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Whole-root iron(III)-reductase activity throughout the life cycle of iron-grown *Pisum sativum* L. (Fabaceae): relevance to the iron nutrition of developing seeds

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Abstract. To understand the whole-plant processes which influence the Fe nutrition of developing seeds, we have characterized root Fe(III)-reductase activity and quantified whole-plant Fe balance throughout the complete 10-week (10-wk) life cycle of pea (*Pisum sativum* L., cv. Sparkle). Plants were grown hydroponically in complete nutrient solution with a continuous supply of chelated Fe; all side shoots were removed at first appearance to yield plants with one main shoot. Root Fe(III)-reductase activity was assayed with Fe(III)-EDTA. Flowering of the experimental plants began on wk 4 and continued until wk 6; seed growth and active seed import occurred during wks 5–10. Vegetative growth terminated at wk 6. Iron(III) reduction in whole-root systems was found to be dynamically modulated throughout the plant's life cycle, even though the plants were maintained on an Fe source. Iron(III)-reductase activity ranged from 1–3 μmol Fe reduced · g^{-1} DW · h^{-1} at early and late stages of the life cycle to 9.5 μmol Fe reduced · g^{-1} DW · h^{-1} at wk 6. Visual assays demonstrated that Fe(III)-reductase activity was localized to extensive regions of secondary and tertiary lateral roots during this peak activity. At midstages of growth (wks 6–7), root Fe(III)-reductase activity could be altered by changes in internal shoot Fe demand or external root Fe supply: removal of all pods or interruption of phloem transport from the reproductive portion of the shoot (to the roots) resulted in lowered root Fe(III)-reductase activity, while removal of Fe from the nutrient solution resulted in a stimulation of this activity. Total shoot Fe content increased throughout the 10-wk growth period, with Fe content in the non-seed tissues of the shoot declining by 50% of their maximal level and accounting for 35% of final seed Fe content. At maturity, total seed Fe represented 74% of total shoot Fe; total Fe in the roots (apoplasmic and symplasmic Fe combined) was minimal. These studies demonstrate that the root Fe(III)-reductase system responds to Fe status and/or Fe requirements of the shoot, apparently through shoot-to-root communication involving a phloem-mobile signal. During active seed-fill, enhanced root Fe(III)-reductase activity is necessary to generate sufficient Fe^{2+} for continued root Fe acquisition. This continuing Fe supply to the shoot is essential for the developing seeds to attain their Fe-content potential. Increased rates of root Fe(III) reduction would be necessary for seed Fe content to be enhanced in *Pisum sativum*.

Key words: Iron (nutrition, uptake) – Iron(III)-reductase – Phloem – *Pisum* – Seed nutrition – Shoot-to-root communication

Introduction

Iron import by developing pea (*Pisum sativum* L.) seeds is determined by two principal factors: the ability of shoot source regions to load Fe into the phloem pathway which supplies the seeds, and the maintenance in these shoot regions of an Fe pool which is available for phloem loading. We have recently demonstrated that leaflets, stipules and pod walls are all capable of phloem Fe loading and transport to developing pea seeds, and have suggested that when sufficient (or excess) Fe is present in the shoot, phloem Fe loading is limited by the synthesis/expression of an endogenous Fe chelator (Grusak 1994). Whereas the available Fe pool may arise, in part, from the mobilization of Fe previously stored in vegetative tissues (Hocking and Pate 1977), it appears that continued Fe influx during reproductive growth is necessary to supply or resupply the shoot Fe pool; plants deprived of exogenous Fe during the seed-fill period have reduced seed Fe concentrations (Grusak 1994). Currently, we have only limited information on root Fe acquisition during this stage of development.

