Nitrate Does Not Result in Iron Inactivation in the Apoplast of Sunflower Leaves[1]

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It has been hypothesized that nitrate (NO_3^-) nutrition might induce iron (Fe) deficiency chlorosis by inactivation of Fe in the leaf apoplast (H.U. Kosegarten, B. Hoffmann, K. Mengel [1999] Plant Physiol 121: 1069–1079). To test this hypothesis, sunflower (*Helianthus annuus* L. cv Farnkasol) plants were grown in nutrient solutions supplied with various nitrogen (N) forms (NO_3^-, NH_4^+ and NH_4NO_3), with or without pH control by using pH buffers [2-(*N*-morpholino)ethanesulfonic acid or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. It was shown that high pH in the nutrient solution restricted uptake and shoot translocation of Fe independently of N form and, therefore, induced Fe deficiency chlorosis at low Fe supply [1 μM ferric ethylenediaminedi(O-hydroxyphenylacetic acid)]. Root NO_3^- supply (up to 40 mM) did not affect the relative distribution of Fe between leaf apoplast and symplast at constant low external pH of the root medium. Although perfusion of high pH-buffered solution (7.0) into the leaf apoplast restricted ^{59}Fe uptake rate as compared with low apoplastic solution pH (5.0 and 6.0, respectively), loading of NO_3^- (6 mM) showed no effect on ^{59}Fe uptake by the symplast of leaf cells. However, high light intensity strongly increased ^{59}Fe uptake, independently of apoplastic pH or of the presence of NO_3^- in the apoplastic solution. Finally, there are no indications in the present study that NO_3^- supply to roots results in the postulated inactivation of Fe in the leaf apoplast. It is concluded that NO_3^- nutrition results in Fe deficiency chlorosis exclusively by inhibited Fe acquisition by roots due to high pH at the root surface.

When grown on highly calcareous soils, most plant species of the so called Strategy I type (Marschner and Römheld, 1994) respond to lack of iron (Fe) by developing characteristic symptoms of Fe deficiency chlorosis, primarily in young leaves. The concentration of Fe expressed on a dry weight leaf basis and the amount of Fe per leaf frequently decreases in chlorotic leaves, although the Fe concentration can sometimes be the same or even higher in chlorotic leaves as compared with green ones, as has been reported in field-grown woody plants ("the chlorosis paradox"; Morales et al., 1998; Römheld, 2000; Nikolic and Römheld, 2002). Various soil factors (e.g. CO_2, ethylene, low temperature, high water content, and drought) resulting in severe inhibition of root growth might be responsible for triggering a restriction in leaf expansion growth, which in turn elevates the Fe concentration in these chlorotic leaves as a consequence of the diminished dilution of Fe concentration (Römheld, 2000, and refs. therein). This explanation would appear to account for the phenomenon of the "chlorosis paradox" that occurs occasionally under field conditions and is always associated with restricted leaf expansion growth. On the other hand, as has been hypothesized by Mengel and coworkers for many years, the higher Fe concentrations in the leaves of chlorotic plants could be caused by an increase in the pH of leaf apoplast induced by bicarbonate (HCO_3^-) or nitrate (NO_3^-) or both of these ions in the soil solution of calcareous soils (Mengel, 1994). These authors argue that because a high leaf apoplastic pH depresses the activity of Fe^{III} reductase, less Fe^{2+} can be transported across the plasma membrane into the leaf symplast, resulting in Fe deficiency chlorosis (Kosegarten et al., 2001). Despite this explanation, however, chlorotic plants with higher concentrations of Fe in younger leaves have never been found so far in nutrient solutions under controlled environmental conditions (Römheld, 2000; Nikolic and Römheld, 2002).

It is well known that at high concentration in the soil solution, HCO_3^- penetrates the root apoplast, thereby neutralizing H^+ ions and lowering Fe uptake by root cells as a consequence of inhibition of plasma membrane-bound Fe^{III} reductase at high apoplastic pH (Fleming et al., 1984; Römheld and Marschner, 1986; Toulon et al., 1992). There is no evidence, however, that free dissolved HCO_3^- can be transported from root to shoot via the xylem to a high enough extent to cause a substantial increase in the pH of the xylem sap (Bialczyk and Lechowski, 1995; Peiter et al., 2001). So far, there is good confirmation that most of the dissolved HCO_3^- taken up is utilized in the processes of organic carbon fixation (e.g. phospho*enol*pyruvate carboxylase) by roots (López-Milán et al., 2000). The presence of high HCO_3^- in the

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nutrient solution was shown to affect neither the bulk pH of apoplastic fluid obtained by centrifugation of intact leaves (Nikolic and Römheld, 1999, 2002) nor the apoplastic pH measured by using a pH-sensitive fluorescent dye (fluorescein isothiocyanate-dextran) after infiltration of xylem sap into excised leaves (Kosegarten et al., 1999). Furthermore, HCO_3^- does not appear as a cause of physiological Fe inactivation in the leaf apoplast nor does it cause an inhibition of Fe uptake into the leaf symplast (Nikolic and Römheld, 2002), which is also in agreement with the recent conclusions of Kosegarten et al. (1999, 2001).

Although the effect of the form of nitrogen (N) supply (NO_3^- versus NH_4^+) on pH changes in nutrient solutions and rhizosphere have been extensively studied over the past two decades, the effect of N form on the pH of leaf apoplast is still a matter of controversial debate (for review, see Sattelmacher, 2001). It has been argued that NO_3^- nutrition induces an alkalization of leaf apoplast (Mengel et al., 1994; Kosegarten et al., 1999, 2001), although there is no clear evidence that this occurs to any great extent (Mühling and Sattelmacher, 1995; Sattelmacher et al., 1998; Mühling and Läuchli, 2001). Also, there are other factors, including NO_3^- reductase activity in the roots (Andrews, 1986) and light-induced pH changes in the leaf apoplast (Mühling and Läuchli, 2001), which may modify any possible potential effect of NO_3^- supply in the nutrient solution on leaf apoplastic pH. According to Mengel's hypothesis, inactivation of Fe in the leaf is caused primarily by NO_3^- supplied from the roots (Mengel, 1994; Mengel et al., 1994), and it is postulated that Fe trapped in the leaf apoplast at high pH can be mobilized by lowering the apoplastic pH that favors Fe^{III} reduction and, thus, Fe uptake into the symplast (Kosegarten et al., 2001). The main support for this Fe inactivation concept comes from the findings that spraying chlorotic leaves with dilute organic or mineral acids is followed by leaf regreening of field-grown kiwifruit (*Actinidia deliciosa*) and peach (*Prunus persica*) trees (Tagliavini et al., 1995; 2000) or sunflower (*Helianthus annuus*) plants grown in nutrient solutions (Kosegarten et al., 2001). However, Nikolic and Römheld (1999) reported that although the pH of leaf apoplast is important in regulation of extracellular Fe^{III} reduction and Fe uptake into the symplast of leaf cells, this effect was of only marginal significance within the physiologically relevant pH range as measured in the leaf apoplastic fluid. In contrast to roots, there is little information in the literature on Fe distribution between apoplast and symplast in leaves (Pich and Scholz, 1991; Becker et al., 1992; Nikolic and Römheld, 2002), and there appears to be a particular lack of experimental data relating to Fe-binding forms in the leaf apoplast caused by various sources of N nutrition.

In this study, we tested Mengel's hypothesis of Fe inactivation in leaves caused by NO_3^- nutrition using the same model plant, sunflower, as has commonly been used in many experiments performed by Mengel et al. (Mengel et al., 1994; Hoffmann and Kosegarten, 1995; Kosegarten et al., 1998a, 1998b, 1999, 2001). The objectives of the work presented here were: (a) to clarify the effect of NO_3^- supply to roots on postulated Fe inactivation in the leaf apoplast as a primary cause of NO_3^--induced Fe deficiency chlorosis; (b) to investigate the influence of form of N supply and pH of nutrient solution stabilized by pH buffers on concentration of Fe in the two-leaf compartments, apoplast, and symplast; and c) to characterize the leaf apoplast for various binding forms of Fe. Furthermore, we studied the direct effect of pH and the presence of NO_3^- in the leaf apoplast of excised leaves on Fe uptake by the symplast of leaf cells. In the present work, a method suitable for determination of different Fe-binding forms in the leaf apoplast, combining extraction of apoplastic washing fluid (AWF) with isolation of cell walls from sunflower leaves has been developed. In this new approach, Fe uptake by leaf symplast as affected by pH and NO_3^- is measured using various radioactive labeled ^{59}Fe solutions infiltrated into the apoplast of excised leaves via petiole by the transpiration stream.

