992) found the highest reduction rate at pH 4.0 in the outer solution. Taking into account the maximal H^+-buffer capacity of cell walls at around pK_a 5 (Sentenac and Grignon, 1981), an apoplastic pH of around 5.0 (Felle, 1998; Kosegarten et al., 1999) with maximal Fe^{3+} reduction rates in the root apoplast is realistic.

Because of experimental difficulties in monitoring Fe^{3+} reduction at the apoplastic side of intact leaf mesophyll (e.g. insensitivity of absorbance measurement at low optical pathlength), Fe^{3+} reduction was recorded in the leaf xylem. Also, the xylem is a part of the apoplast that is separated by the plasmalemma from the leaf cells surrounding the xylem vessels. It is assumed that also these plasma membranes are equipped with Fe^{3+} reductases and, in analogy to the monitoring of reduced formation of the Fe^{2+}-ferrozine complex in the xylem vessels at high xylem apoplastic pH in the presence of HEPES buffer (Table V), we suggest that at apoplastic microsites of the interveinal leaf area with high apoplastic pH $\geq$ 6.3 (Figs. 2C and 3A) under alkaline nutritional conditions, Fe^{3+} reduction is clearly decreased. At about pH 7.0 in the xylem apoplast, Fe^{3+} reduction was reduced by about 50% (Table V) and at microsites with pH $\geq$ 7.0 (Fig. 2C), the rate of Fe^{3+} reduction should be even lower (Table V). Such an analogous conclusion is justified, because a similar pH-dependent pattern between Fe^{3+} reduction and outer solution pH was found in intact roots with maximal rates of Fe^{3+} reduction at low pH and a concomitant decrease with increasing pH (Toulon et al., 1992; Susin et al., 1996).

Using the experimental approach of microscope imaging, we monitored, for the first time to our knowledge, Fe^{3+} reduction at the apoplastic side in intact leaf tissue. It is interesting that here a similar apoplastic pH dependency of Fe^{3+} reduction prevails, as is the case for intact roots. With this experimental setup, a nonenzymatic, spontaneous Fe^{3+} reduction (e.g. by ascorbate) cannot be excluded. However, the pH-dependent response, as shown in Table V, is pronounced and therefore strongly indicates an enzymatic mechanism of Fe^{3+} reduction.

Phenomenon of Intercostal Chlorosis

The influence of chlorosis on leaf apoplast pH was investigated and the results are shown in Table IV. The striking pH difference between the apoplast of the green leaf veins (pH 4.5–4.7) and the chlorotic intercostal area (pH 5.3) presumably reflects the overall lower energetic status of chlorotic intercostal leaf regions. In contrast to the apoplastic pH measurements in young leaves before leaf chlorosis occurred (Fig. 2), the apoplastic pH of leaves with intercostal chlorosis was measured at the cellular level in the absence of NO_3^- or other N forms (Table IV). Therefore, the apoplastic pH in the intercostal area of the chlorotic and, in particular, in the green control leaves, was relatively low and no microsites with high apoplastic pH were measured. Supplying chlorotic leaves with NO_3^- may cause the apoplastic pH to increase at sites of high N demand. Since growth of chlorotic leaves is restricted, the need for NO_3^- is also reduced and, in particular, in fully developed chlorotic leaves high apoplastic pH levels restricting Fe^{3+} reduction may not necessarily prevail.

Compared with the chlorotic intercostal area, the apoplast pH of the green leaf veins (Fig. 4) was particularly low (Table IV), presumably because of: (a) the influx of xylem liquid, which had a relatively low pH (Table I), and (b) the efficient pH regulation via H^+-ATPase at the site of xylem vessels (Michelet and Boutry, 1995). NO_3^- nutrition did not increase the xylem apoplastic pH compared with the NH_4NO_3 treatment, and light/dark changes had only a minor effect (Table II). Therefore, Fe^{3+} reduction in the area of green veins of interchlorotic leaves is presumably still optimal for continuous Fe supply of the neighboring cells adjacent to the xylem vessels, and this may be the reason that during leaf yellowing the tissue around the leaf xylem remains green (Fig. 4).

Several studies have shown that upon NO_3^- nutrition, leaf chlorosis will be induced (e.g. Aktas and Van Egmond, 1979; Mengel and Geurtzen, 1988). As the xylem liquid enters the intercostal area of young, still green leaves under alkaline nutritional conditions, high apoplastic pH levels prevailed at microsites (Figs. 2C and 3A) over the whole leaf blade, presumably related to the growing sites due to increased uptake of NO_3^- via proton cotransport. When averaged across the leaf, these substantial apoplastic pH changes were limited to about 10% of the whole interveinal leaf apoplast in this study, and were shown to be a small overall apoplastic pH change (Table I). However, the high apoplastic pH at these interveinal microsites may depress Fe^{3+} reductase activity by about 50% (Table V). Such a restriction is not small, in particular because at these growing microsites, different reactions such as DNA synthesis (Reichard, 1993) and chlorophyll synthesis (Terry and Abadia, 1986) compete for Fe. We therefore suggest that the uptake of Fe^{2+} may be depressed at these interveinal microsites and may be sufficient to induce leaf yellowing and growth retardation under alkaline conditions (Kosegarten et al., 1998).

From these argumentations it is clear that future research is needed to clarify the induction of leaf yellowing and to investigate apoplastic pH throughout the leaf chlorosis process. Interestingly, leaf yellowing of young green leaves is a slowly continuing process that starts at minute areas over the whole leaf surface and not simultaneously at all interveinal sites. This observation fits with the distribution of high apoplastic pH in the leaf at interveinal microsites (Figs. 2C and 3A) and with our hypothesis that at these sites of high apoplastic pH, with with Fe^{3+} reduction is inhibited, which may induce leaf yellowing. Since apoplastic pH is a dynamic rather than a static parameter (see Hoffmann and Kosegarten, 1995) future studies would be of particular interest to correlate leaf paling with apoplastic pH throughout the process of leaf chlorosis at the cellular level to understand the complex nature of leaf yellowing. Also, a Fe^{3+}-sensitive fluorochrome that can be loaded into the leaf apoplast would realize measurements of Fe^{3+} reduction at interveinal microsites.

In the present study, apoplastic pH during leaf yellowing and apoplastic pH compared with Fe^{3+} reduction in yellowing leaves were not examined. It is quite possible that, in contrast to young green leaves, in growing but yellowing leaves microsites with high apoplastic pH may be increased, because at lower photosynthetic rates plasmalemma H^+-ATPase activity may be restricted. Due to low photosynthetic rates of chlorotic leaves (Kosegarten et al., 1998), the amount of reducing equivalents may also be restricted and therefore Fe^{3+} reduction as well.

OUTLOOK

Plants grown on calcareous soils suffer from a physiological Fe deficiency, and a substantial amount of Fe is presumably trapped in the apoplast of leaves and roots. The supply of Fe has to overcome two critical steps: (a) the high pH in the leaf apoplast, which hampers Fe^{3+}-citrate reduction (Table V), and (b) the high pH in the root apoplast (Toulon et al., 1992; Kosegarten et al., 1999), which may hamper Fe^{3+}-siderophore reduction. The pH dependence of Fe^{3+} reduction in the root apoplast remains to be proven.

ACKNOWLEDGMENT

We thank Dr. F. Grolig (Botanik, Philipps Universität, Marburg, Germany) for critically reading the manuscript.

Received June 7, 1999; accepted September 4, 1999.

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