The acquisition of Fe by pea, as for other dicotyledonous species growing in an oxygenated soil environment, requires the reduction of Fe(III) to Fe^{2+} prior to influx of the divalent cation (Chaney et al. 1972). This

Abbreviations: BPDS = bathophenanthrolinedisulfonic acid; DAF = days after flowering; DW = dry weight; EDDHA = *N*,*N*'-ethylenebis[2-(2-hydroxyphenyl)-glycine]; wk = week

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reduction has been shown to occur predominantly via a membrane-localized reductase system (Sijmons et al. 1984; Grusak et al. 1989; Brüggemann et al. 1990; Schmidt et al. 1990; Holden et al. 1991). In pea seedlings, 2 weeks (wks) old, the reduction of Fe has been implicated as the physiological rate-limiting step in the overall Fe-acquisition process (Grusak et al. 1990b). Thus, the activity of this root-system protein during reproductive growth will be an important determinant of root Fe influx and transport to the shoot.

Our current understanding of the regulation of the root Fe(III) reductase is based primarily on the study of Fe-sufficient vs. Fe-deficient plants. When various species are challenged with external Fe-deficiency stress, the root system responds by increasing its Fe(III) reduction capacity over that of the Fe-sufficient condition (Römheld and Marschner 1986). Although the signal for this response (or its site of origin) has yet to be firmly established, results with Fe-deficient sunflower (*Helianthus annuus* L.) demonstrate that shoot-to-root communication, via a phloem-mobile signal, may be involved (Landsberg 1982, 1984); root cytoplasmic Fe levels might also serve as a regulatory signal (Bienfait 1988). For roots maintained on an external Fe source, however, it is unclear whether the reductase system can respond to internal shoot Fe demand. For instance, can an Fe-grown plant in midreproductive growth, with a potentially elevated shoot Fe requirement, communicate this demand to the roots and modulate root Fe(III)-reductase activity in order to generate sufficient Fe^{2+} for root influx? Interestingly, because most Fe(III)-reductase studies have utilized roots obtained from young plants, it is unclear whether roots of various species even have the potential for elevated activity during reproductive growth.

Anemia induced by iron-deficiency is a severe health problem in various human populations (Yip 1994). Therefore, we have been interested in developing strategies which will enable us to increase the Fe content of edible seeds, so that plant foods of enhanced nutritional quality can be provided for human consumption. In order to achieve this goal, we must understand the function and limitations of those whole-plant processes responsible for supplying Fe to the site of phloem loading, prior to seed Fe import. In this investigation, we present the first characterization of whole-root system Fe(III)-reductase activity throughout the complete developmental maturation of a dicotyledonous plant (*Pisum sativum* L., cv. Sparkle). We also report root Fe(III)-reductase results for reproductive-age plants following alterations in external root Fe supply or internal shoot Fe demand. These data, along with measurements of total Fe pools in vegetative and reproductive tissues, will be used to discuss the shoot regulation of root Fe(III)-reductase activity and the relevance to seed Fe content of continued Fe(III) reduction and influx during reproductive growth.

Materials and methods

Plant material and culture. Seeds of pea (*Pisum sativum* L., cv. Sparkle) were imbibed, planted, and grown hydroponically in aerated, buffered nutrient solution as previously described (Grusak 1994). Plants were grown in a controlled environmental chamber with a 16-h, 20°C, and 8-h, 15°C, day-night regime under a mixture of incandescent and fluorescent lamps. The photon flux density of photosynthetically active radiation was 500 μmol photons $\cdot m^{-2} \cdot s^{-1}$ at the top of mature plants (average height 1.0 m) and 350 μmol photons $\cdot m^{-2} \cdot s^{-1}$ at the level of the lowest leaf. Plants were grown individually in polyethylene vessels containing 3.5 L of nutrient solution; all side shoots were excised at first appearance with a razor blade to yield plants with one main shoot. Side shoots arose during the third and fourth wk of growth and only at nodes 2–9; all excisions were thus confined to this 2-wk period. Pod and seed ages were ascertained by tagging flowers as previously described (Grusak 1994). Nutrient solutions were replaced twice weekly and were buffered with 2 mM 2-(N-morpholino)ethanesulfonic acid (Mes) buffer (adjusted with KOH), which maintained pH between 5.5 and 6.0. For older plants (primarily between wks 6 to 10), solution levels in the growth vessels were checked daily and were adjusted to 3.5 L with nutrient solution, as needed. Iron was added as 5 μM Fe(III)-*N*,*N*′-ethylenebis[2-(2-hydroxyphenyl)-glycine] (Fe(III)-EDDHA); the frequent solution changes ensured that Fe concentrations never dropped below 4.5 μM.