RESULTS

Effects of N Form and Nutrient Solution pH on Chlorophyll, Growth, and Fe Utilization

The pH of unbuffered nutrient solution supplied with NO_3^- shifted from 5.6 in fresh nutrient solution to about 6.8 before the next solution change (data not shown). However, the pH of NH_4NO_3-supplied nutrient solution without pH buffering was continually low, alternating between 5.5 and 4.5. As shown in Figure 1 and Table I, Fe deficiency chlorosis on the leaves of sunflower plants only occurred in the treatments with a high pH in the nutrient solution (NO_3^-, unbuffered; NH_4^+ and NH_4NO_3, buffered at pH 7.5) at low Fe supply (1 μM). Although severe chlorosis symptoms were found in either NH_4^+ or NH_4NO_3 treatment with pH stabilization at 7.5 using HEPES buffer, NO_3^- treatment without pH control (about pH 6.8) showed only moderate chlorosis (Fig. 1; Table I). Maintaining low pH at 5.0 using MES buffer, even at an extremely high concentration of NO_3^- (40 mM), did not result in any decrease in leaf chlorophyll content compared with the unbuffered NH_4NO_3 treatment (about pH 4.5).

The growth of sunflower plants, estimated as dry weights of shoot and roots, were significantly reduced in all treatments with high pH (NO_3^-, unbuffered; NH_4^+ and NH_4NO_3, pH 7.5, HEPES). Furthermore, this was closely related to chlorophyll content, especially in severely chlorotic plants (Table I). The NH_4NO_3 treatment with pH of the nutrient solution buffered to 7.5 showed strong inhibition of leaf expansion growth, whereas leaf area was only slightly

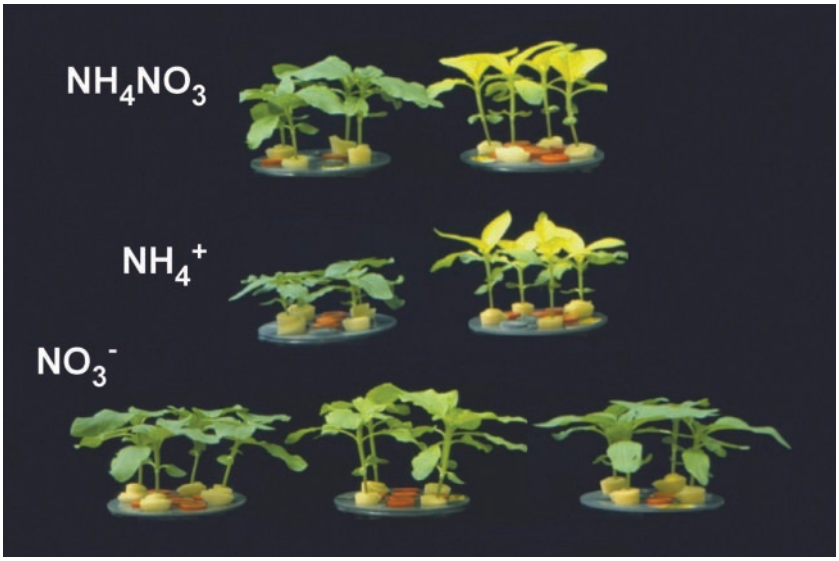

Figure 1. Effects of N form and nutrient solution pH on Fe deficiency chlorosis occurrence in sunflower plants after 2 weeks of growth in nutrient solution supplied with 1 μM Fe. Top (left to right): 2 mM NH_4NO_3 (pH $\cong$ 4.5, unbuffered) and NH_4NO_3 (pH 7.5, HEPES). Middle (left to right): 4 mM NH_4^+ (pH 5.0, MES) and 4 mM NH_4^+ (pH 7.5, HEPES). Bottom (left to right): 4 mM NO_3^- (pH 5.0, MES), 4 mM NO_3^- (pH $\cong$ 6.8, unbuffered), and 40 mM NO_3^- (pH 5.0, MES).

decreased in NO_3^--treated plants without pH control (Table I). However, plant growth was significantly reduced in green plants too but only when the NH_4^+-N was the sole form supplied at low pH. Root Fe concentration and particularly Fe amount were significantly lower in chlorotic plants than in green ones (Table II). Both concentration and amount of leaf Fe also markedly decreased in chlorotic plants (2–3 times) grown in various N treatments with high pH in the nutrient solution. It is obvious that in all treatments where nutrient solution pH was high, NH_4^+ and NH_4NO_3 treatments with pH of nutrient solution buffered to 7.5, and NO_3^- treatment without pH buffering, Fe uptake was strongly restricted (Table II). Also, as clearly shown in Figure 2, Fe concentration in xylem sap significantly decreased in both NO_3^-- and NH_4NO_3-fed plants grown at high external pH in the nutrient solution supplied with various N forms (NO_3^-, unbuffered; NH_4NO_3, pH 7.5, HEPES) as compared with plants grown at low external pH (NO_3^-, pH 5.0, MES; NH_4NO_3, unbuffered).

Effects of N Form and pH of Nutrient Solution on pH and NO_3^- Concentration in Xylem Sap and Leaf Apoplastic Fluid

No significant pH differences were found in xylem sap among treatments with different forms of N supply (Table III). NO_3^--treated plants always showed higher pH of bulk apoplastic fluid for 0.2 to 0.3 pH units than other N treatments (i.e. NH_4^+ and NH_4NO_3), although these differences were not significant (Table III). However, no differences in the pH of apoplastic fluid were found between green and chlorotic leaves of plants solely fed with NO_3^- with or without pH control. Moreover, xylem sap and leaf apoplastic pH were not decreased even in the plants where Fe deficiency chlorosis was alleviated by adding 25% of N as NH_4^+ (data not shown).

Table I. *Chlorophyll content and growth characteristics of sunflower as affected by N form and external pH*

Plants were grown for 2 weeks in nutrient solutions supplied with 4 or 40 mM N as NO_3^- or with 4 mM N as either NH_4^+ or NH_4NO_3. In all treatments, Fe was applied as Fe^{III}EDDHA at 1 μM. Data are means ± SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

Treatments	Chlorophyll	Biomass		Leaf Area
		Roots	Shoot	
	Spectral plant analysis diagnostic units	*mg*		*cm^{-2}*
NO_3^-; pH 5.0 (green)	34.3 ± 0.7a	147 ± 17a	647 ± 49a	211 ± 8a
NO_3^-, 40 mM; pH 5.0 (green)	31.7 ± 0.7a	153 ± 25a	760 ± 58a	228 ± 16a
NO_3^-; unbuffered (chlorotic)	16.8 ± 4.2b	128 ± 9a	580 ± 42ab	183 ± 25b
NH_4^+; pH 5.0 (green)	35.0 ± 2.2a	90 ± 12b	356 ± 5b	128 ± 8c
NH_4^+; pH 7.5 (chlorotic)	3.9 ± 0.6c	56 ± 21b	345 ± 3b	110 ± 12c
NH_4NO_3; unbuffered (green)	35.6 ± 1.1a	157 ± 26a	724 ± 54a	266 ± 33a
NH_4NO_3; pH 7.5 (chlorotic)	4.2 ± 0.5c	42 ± 5c	335 ± 17b	123 ± 9c

Table II. *Effects of N form and nutrient solution pH on concentration, amount, and total Fe uptake of sunflower*