Iron(III) reductase measurements. Root-associated iron(III) reduction was determined for whole-root systems of intact plants using the spectrophotometric measurement of Fe(II) chelated to bathophenanthrolinedisulfonic acid (BPDS). Plants were brought into a darkened laboratory and roots were given two 5-min rinses in nutrient solution (3.5 L), without Fe or Mes buffer. Roots (on intact plants) were then transferred to assay solutions containing all growth nutrients except Fe(III)-EDDHA, with the addition of 0.1 mM Fe(III)-EDTA, 0.1 mM Na_2-BPDS and 5 mM Mes buffer (pH 5.5); roots were assayed in volumes from 1.5 to 3.5 L, depending on the age of the plant. Assays were conducted in the dark at 22°C for 30 min; time course studies using 7- and 9-wk-old plants confirmed that Fe(III) reduction in intact roots was linear for up to 60 min (data not shown). All rinse and assay solutions were gently aerated with four bubbling tubes to provide oxygen to the roots and mixing of the solutions. Absorbances of the assay solutions (at 535 nm) were determined spectrophotometrically with a Gilford Response II, UV-VIS spectrophotometer (Ciba-Corning Diagnostics, Medfield, Mass., USA); Fe(II)-$BPDS_3$ was quantified using a molar extinction coefficient of 22.14 $\cdot mM^{-1} \cdot cm^{-1}$. Because of the difficulty in reliably blotting surface moisture from large root systems, reductase values were calculated on a dry-weight (DW) basis.

The amount of Fe(III) reduction attributable to root reductant release was determined as previously described (Grusak et al. 1990a). Iron(III)-reductase activity was visualized in excised, primary-lateral roots using the agarose-BPDS technique as described by Grusak et al. (1993).

Iron-deficiency treatment and plant manipulations. For selected plants, we investigated the effect of short-term root Fe deficiency or altered shoot Fe demand on whole-root system Fe(III)-reductase activity. Treatments were initiated at 6 wks of age; reductase measurements were performed at wk 7. Plants labeled as iron-deficient were maintained during wk 7 on nutrient solution without added Fe. Roots were first rinsed with two 30-min changes of nutrient solution without Fe; then, they were provided and maintained on buffered nutrient solution containing 0.3 mM Na_2-BPDS. The BPDS was added to minimize the uptake (following reduction) of any trace amounts of Fe which might have precipitated on root cell walls during the first 6 wks of growth. The midweek solution replacement also included 0.3 mM Na_2-BPDS.

Shoot manipulations consisted of (i) the removal of all visible pods or flowers by excision (with a razor blade) at the point of attachment to the peduncle, or (ii) steam-girdling a 1-cm internodal region of the main stem of the plant, two internodes below the first flowering node (i.e., between nodes 8–9 or 9–10). This second procedure killed the phloem and other living cells of the stem, thus preventing the phloem transport of any substances from the

reproductive portion of the shoot to the roots (see Grusak 1994 for details); xylem transport to the upper shoot was maintained. Carbohydrate nutrition of the roots was supported via phloem transport from the lower eight or nine nodal units below the point of steam-girdling. To assess the effect of the steam-girdling treatment on general root ion influx, net influx of NO_3^- and K^+ was determined for roots of control and steam-girdled plants for the culture period from day 45 to day 48. Initial and final (following readjustment to 3.5 L) nutrient solution concentrations of NO_3^- and K^+ were measured with ion-selective electrodes, and net ion influx was calculated on a root DW basis (Grusak and Pezeshgi 1994), using the root weight at wk 7.