Data are means ± SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

Treatments	Fe concentration		Fe Amount		Fe Uptake
	Roots	Leaves	Roots	Leaves	
	μg g⁻¹ dry wt		*μg organ⁻¹*		*μg plant⁻¹*
NO_3^-; pH 5.0 (green)	266 ± 20a	81 ± 11a	39 ± 3a	34 ± 4a	81 ± 7a
NO_3^-, 40 mM; pH 5.0 (green)	245 ± 34a	78 ± 8a	35 ± 12a	38 ± 5a	79 ± 8a
NO_3^-; unbuffered (chlorotic)	175 ± 30b	49 ± 4b	22 ± 4b	19 ± 2b	45 ± 2b
NH_4^+; pH 5.0 (green)	318 ± 28a	100 ± 14a	26 ± 5a	25 ± 3ab	58 ± 5ab
NH_4^+; pH 7.5 (chlorotic)	207 ± 36b	34 ± 3c	10 ± 2c	9 ± 1c	23 ± 4 c
NH_4NO_3; unbuffered (green)	239 ± 44ab	85 ± 10a	33 ± 2a	42 ± 6a	83 ± 6a
NH_4NO_3; pH 7.5 (chlorotic)	229 ± 51b	35 ± 4c	9 ± 1c	8 ± 1c	21 ± 2c

In all N treatments, the NO_3^- concentration in leaf apoplastic fluid was much lower than in xylem sap (Fig. 3). The NO_3^- concentration did not differ either in xylem sap or was the same in the apoplastic fluid of both green and chlorotic plants grown in either NO_3^- or NH_4NO_3 treatment supplied with 4 mM N. However, when plants were grown in nutrient solution with 40 mM NO_3^-, the NO_3^- concentration in both xylem and apoplastic sap increased 2 to 3 times (Fig. 3), but high apoplastic NO_3^- did not result in any decrease of chlorophyll content (Table I; Fig. 1). Almost no NO_3^- was detected in xylem sap (below 0.5 mM) and apoplastic fluid (below 0.1 mM) in NH_4^+ treatments (data not shown).

Effects of N Form and Nutrient Solution pH on Fe Compartmentation in the Leaves

The Fe concentrations in both leaf compartments, apoplast and symplast, significantly decreased in chlorotic leaves of sunflower plants grown at high external pH in nutrient solutions supplied with various N forms (NO_3^-, NH_4^+, and NH_4NO_3) as compared with green leaves of plants grown at low external pH (Table IV). Although the absolute apoplastic Fe concentration was about 2-fold higher in green leaves as compared with chlorotic ones, the relative proportion of apoplastic Fe remained almost unchanged (22%–28%) in all treatments. Thus, there were only relatively small differences in distribution of symplastic Fe and various apoplastic Fe binding forms between green (low external pH) and chlorotic (high external pH) leaves, regardless of whether $NH_4NO_3^-$ or sole NO_3^--N form was supplied (Fig. 4). In contrast, the relative proportion of soluble Fe presents in AWF was about 2 times lower in chlorotic leaves (all N treatments with high external pH) as compared with green ones (all N treatments with low external pH), but this fraction only represents 0.4% to 1% of total leaf Fe. Total cell wall Fe concentration and concentrations of both Fe-binding forms in the cell walls (i.e. weakly and strongly bound) decreased about 2-fold in chlorotic leaves in all N treatments when nutrient solution pH was kept high (Table V).

Maintaining a constant low pH (pH 5.0, MES), plants grown even at an extremely high NO_3^- concentration in the nutrient solution (40 mM) did not

Table III. *The pH of xylem sap and leaf apoplastic fluid of young fully expanded sunflower leaves as affected of N form and nutrient solution pH*

Data are means ± SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

Treatments	Xylem sap	Leaf apoplastic fluid
NO_3^-; pH 5.0 (green)	5.6 ± 0.1a	6.7 ± 0.1a
NO_3^-, 40 mM; pH 5.0 (green)	5.8 ± 0.2a	6.6 ± 0.0a
NO_3^-; unbuffered (chlorotic)	5.8 ± 0.1a	6.7 ± 0.0a
NH_4^+; pH 5.0 (green)	5.7 ± 0.2a	6.4 ± 0.1a
NH_4^+; pH 7.5 (chlorotic)	5.6 ± 0.1a	6.5 ± 0.1a
NH_4NO_3; unbuffered (green)	5.8 ± 0.1a	6.5 ± 0.1a
NH_4NO_3; pH 7.5 (chlorotic)	5.7 ± 0.2a	6.4 ± 0.1a

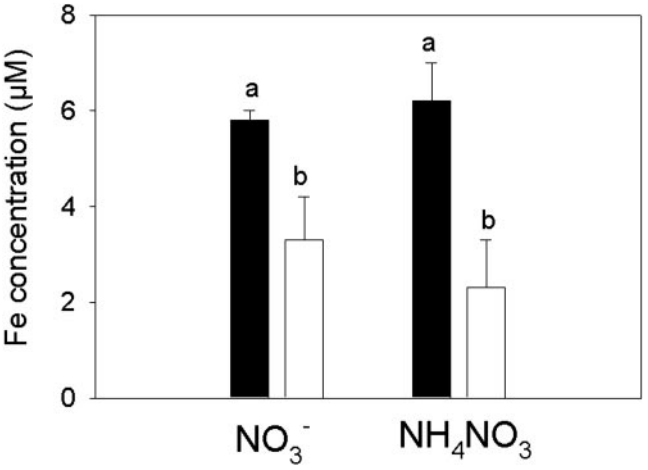

Figure 2. Effect of N form and pH of the nutrient solution on Fe concentration in xylem sap. Plants were grown for 2 weeks in nutrient solutions at 4 mM N supplied with either NO_3^- or NH_4NO_3. Black bars, Green (NO_3^-, pH 5.0; NH_4NO_3, unbuffered); white bars, chlorotic (NO_3^-, unbuffered; NH_4NO_3, pH 7.5). Data are means ± SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

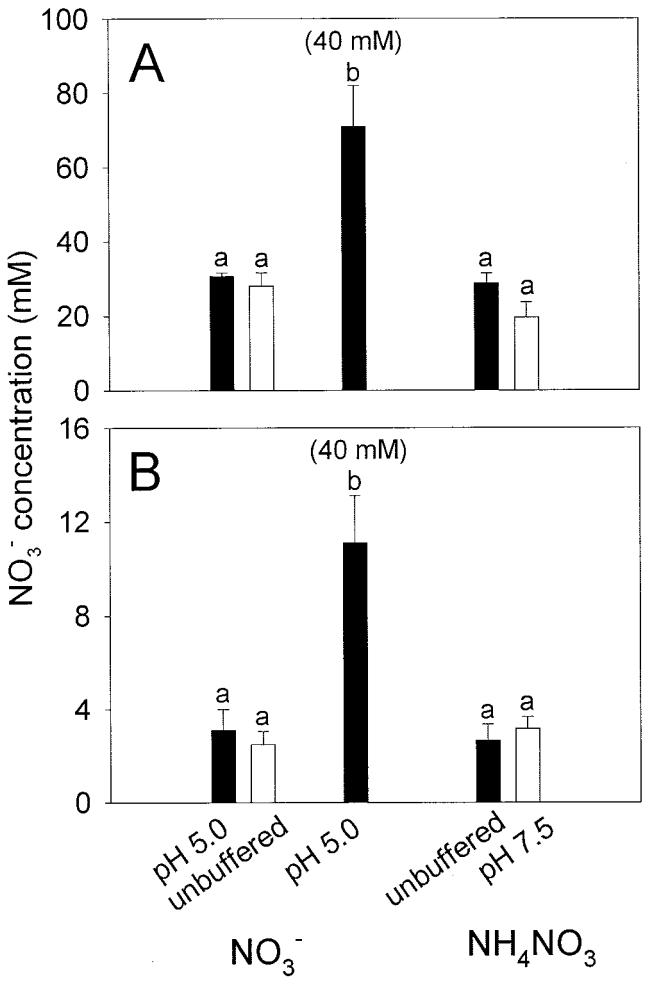

Figure 3. Concentration of NO_3^- in xylem sap (A) and leaf apoplastic fluid (B) as affected by N forms and external pH. Plants were grown for 2 weeks in nutrient solutions supplied with NO_3^- (4 or 40 mM N) or NH_4NO_3 (4 mM N). Black bars, Green (NO_3^-, pH 5.0; NH_4NO_3, unbuffered); white bars, chlorotic (NO_3^-, unbuffered; NH_4NO_3, pH 7.5). Data are means ± SD of four replications. Significant differences between treatments ($P < 5\%$) are indicated by different letters.

differ in Fe concentration in leaf cell wall from those grown in a solution at a 10 times lower NO_3^- concentration (4 mM; 3.9 and 4.2 μmol g^{-1} dry weight, respectively) and, as a consequence, the leaves remained green (Fig. 1; Tables I and V). The concentration of leaf cell wall Fe, however, was significantly lower in plants grown in at high pH (about 6.8) in nutrient solution without pH regulation (1.7 μmol g^{-1} dry weight). Concentration of Fe in leaf cell walls showed a close correlation with leaf chlorophyll content regardless of N form, whereas increase in apoplastic NO_3^- concentration did not affect Fe concentration in the cell walls (Fig. 5, A and B). This clearly indicates that high leaf apoplastic NO_3^- concentration (up to 12 mM) did not induce any excessive Fe trapping in the cell walls (Fig. 5B).