Tissue Fe content and biomass analysis. Individual plants were harvested immediately following the root assay and were separated into seeds, pod walls, peduncles and flowers, shoot remainder (i.e., main stem, stipules and leaflets), and roots. Developing seeds from pods younger than 9 days after flowering (DAF) were not separated from the pod walls; the age of all harvested seeds was noted. All material was dried at 60°C to constant dry weight before weighing. Shoot parts (except seeds) from all plants were ground to uniformity in a stainless-steel mill, prior to aliquot sampling for tissue digestion and Fe analysis. Seeds from wk-10 plants were also ground and digested for Fe analysis. For small samples, all of the tissue was digested; for larger samples, three aliquots (approximately 0.25 g DW each) were digested. Samples were wet-digested in borosilicate tubes by adding 2 mL of concentrated perchloric acid and 5 mL concentrated nitric acid, heating to 150°C for 1 h, and then heating to 200°C until samples were near dryness. Blanks and standards were run in parallel. Digests were redissolved in 1 mL of 2 M HNO_3 and, after 1 h, were brought to 10 mL with deionized water. Analysis of Fe content was performed with the BPDS colorimetric technique (Isaac 1990), following pH adjustment of the digestate aliquots to pH 4.0 with 2 M sodium acetate, and using BPDS as the Fe^{2+} chelator instead of *o*-phenanthroline. Selected digestates were re-analyzed using atomic absorption (AA) spectrometry; AA values varied by no more than 3% from the colorimetrically determined values (data not shown).

Iron content was also determined for the apoplasmic and symplasmic compartments of roots harvested from 4-, 7-, and 10-wk-old plants. Whole-root systems were collected from plants not measured for root Fe(III)-reductase activity [i.e., not placed in 0.1 mM Fe(III)-EDTA]; all plants of a given age were of comparable size to those used for reductase assays. Apoplasmic Fe was removed and quantified using a modification of the free-space technique of Bienfait et al. (1985). Briefly, roots were given two 10-min rinses of 0.2 mM $CaSO_4$. They were then transferred to a solution (3 L) that was purged continuously with N_2 and consisted of 0.5 mM $MgSO_4$, 5 mM Mes buffer (pH 5.5) and 0.1 mM Na_2-BPDS. After 5 min, 100 mL of 341 mM $Na_2S_2O_4$ was added, yielding an $Na_2S_2O_4$ concentration of 11 mM; roots were removed after an additional 10 min. Aliquots of the solution were measured spectrophotometrically to determine apoplasmic Fe release; Fe(II)-$BPDS_3$ was quantified as described above. Roots were then dried, digested and analyzed (see above) to quantify the remaining symplasmic Fe.

Data averages and statistical analysis. All reported reductase values were based on a minimum of six root systems. Iron-content data for plant parts were based on material from a minimum of three plants for each time point. Seed Fe content was calculated for each harvested plant, using that plant's total seed DW (from seeds of various ages) and a seed Fe concentration of 70 μg · g^{-1} DW (the measured wk-10 seed average). This approach is valid because we have previously shown that seed Fe concentration remains constant throughout seed development (Grusak 1994). Statistical significance of differences between mean values was performed using the Student *t*-test.

Results

Root Fe(III) reductase activity. Iron(III)-reductase activity was measured in whole-root systems of intact, Fe-grown pea plants (cv. Sparkle) throughout their developmental maturation, using an assay solution containing 0.1 mM Fe(III)-EDTA. Activities ranged from 2–3 μmol Fe reduced · g^{-1} DW · h^{-1} on wks 2–4, to 9.5 μmol Fe reduced · g^{-1} DW · h^{-1} on wks 6–7, and returned to low reduction rates by wks 9–10 (Fig. 1). Root biomass increased throughout the 10-wk period (Fig. 1); DW gains were gradual after wk 5. Vegetative and reproductive development at any specific age was quite uniform for the plants used in this study. Leaflet expansion at the uppermost nodes (nodes 20–22) occurred by wk 6; prior to this time, mean values (± SE) for the number of nodes with fully expanded leaves were: 5.3 ± 0.3, wk 2; 9.8 ± 0.3, wk 3; 13.0 ± 0.0, wk 4; 16.8 ± 0.3, wk 5. Flowering began at wk 4 and continued through wk 6. The average number of pods on older plants was 17.5 ± 0.5 (± SE).