Fe Uptake by the Symplast of Intact Leaves as Dependent on Apoplastic pH and NO_3^- Concentration

Uptake solutions with radiolabeled ^{59}Fe citrate were infiltrated into the leaf apoplast by the transpiration stream via the petioles of excised leaves. This approach was used to clarify using short-term experiments whether high leaf apoplastic pH and/or high apoplastic NO_3^- concentration can inhibit Fe uptake by leaf symplast. To maintain the apoplastic pH at a defined value, various pH-buffered ^{59}Fe-labeled solutions were perfused into the xylem apoplast for 2 h. As shown in Figure 6, unbuffered solution, representing the indigenous pH conditions in the apoplast, and solutions buffered at low pH (5.0 and 6.0, respectively) did not differ significantly, whereas at a high buffered pH (7.0), Fe uptake was substantially decreased. These differences were much more pronounced when leaves were exposed to high light intensity (500 μmol m^{-2} s^{-1}) than in darkness (Fig. 6). Furthermore, regardless of the pH of the uptake solutions, light strongly affected Fe uptake by the symplast of leaf cells. It is interesting to note that Fe uptake rate was even higher at pH buffered to 7.0 under light as compared with the uptake rate in darkness at low pH.

To study the effect of apoplastic NO_3^- on Fe uptake, 6 mM NO_3^- was infiltrated together with ^{59}Fe citrate as described previously (Fig. 7; see "Materials and Methods"). To avoid any effect of NO_3^- present in the leaf apoplast, plants were precultured in NO_3^--free nutrient solution 48 h before the experiment. The presence of NO_3^- in the apoplastic solution showed no effect on Fe uptake by leaf symplast, either in darkness or when leaves were exposed to high light intensity (Fig. 7). However, light strongly stimulated ^{59}Fe uptake, regardless of the presence of NO_3^- in the apoplastic solution.

DISCUSSION

To clarify any possible influence of NO_3^- (and other N forms) on the availability of Fe in sunflower leaves, it is necessary to distinguish the effects of changes in pH at the root surface induced by the various N forms on the uptake and translocation of Fe by the plant from the comparative effects of pH per se. To achieve this, the pHs of the nutrient solutions supplied with various N forms were stabilized using organic pH buffers (see "Materials and Methods"). These pH buffers diffuse from the nutrient solution to penetrate the root apoplast and stabilize it at desired values, thus counteracting possible pH changes modulated by the plasma membrane proton pump (Logan et al., 1997) or by proton cotransport of NO_3^- (Meharg and Blatt, 1995). From the literature, the buffers used (MES, acid dissociation constant$_a$ = 6.15; and HEPES, pK_a = 7.55) at 5 mM concentration are effective enough to maintain the desired pH in the root apoplast either at 5.0 or 7.5, taking into

Table IV. *Effects of N form and nutrient solution pH on absolute and relative (share of total Fe) concentrations of symplastic and apoplastic Fe fractions in sunflower leaves*

Data are means ± SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

Treatments	Symplastic Fe		Apoplastic Fe	
	Absolute	Relative	Absolute	Relative
	μg g⁻¹ dry wt	*%*	*μg g⁻¹ dry wt*	*%*
NO_3^-; pH 5.0 (green)	67.4 ± 3.2a	73	24.7 ± 2.7a	27
NO_3^-,40 mM; pH 5.0 (green)	54.1 ± 3.2a	72	21.5 ± 3.2a	28
NO_3^-; unbuffered (chlorotic)	42.1 ± 5.0b	76	12.9 ± 2.0b	24
NH_4^+; pH 5.0 (green)	58.3 ± 14.4a	75	18.3 ± 1.8a	25
NH_4^+; pH 7.5 (chlorotic)	29.0 ± 2.8c	72	11.4 ± 1.3b	28
NH_4NO_3; unbuffered (green)	63.8 ± 5.0a	72	24.1 ± 5.6a	28
NH_4NO_3; pH 7.5 (chlorotic)	35.3 ± 3.3c	74	12.2 ± 1.0b	26

account the buffering capacity of the isolated cell walls (Sentenac and Grignon, 1981; Wang et al., 1992).

Fe deficiency chlorosis always occurred when the pH of the nutrient solution was high regardless of the form of N supply (Fig. 1; Table I). When the pH of the nutrient solution was maintained low (pH 5.0, MES), the growth of NH_4^+-fed plants was strongly inhibited in comparison with plants supplied with NO_3^- (Fig. 1; Table I), an observation reported for other plant species (Kirkby and Hughes, 1970; Raab and Terry, 1994; Walch-Liu et al., 2000). Strong growth inhibition was also observed in plants cultivated in high pH-buffered nutrient solution (pH 7.5, HEPES) supplied with NH_4^+ or NH_4NO_3 and was associated with severe Fe deficiency (Fig. 1; Table I). The inhibition of both chlorophyll synthesis and plant growth was more prominent in both NH_4^+- and NH_4NO_3-fed plants grown in the nutrient solutions pH buffered at 7.5 as compared with NO_3^--fed plants grown in the nutrient solution without pH control (Fig. 1; Table I). This can be explained by the fact that the pHs of the buffered NH_4NO_3- and NH_4^+-supplied nutrient solutions were constantly high (7.5), whereas the NO_3^--fed plants were subjected to an alternating pH from 5.6 (initial pH in the renewed solution) to about 6.8 as measured before the nutrient solution change (data not shown), probably because of H^+ consumption by roots via H^+/NO_3^- symport (Meharg and Blatt, 1995). Thus, this NO_3^- treatment could provide a few hours of unrestricted Fe uptake at low pH after renewal of the nutrient solution every 2 d (see "Materials and Methods"). However, when the pH of NO_3^--supplied nutrient solution was maintained high (pH 7.5, HEPES), plants showed severe chlorosis and rapid inhibition in shoot and root growth as a consequence of restricted Fe uptake (data not shown).

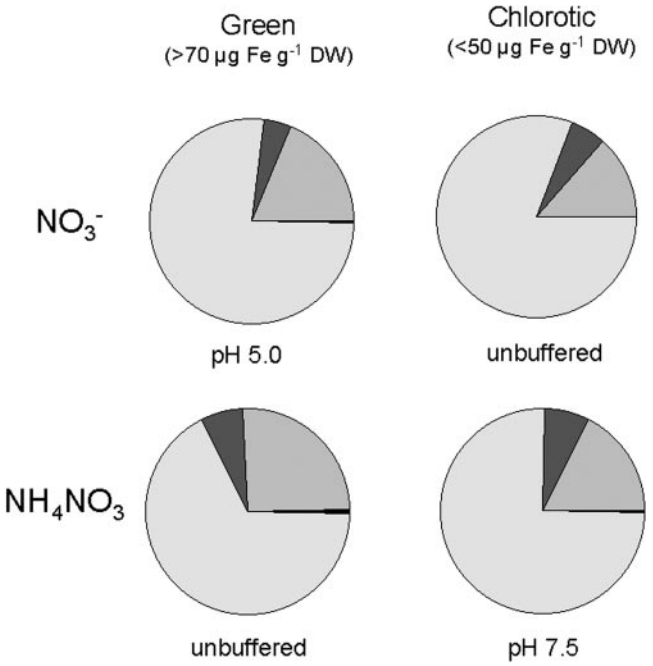

Figure 4. Effects of N forms and external pH on relative amount of Fe fractions (symplastic and apoplastic) and apoplastic Fe binding forms of sunflower leaves. Light gray, Symplastic Fe; gray, apoplastic Fe, weakly bound (cell walls); dark gray, strongly bound (cell walls); black, soluble (AWF).

The higher root apoplastic pH caused either by the unbuffered NO_3^- supply or maintained by a HEPES buffer in NH_4^+- or NH_4NO_3-fed plants significantly decreased both the concentration and the amount of Fe in roots and shoots, compared with the low pH treatments (Table II). Thus, restricted Fe uptake due to inhibited Fe^{III} reduction at high root apoplastic pH (Römheld and Marschner, 1986; Toulon et al., 1992) is most likely the primary cause of Fe deficiency chlorosis in sunflower plants. This conclusion is supported by the significantly lower Fe concentration in xylem sap in both NO_3^--fed plants grown in the nutrient solution without pH control or either NH_4^+- or NH_4NO_3-fed plants with buffered pH at 7.5 as compared with plants grown at low external pH (Fig. 2).

In contrast to our findings, Kosegarten et al. (1998a, 1998b) found that at a low-nutrient solution pH (from 4.0–5.0), controlled by a pH-stat device, NO_3^--fed sunflower plants had a significantly lower chlorophyll concentration or even reduced leaf growth

Table V. *Effects of N forms and nutrient solution pH on Fe-binding forms in cell walls of sunflower leaves*

Total cell wall Fe was calculated as the sum of strongly bound and weakly bound fractions. Data are means ± SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

Treatments	Total Fe	Weakly Bound Fe	Strongly Bound Fe
		μmol g⁻¹ dry wt	
NO_3^-; pH 5.0 (green)	3.4 ± 0.9a	2.2 ± 0.7a	1.2 ± 0.1a
NO_3^-, 40 mM; pH 5.0 (green)	3.9 ± 1.3a	2.6 ± 0.8a	1.3 ± 0.5a
NO_3^-; unbuffered (chlorotic)	1.7 ± 0.4b	1.2 ± 0.4b	0.5 ± 0.1b
NH_4^+; pH 5.0 (green)	4.2 ± 2.1a	2.8 ± 1.5c	1.4 ± 0.6a
NH_4^+; pH 7.5 (chlorotic)	2.4 ± 0.3c	1.2 ± 0.3b	1.2 ± 0.1a
NH_4NO_3; unbuffered (green)	3.8 ± 0.9a	2.5 ± 0.6a	1.3 ± 0.5a
NH_4NO_3; pH 7.5 (chlorotic)	1.9 ± 1.0b	1.5 ± 0.5b	0.4 ± 0.2b

compared with those grown with NH_4NO_3. It would seem that the limited capability of the pH stat system to maintain constant low pH in the root apoplast of NO_3^--fed plants used in these studies could be the major reason for a latent Fe deficiency before a decrease in leaf Fe concentration. Furthermore, Kosegarten at al. (1998a) and Zou et al. (2001), respectively, reported a significantly higher root Fe concentration in chlorotic sunflower and maize plants grown in nutrient solutions supplied with NO_3^- as compared with green, NH_4^+-, or NH_4NO_3-fed plants. In both these studies, Fe concentration may have been overestimated as a consequence of Fe precipitation because of low chelate stability of FeEDTA above pH 6.5 (Strasser et al., 1999). In the present study, precipitation of Fe was avoided by growing the plants in the nutrient solutions supplied with ferric ethylenediaminedi(O-hydroxyphenylacetic acid), which is much more stable at high pH (up to 9.0) in well-aerated hydroponic solutions (Halvorson and Lindsay, 1972). Moreover, washing the roots by the method of Bienfait et al. (1985) ensured greater removal of extraplasmatic Fe, thus minimizing the possibility of overestimation of root Fe concentration (Table II; see also Strasser et al., 1999).

The pH of apoplastic fluid collected by the centrifugation technique from intact leaves (Table III) represents the bulk pH of the whole leaf apoplast as an average of various cells (i.e. parenchyma, adaxial, and abaxial epidermal cells) with different ion concentrations (Karley et al., 2000) so that any possible spatial pH changes are not revealed. The pH of apoplastic fluid from leaves of NO_3^--fed plants collected 3 h after light onset was always higher by about 0.1 to 0.3 units than in NH_4^+- or NH_4NO_4-fed plants (Table III); however, these differences were not statistically significant. These findings are in accord with the results of Kosegarten et al. (1999) showing mean data of the apoplastic pH determined by the fluorescence microscope imaging technique. However, a clear increase in apoplastic pH above 6.5 found in their study was only in the small intervenial area of young leaves (about 10% of the total apoplastic space),

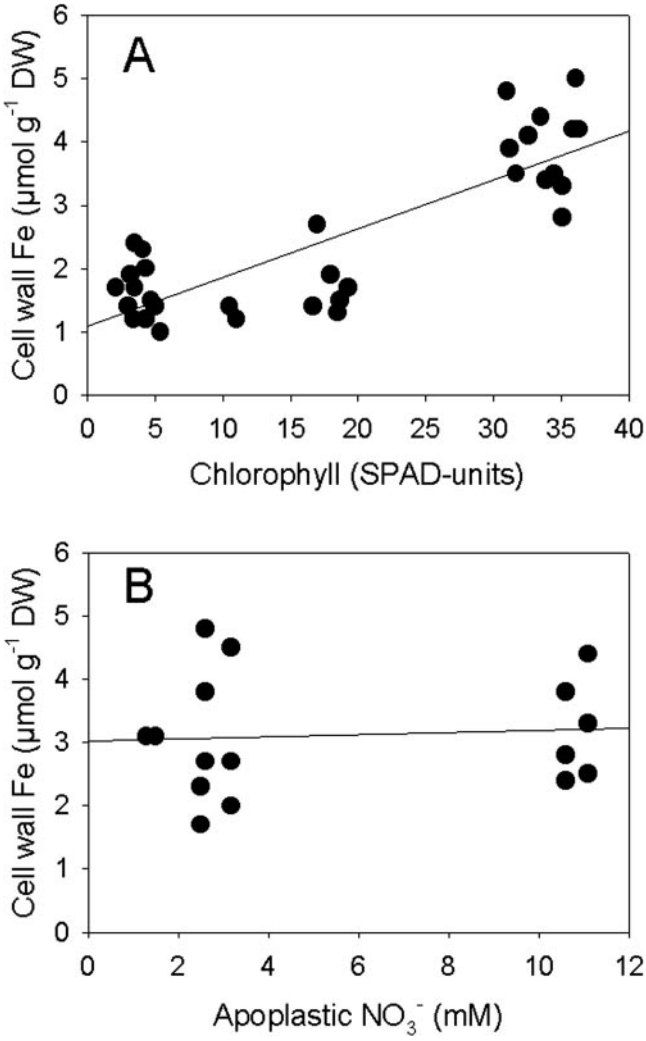

Figure 5. Cell wall Fe concentration in relation to chlorophyll content (A) and NO_3^- concentration in leaf apoplastic fluid (B) in young, fully expanded leaves of sunflower. Plants were grown for 2 weeks in nutrient solutions supplied with NO_3^- (4–40 mM N) or NH_4NO_3 (4 mM N).