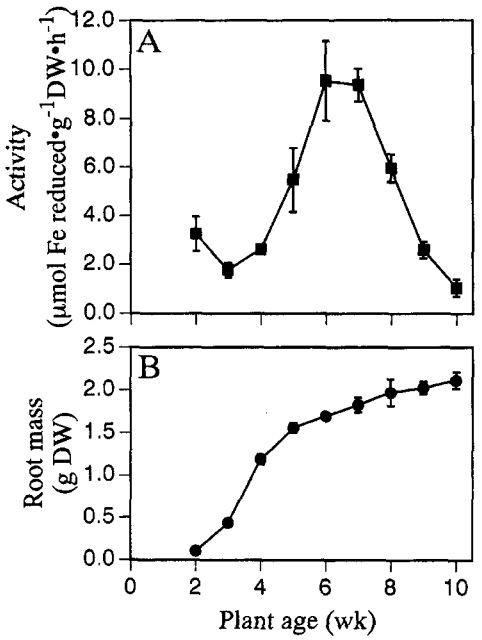

Fig. 1A, B. Iron(III) reductase-activity (**A**) and root mass (**B**) of whole-root systems of Fe-grown pea plants throughout a complete life cycle. Plants were grown hydroponically and maintained on 5 μM Fe(III)-EDDHA. Iron (III) reduction was measured using 100 μM Fe(III)-EDTA. Data are means ± SE

The observed Fe(III) reduction was primarily due to membrane-associated reductase activity. Soluble reductant release from wk-7 plants accounted for only 3.2% of the total reductase activity. The regions of the root system responsible for Fe reduction were assessed with the agarose-BPDS technique, using wk-6 and wk-7 plants. Staining patterns, indicative of Fe(III) reduction, were localized predominantly to secondary and tertiary lateral roots (data not shown); these roots exhibited patchy or continuous staining from their point of attachment with the next larger root (i.e., basal end) to within 1–5 mm of the root apex. Secondary lateral roots ranged in length from 10 to 20 cm; tertiary lateral roots ranged in length from 1 to 8 cm. Variable staining was found along the length of primary lateral roots, depending on their developmental maturity. Older primary lateral roots (up to 90 cm in length) exhibited staining only along more apical

Table 1. Whole-root system Fe(III)-reductase activity in wk-7 pea plants following a treatment at wk 6 to alter shoot Fe demand or root Fe supply. Iron(III) reduction was measured using 100 μM Fe(III)-EDTA. Data are means ± SE

Treatment	Fe(III)-reductase activity		Root system (g DW)
	(μmol · g^{-1} DW · h^{-1})	% of control	
Control	9.45 ± 1.05	100	1.82 ± 0.09
Pod removal	3.07 ± 0.23	33	2.78 ± 0.10
Steam-gridle	4.22 ± 0.80	45	1.74 ± 0.03
Fe-deficiency	45.87 ± 4.26	485	1.77 ± 0.13

Table 2. Net influx of NO_3^- and K^+ for pea plants measured during the growth period days 45–48. Data are means ± SE

Treatment	Net NO_3^- influx (μmol $NO_3^- \cdot g^{-1}$ DW·h^{-1})	Net K^+ influx (μmol $K^+ g^{-1}$ DW·h^{-1})
Control	16.04 ± 1.06	4.32 ± 0.21
Steam-girdle	9.89 ± 0.78	4.00 ± 0.19

regions, but no closer than 10 cm from the apex. Younger primary lateral roots (10–20 cm in length), which had no secondary lateral roots, exhibited staining from their basal end to within 2 cm of the apex.