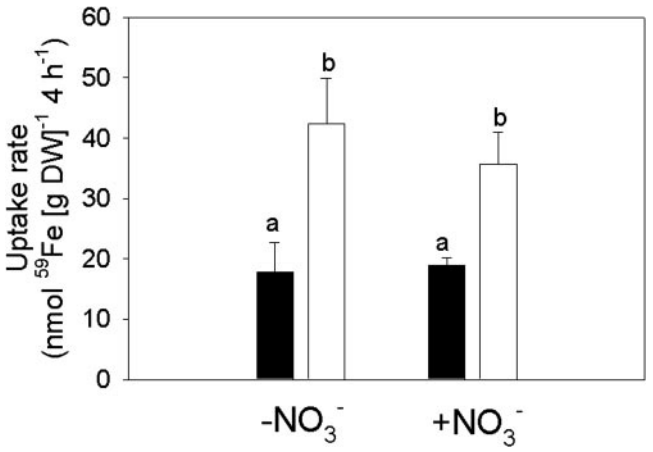

Figure 6. Uptake of ^{59}Fe by the leaf symplast of sunflower, as depending of apoplastic NO_3^-. $-NO_3^-$, Plants were precultured in NO_3^--free nutrient solution for 48 h, and less than 0.5 mM NO_3^- was measured in the apoplastic fluid obtained by centrifugation of intact leaves; no NO_3^- was added in ^{59}Fe-labeled solution. $+NO_3^-$, Plants were grown in NO_3^--supplied nutrient solution (4 mM), resulting in up to 5 mM NO_3^- in the leaf apoplastic fluid; 6 mM NO_3^- was added to the ^{59}Fe-labeled solution. After perfusion of radioactive labeled $\pm$ NO_3^- solution into the leaf apoplast of excised leaves via the petiole (2 h) by the transpiration stream, leaves were further incubated in darkness or under light (500 μmol m^{-2} s^{-1}) for 2 h. Black bars, Dark; white bars, light. Data are means $\pm$ SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

which they called microsites. Kosegarten et al. (1999) supposed that such increases in apoplastic pH of growing but chlorotic leaf microsites were a consequence of high NO_3^- uptake by rapidly expanding cells, presumably caused by the removal of protons from the apoplast. Experimental evidence is still lacking, however, that these microsites of high apoplastic pH represent areas of growth.

As evident from Figure 3, the NO_3^- concentration either in xylem sap or in apoplastic fluid collected from young leaves was not significantly different between NO_3^-- and NH_4NO_3-fed plants. Measured NO_3^- concentration in both xylem and leaf apoplastic fluids was in the range reported for sunflower plants in various studies (Dannel et al., 1995; Kosegarten et al., 1999; Sattelmacher et al., 1998). Higher NO_3^- concentrations in the xylem sap than in the apoplastic fluid (Fig. 3; Dannel et al., 1995) could presumably be due to NO_3^- uptake into the vacuoles of xylem parenchyma cells or because the exudates were from decapitated plants and, hence, obtained in the absence of a transpiration stream (Mühling and Läuchli, 2001). Interestingly, although NO_3^- was supplied in the nutrient solution in an excessive amount (40 mM), leading to a 2- to 3-fold increase in apoplastic NO_3^- concentration, no Fe chlorosis was found in young leaves when the pH of nutrient solution was maintained low with MES buffer (Table I; Figs. 3 and 5A).

Because nearly all of the assimilated NH_4^+ is translocated to the shoot in organically bound form (e.g. amino acids), predominantly via the xylem, no marked uptake of NH_4^+ ions from the apoplast into the symplast of leaf cells is to be expected (Dannel et al., 1995); therefore, a pH fall in the leaf apoplast appears unlikely if NH_4^+ is supplied to roots (Mühling and Läuchli, 2001). Apoplastic pH may even be raised as a consequence of the removal of protons, presumably via proton/amino acids cotransport (Williams et al., 1990; Logan et al., 1997).

Loading of 6 mM NO_3^- into the leaf apoplast via the petiole of excised leaves of sunflower plants precultured in NO_3^- free nutrient solution did not affect ^{59}Fe uptake into the leaf symplast (Fig. 6). Infiltration of various buffer solutions into the excised leaves was very efficient in controlling the apoplastic pH of xylem vessels, allowing only minor pH changes (Kosegarten et al., 1999). As shown in Figure 7, perfusion of high pH-buffered solution (50 mM HEPES, pH 7.0) into the leaf apoplast restricted ^{59}Fe uptake rate by about 35% to 40% of that found at low pH (unbuffered solution; 50 mM MES, pH 5.0 and 6.0, respectively). These results are in agreement with the results of Nikolic and Römheld (1999) showing the pH dependence of Fe^{III} citrate reduction and ^{59}Fe uptake by leaf discs infiltrated by the solutions buffered at various pHs (5.0–7.0) and incubated in darkness.

A decrease in reduction of Fe^{III} citrate occurring only at high apoplastic pH caused by the infiltration of HEPES buffer (e.g. pH 7.0) into the apoplast of sunflower leaves has been demonstrated by two dif-

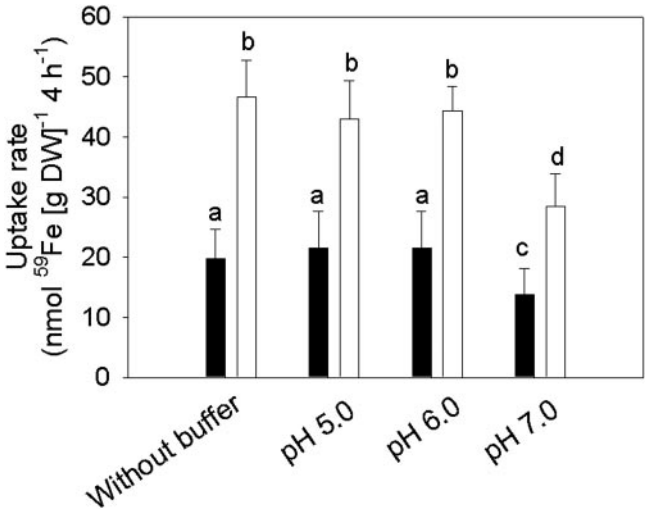

Figure 7. Effect of apoplastic pH on ^{59}Fe uptake by the symplast of intact sunflower leaves. ^{59}Fe-labeled solution (10 μM Fe citrate, 1:100 molar ratio) without or with various pH buffers (50 mM MES [pH 5.0 and 6.0] and 50 mM HEPES [pH 7.0]) was infiltrated into the leaf apoplast of excised leaves (2 h) via the petiole by the transpiration stream. Leaves were further incubated in darkness or under light (500 μmol m^{-2} s^{-1}) for 2 h. Black bars, Dark; white bars, light. Data are means $\pm$ SD of four replications. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

ferent experimental approaches, i.e. in leaf discs by measuring the absorbance of the ferrous biphenylphenanthrolinedisulfonic acid complex (Nikolic and Römheld, 1999) or in the excised leaves determined by microscope image analysis as formation of the Fe^{II}-ferrozine complex (Kosegarten et al., 1999). Considering that such a high pH (≥7.0) accounts for less than 3% of the total intervenial leaf apoplast of NO_3^--fed plants (Kosegarten et al., 1999), it seems difficult to explain how such a small space can affect Fe uptake in the range sufficient to induce leaf chlorosis. In the remainder, which makes up about 90% of total intervenial apoplastic space, Fe^{III} reduction reactions are not inhibited (see Kosegarten et al., 1999), presumably providing enough Fe for that required for metabolic processes. Thus, quite regardless of the presence of high concentrations of NO_3^- in the leaf apoplast, Fe chlorosis does not appear if sufficient Fe is taken up by roots (e.g. at low pH-buffered nutrient solution) or if it has been loaded into the leaf xylem apoplast (Tables I and II; Figs. 3 and 5A).

As clearly shown in the presented study, light strongly increased Fe uptake rates into the symplast of leaf cells apart from the effect of apoplastic pH. Hence, the Fe uptake rate at high apoplastic pH (7.0) under high light intensity was even greater than that found at low apoplastic pH (5.0 and 6.0, respectively) in the dark (Fig. 7). Temporal variations in H^+ concentration resulting from changes in metabolic activity caused by a dark/light transition leads to a biphasic apoplastic pH response (Mühling and Sattelmacher, 1995; Sattelmacher, 2001). Increase in apoplastic pH under dark conditions probably might be caused by inhibition of light-induced H^+ extrusion into the leaf apoplast (Mühling and Läuchli, 2001). Light might also directly affect Fe uptake into the symplast of leaf cells by nonenzymatic photoreduction of Fe^{III} citrate in the leaf apoplast (Brown et al., 1979; Schmidt, 1999). In vitro photoreduction of Fe^{III} citrate by high light intensity (500 μmol m^{-2} s^{-1}) was much less pH regulated in the range from 5.0 to 7.5 as compared with reduction by leaf tissue in darkness (M. Nikolic, unpublished data; Nikolic and Römheld, 1999).

Once taken up by the roots, Fe is transported to the shoots via the xylem, mainly in the form of Fe^{III} citrate complexes (Tiffin, 1966), and through the petioles, the xylem stream enters the leaf via the veins and moves toward the sites of high evaporation such as leaf margins (Sattelmacher, 2001). In accordance with this accepted concept, Fe^{III} can be bound to cell wall components, presumably to free carboxyl groups (e.g. pectins) before being taken up by the symplast of mesophyll cells. Thus, the relative proportion of soluble Fe present in AWF as various complexes with organic anions (e.g. citrate and malate) is very low, and most of the apoplastic Fe is bound to the cell wall (over 95%) and held at different binding strengths (Fig. 4). Because of spatial limitations for cations in vivo, the cation exchange capacity of isolated cell wall material is much higher than the cell walls of intact plant tissue (Sattelmacher, 2001). Thus, during the isolation procedure, the cell walls are able to fix a certain portion of Fe of symplastic origin (e.g. soluble Fe-containing proteins and Fe in vacuoles), which leads to an overestimation of the proportion of Fe located in the cell walls.

In contrast to what would have been expected from Mengel's hypothesis, NO_3^- supply to the unbuffered nutrient solution resulted in significant decreases in concentration of leaf Fe in both compartments, symplast and apoplast, with a tendency to maintain of the relative proportion of apoplastic Fe unchanged (Table IV). These findings are in agreement with results of Nikolic and Römheld (2002) reported for both green and chlorotic grapevine (*Vitis vinifera*) plants grown on calcareous soil under field conditions. Moreover, the relative proportions of symplastic and the various apoplastic binding forms of leaf Fe did not differ greatly between green (low external pH) and chlorotic (high external pH) leaves in both NH_4NO_3- and NO_3^--fed plants (Fig. 4).

Considering further Mengel's hypothesis that high apoplastic pH due to high NO_3 uptake in young developing leaves depresses Fe^{III} reduction and thereby Fe^{2+} uptake into symplast (Kosegarten et al., 2001), the question has to be raised as to the fate of this nonutilized Fe. Presumably, it must be accumulated outside the plasma membrane and most probably trapped in the cell walls, which implies a relative increase in the concentration of Fe. Our results, however, were unable to confirm this expected increase in the cell wall Fe isolated from leaves of NO_3^--fed plants. They showed the contrary: Both binding forms of leaf cell wall Fe (weakly and strongly bound) were significantly lower in all chlorotic plants grown in the high-pH nutrient solutions regardless of N form (Table V) due to restricted Fe uptake caused by high pH in the root apoplast (Table II; Nikolic et al., 2000). The lack of correlation between leaf apoplastic NO_3^- concentration and Fe concentration in the cell walls (Fig. 5B) also provides further evidence against the postulated hypothesis of Fe inactivation. Therefore, we suggest that the reported regreening of chlorotic leaves after spraying with citric acid (Tagliavini et al., 2000; Kosegarten et al., 2001) may be explained by the effect of citric acid in strongly extracting Fe bound in the cell walls, thereby increasing soluble Fe^{III} citrate in the leaf apoplastic fluid, rather than by the postulated enhanced reduction of Fe^{III} and, thus, Fe uptake into the symplast due to lowering of apoplastic pH (Kosegarten et al., 2001).

Despite the fact that NO_3^- is a predominant N form in most crop soils (Römheld, 2000), Fe deficiency chlorosis does not appear in perennial woody plants (e.g. grapevine, pear [*Pyrus communis*], peach, etc.) grown on non calcareous soils and in the case of

sunflower is rare even on calcareous soils. Thus, the recently reported NO_3^--induced Fe deficiency chlorosis in sunflower grown in the nutrient solution culture at low Fe supply (Kosegarten et al., 1998b, 1999, 2001) is an interesting physiological phenomenon but of little relevance to Fe deficiency in field grown plants. Nevertheless, even in this case, Fe chlorosis is mainly a consequence of lower uptake and shoot translocation of Fe by NO_3^--fed plants as compared with plants grown in the nutrient solutions with NH_4^+-N supply (Table II; Mengel and Geurtzen, 1986; Kosegarten et al., 1998b). Furthermore, the results from Kosegarten's experiments with the controlled pH of nutrient solutions do not differ in principal whatsoever from ours because the uptake of Fe by roots was influenced by pH rather than by N form.

CONCLUSION

High-pH buffers (e.g. HCO_3^- and HEPES) restrict uptake and shoot translocation of Fe by nutrient solution grown plants regardless of whether N is supplied solely as NH_4^+ or as NO_3^- or as both forms as NH_4NO_3. Increasing N supply to roots solely as NO_3^- (up to 40 mM) does not change the relative distribution of Fe between leaf apoplast and symplast, and there are no differences in either total Fe leaf concentrations or Fe distribution between various binding fractions in the cell walls if the external pH of the root medium is kept constantly low (5.0). In unbuffered nutrient solution, however, NO_3^- supply decreases Fe uptake due to an increase in root surface pH. Similarly, uptake of Fe is depressed if the nutrient solution is supplied with NH_4^+ as the sole N source and buffered at high pH (7.5). We found no evidence that NO_3^- supply to roots inactivates Fe in the leaf apoplast as postulated by Mengel and coworkers. The results presented here clearly show that occurrence of NO_3^--induced Fe deficiency chlorosis is exclusively caused by inhibited uptake and translocation of Fe from roots to shoots as a consequence of high pH at the root surface.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sunflower (*Helianthus annuus* L. cv Frankasol) was grown under controlled environmental conditions in a growth chamber with light/dark regime of 16/8 h, temperature regime of 24°C/20°C, photon flux density of approximately 300 $\mu mol^{-2}\ s^{-1}$ at plant height, and relative humidity of about 70%. After soaking in 1 mM $CaSO_4$ overnight, seeds were germinated in quartz sand moistened with saturated $CaSO_4$ solution in darkness for 3 d. The 5-d-old seedlings were then transferred to full-strength nutrient solutions (four plants per 2.5-L plastic pot) containing: 0.7 mM K_2SO_4, 0.1 mM KH_2PO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.2 μM $CuSO_4$, 0.1 μM $ZnSO_4$, and 0.01 μM $(NH_4)_6Mo_7O_{24}$. N was applied in the different forms as $Ca(NO_3)_2$, $(NH_4)_2SO_4$, and NH_4NO_3, at a concentration of 4 mM N for each treatment, unless mentioned separately in the text. For NH_4^+ and NH_4NO_3 treatments, $CaCl_2$ was added at a final concentration of 2 mM to compensate for Ca supplied in the NO_3^- treatment. In all experimental sets, Fe was supplied as ferric ethylenediaminedi(O-hydroxyphenylacetic acid) at 1 μM. In addition, the pH of nutrient solutions (adjusted with NaOH) was kept constant by buffering either at 5.0 with 2-(*N*-morpholino)ethanesulfonic acid or 7.5 with HEPES, both at final concentration of 5 mM. If used at such low concentrations, these organic buffers appeared to be biologically inert without adverse effects on plant growth (Bugbee and Salisbury, 1985). The nutrient solutions were renewed completely every 2 d to avoid nitrification (Walch-Liu et al., 2001) and continuously aerated. Two weeks after transfer to nutrient solutions, plants were harvested.

Chlorophyll Determination

Leaf chlorophyll content was determined nondestructively using a portable Chlorophyll Meter SPAD-502 device (Minolta Camera Co., Osaka).