Iron deficiency during reproductive growth (wks 6–7) resulted in a nearly fivefold stimulation in Fe(III)-reductase capacity (Table 1). Shoot manipulations had an opposite effect (Table 1): the removal of all pods and flowers at wk 6 lowered root reductase activity by 67% within 1 wk, while steam-girdling the main stem (two nodes below the first flowering node) also resulted in a decline (55%). Root-system DW was unaffected by root Fe deficiency or main-stem steam-girdling; pod removal promoted a significant increase in root system DW (Table 1). Although roots of steam-girdled plants appeared to gain mass during the treatment week (cf. Fig. 1, wk-6 values and Table 1), implying they had sufficient energy for Fe(III) reduction, we further investigated whether this treatment affected other root membrane processes. Net NO_3^- influx of steam-girdled plants was reduced to 62% of controls, while net K^+ influx values for control and treated plants were not significantly different (Table 2).

Iron content of shoot and root tissues. Total Fe content (μg Fe) was calculated independently for all non-seed shoot tissues, and these were pooled to yield total shoot non-seed Fe (Fig. 2). Iron content in this fraction peaked around wk 7 at 1581 μg Fe and dropped to 50% of this value by wk 10. Total seed Fe was 2244 μg at plant maturity, which represented 74% of total shoot Fe (Fig. 2). Root Fe content increased throughout the 10-wk growth period in both the apoplasmic and symplasmic fractions (Table 3). On average, apoplasmic Fe represented 77% of the total root Fe pool.

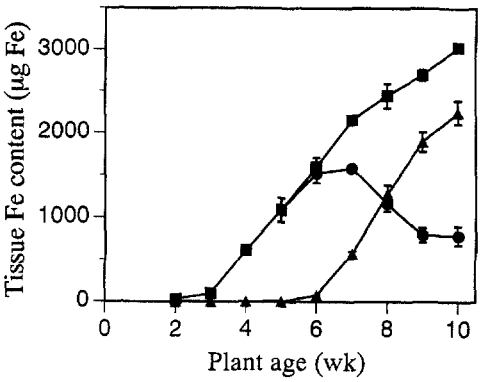

Fig. 2. Total Fe content of shoot fractions throughout a complete life cycle in pea. Plants were grown hydroponically and maintained on 5 μM Fe(III)-EDDHA; plants consisted of only one main stem. ■—■, total shoot Fe; ●—●, total shoot non-seed Fe; ▲—▲, total seed Fe. Data are means ± SE

Discussion

Regulation of root Fe(III) reductase activity. Whole-root Fe(III)-reductase activity in pea was found to be dynamically modulated throughout the life cycle of the plant (Fig. 1). Iron(III)-reductase activity (DW basis) increased nearly fivefold by midreproductive growth (relative to preflowering vegetative growth) and decreased throughout late stages of maturity. These changes occurred even though plants were maintained on a constant source of external Fe [4.5–5.0 μM Fe(III)-EDDHA]. Previous studies, from a number of plant species, have revealed alterations in root Fe(III)-reductase activity only when plants were challenged with Fe-deficiency stress (e.g., Bienfait et al. 1983; Römheld and Marschner 1983; Landsberg 1986; Bienfait et al. 1987) or other micronutrient deficiencies (Jolley and Brown 1991; Welch et al. 1993); plants supplied with Fe and other micronutrients generally exhibited stable and low Fe(III) reductase activities. However, most earlier studies utilized young plants or focused on only a single developmental age. The current investigation is the first to characterize root Fe(III)-reductase activity throughout the complete life cycle of a plant.