Determination of Total Fe in Plant Tissues

After 14 d of growth in the nutrient solutions, sunflower plants were harvested and separated to shoots (leaves + stems) and roots. To remove extraplasmatic Fe, roots were washed with bipyridyl and sodium dithionite as described by Bienfait et al. (1985). Roots and shoots were weighted after oven drying at 65°C for 48 h, ashed at 550°C for 8 h, and digested in 5 M HNO_3. Fe was determined by atomic absorption spectrometry (AAS; UNICAM 939, Unicam, Kassel, Germany) after dissolving the residues in 1% (w/v) HCl.

Determination of Apoplastic and Symplastic Fe in Leaves

Apoplastic Fe in intact leaf discs was determined according to the method of Nikolic and Römheld (2002). Samples containing 15 leaf discs (1.5-cm diameter) were vacuum infiltrated in solution containing 1.5 mM 2,2′-bipyridyl and 10 mM MES (pH 6.0) and than incubated for 20 min under reductive conditions by adding sodium dithionite at a final concentration of 5 mM under continuous N_2 bubbling through the solution. Apoplastic Fe was removed as $Fe^{II}[bipyridyl]_3$ complex, and Fe concentration was determined by measuring A_{520} (spectrophotometer U-3300, Hitachi, Tokyo) and using extinction coefficient of 8.65 mM^{-1} (Bienfait et al., 1985). After removal of apoplastic Fe, leaf discs were shortly washed several times in deionized water, and remaining Fe (symplastic) was determined as previously described by Nikolic and Römheld (2002).

Isolation of Cell Walls

The young, fully expanded leaves were excised, and major veins were removed. Leaf discs (0.5-cm diameter) were taken and plasmolyzed under vacuum (−40 kPa) in 20 mL of 0.4 M Suc solution for 20 min. The samples (about 1 g fresh weight) were homogenized in 5 mL of 0.4 mM Suc solution using precooled mortar and pestle. Leaf homogenate was brought to 15 mL, and the pellet obtained after centrifugation at 1,000*g* for 15 min was resuspended and successively pelleted in 15 mL of 0.4, 0.6, and 1 M Suc solutions, respectively (1,000*g*, 10 min each). The final pellet was washed three times with 10 mL of 0.5% (w/w) SDS and centrifuged at 1,000*g* for 10 min, followed by several washings (at least six times) with deionized water, until these cell wall materials became free of plasma membranes and other cytoplasmic fragments as observed by a light microscope. All procedures were carried out at 4°C except plasmolysis and washing with SDS, which were performed at room temperature.

Determinations of Different Fe-Binding Forms in the Leaf Apoplast

For determination of water-soluble Fe in the leaf apoplast, young expanded leaves were excised, and central veins were removed. Leaflets were briefly washed and incubated for 30 min in deionized water to prevent water uptake during further vacuum infiltration (Mühling and Sattelmacher, 1995). Leaves were infiltrated with ice-cold deionized water in a vacuum desiccator several times reducing the pressure to −45 kPa, followed with slow relaxation to atmospheric pressure within 2 to 3 min. Infiltrated

leaves that become darker than noninfiltrated were carefully blotted dry, rolled over a plastic tube, orientated with the cut edge up and fixed into a plastic 50-mL syringe. The leaves-filled syringes were then centrifuged at 1,000*g* for 15 min at 4°C. Collected AWF samples were evaporated in a rotary evaporator, and dry residue was dissolved in 1% (w/v) HCl for determination of Fe by AAS. Leaves remaining after centrifugation were dried at 65°C overnight and weighted. Concentration of soluble Fe fraction was calculated per leaf dry weight basis. All samples were tested for possible cytoplasmic contamination using the malate dehydrogenase (MDH) activity assay (Husted and Schjoerring, 1995) as a cytosolic (i.e. mitochondria) marker enzyme.

Isolated cell wall materials were resuspended in 10 mL of solution containing 1.5 mM 2,2′-bipyridyl and 0.5 mM $CaSO_4$, and weakly bound Fe fraction was removed during reductive extraction in the presence of sodium dithionite at final concentration of 10 mM under continuous flow of N_2. After incubation for 30 min, the A_{520} was measured in a 3-mL aliquot, and cell wall materials were washed several times with deionized water for determination of Fe remaining in the cell walls (strongly bound Fe fraction). Finally, the pellet was resuspended in deionized water and transferred to a glass beaker, evaporated, dried, weighted, and ashed. The residue was digested in 5 M HNO_3 and dissolved in 1% (w/v) HCl for Fe determination by AAS.

Collection and Analysis of Xylem Sap and Leaf Apoplastic Fluid

Xylem sap was obtained by exudation after sunflower plants were decapitated at the stem about 2 cm above the root base. Silicon tubes were fixed over the decapitated stem, and xylem sap was collected for 2 h after discarding the exudates obtained during the first few minutes. The apoplastic fluid from intact fully expanded young leaves was collected 3 h after light onset by the centrifugation method described previously by Dannel et al. (1995). The first fraction, obtained at low centrifugal speed of 1,500*g* to remove xylem sap, was discarded, and the apoplastic fluid was collected by the second centrifugation at 2,500*g* for 15 min at 4°C. Cytoplasmic contamination of all samples was quantified by comparing the MDH activity and K^+ concentration in the apoplastic fluid with total leaf homogenate. The MDH activity of about 2% of the total leaf homogenate and measured K^+ concentration up to 15 mM indicated that there was no symplastic contamination of the apoplastic fluid (Dannel et al., 1995; Nikolic and Römheld, 2002).

The pH measurements were carried out in the samples immediately after their collection using a glass microelectrode (InLab 423, Mettler Toledo, Giessen, Germany). After pH measurements, the samples of xylem sap and leaf apoplastic fluid were frozen in liquid N_2 and stored at −20°C for further analysis of NO_3^- and Fe. NO_3^- was determined using an RQflex reflectometer (Merck, Darmstadt, Germany). Fe was determined by flame AAS using a microinjection device (50 μL) after dilution of the samples at a ratio of 1:1 (v/v) with 5 M HCl.

Measurement of ^{59}Fe Uptake by Leaf Symplast in Intact Leaves

Radiolabeled ^{59}Fe citrate was prepared by mixing $^{59}FeCl_3$ with citric acid with a molar ratio of 1:100. Young, fully expanded young leaves were excised, and the cut ends of petioles were placed in a 2-mL plastic tube containing ^{59}Fe citrate solution (10 μM Fe; specific activity of 10 μCi μmol^{-1} Fe) in the presence of various buffers: 50 mM MES (pH 5.0 and 6.0) and 50 mM HEPES (pH 7.0). Unbuffered ^{59}Fe-labeled uptake solutions in the presence or absence of 6 mM NO_3^- were used in an alternative experiment. Radioactive labeled solution was perfused (2 h) via the petiole by transpiration stream into the leaf apoplast under light (500 $\mu mol\ m^{-2}\ s^{-1}$). Thereafter, radioactive solutions were replaced with non-radiolabeled solutions, and leaves were incubated for a further 2 h under light or in darkness. To prevent photoreduction of Fe^{III} citrate under light exposure, plastic tubes were wrapped with aluminum foil and covering the leaf lamina with a light transparent plastic bag regulated water losses by transpiration. For determination of ^{59}Fe uptake by leaf symplast, leaf discs (1-cm diameter) were punched by a calibrated cork borer from the leaf area devoid of major veins and washed twice in ice-cold nonradioactive solution containing 10 mM MES (pH 6.0), 0.5 mM $CaSO_4$, and 10 μM $FeCl_3$ for 10 min each. Apoplastic ^{59}Fe was removed with bipyridyl and sodium dithionite during 30 min of reductive incubation as mentioned above. After incubation, leaf discs were finally washed twice in 0.5 mM $CaSO_4$ for 5 min each, oven dried, ashed, and residue was dissolved in 1% (w/v) HCl for measuring of ^{59}Fe radioactivity by a liquid scintillation counter (Wallac 1414 Win Spectral, Wallac Oy, Turku, Finland).

Statistics

Data were subjected to analysis of variance using the statistical software SigmaStat for Windows (version 2.0, 1997, SPSS Inc., Chicago). Means were compared by Duncan's test at $P < 5\%$.

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