The elevated reductase activities observed during wks 6–7 were attributable to plasma-membrane-localized protein activity (Grusak et al. 1989), because soluble reductant release accounted for only 3.2% of the total Fe(III)

Table 3. Iron content in whole-root fractions of pea plants. Data are means ± SE

Plant age (wk)	Root apoplasm (μg Fe)	Root symplasm (μg Fe)	Total root (μg Fe)
4	109.3 ± 23.6	26.2 ± 2.7	135.5 ± 21.6
7	151.4 ± 20.0	44.0 ± 5.6	195.4 ± 18.2
10	232.1 ± 19.5	83.6 ± 15.0	315.7 ± 30.6

reduction (wk 7, see *Results*). The level of activity of this protein was apparently regulated by internal plant factors (e.g., nutritional or developmental status), rather than by external conditions. Zinc deficiency (Jolley and Brown 1991) and Cu deficiency (Welch et al. 1993) have each been shown to stimulate Fe(III)-reductase activity, but neither was responsible for the increased activity in the present study. Analysis of nutrient solutions used to nourish plants during the 6th and 7th wk of growth indicated that none of the micronutrient concentrations was reduced by more than 10% (data not shown). In addition, root growth, per se, was not correlated with the observed reductase changes (Fig. 1). Weekly gains in root mass were relatively constant from wks 5–10, yet Fe(III)-reductase activity increased and then declined during this period.

At the time of peak activity (wks 6–7), root Fe(III) reduction was localized primarily to secondary and tertiary lateral roots; Fe(III) reduction occurred throughout most of the length of these roots, except for the apices (see *Results*). This extensive localization was similar to that observed in primary (and secondary) lateral roots of Fe-deficient pea plants up to 2 wks of age (Grusak et al. 1993). We reiterate, however, that the plants used in this study were not maintained under Fe-deficient conditions. The occurrence of Fe(III)-reductase activity throughout most of the root system was, therefore, coordinated by internal factors. These factors appear related to shoot Fe demand or shoot Fe requirements, rather than being a programmed, age-dependent response of the root system itself. If roots were genetically programmed to exhibit a specific Fe(III)-reductase activity at a given developmental age, treatments such as pod removal (Table 1) should not have altered this activity.

Because a steam-girdle treatment to interrupt shoot-to-root communication from the reproductive region of the shoot (i.e., via the phloem), also resulted in a decline in root Fe(III)-reductase activity (Table 1), we propose that the root's ability to perceive shoot Fe status occurs via a phloem-mobile signal. It does not appear that the steam-girdle effect was due merely to a decline in energy supply from the shoot; root DW gain continued following this treatment (Table 1) as did net NO_3^- and K^+ influx (Table 2), although NO_3^- influx was partially reduced. Previous results with sunflower (Landsberg 1982, 1984) have suggested the existence of a phloem-mobile signal which can modulate root Fe(III)-reductase activity. Furthermore, studies with split-root systems (Romera et al. 1992) have shown that making half the root system Fe-deficient can induce Fe-deficiency responses in the Fe-sufficient half, presumably due to signal transmission between distant parts of the plant. This signal may be hormonal in nature

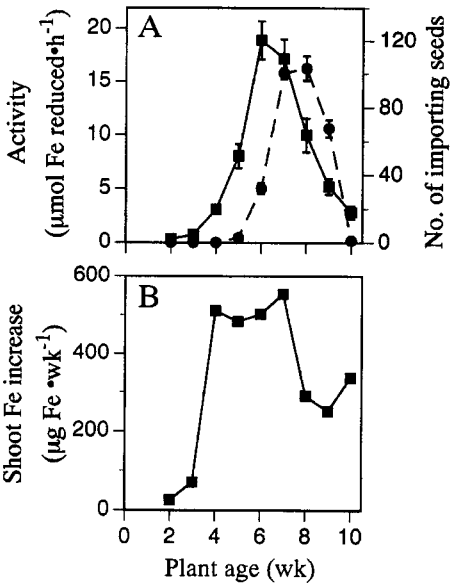

Fig. 3A, B. Iron(III)-reductase capacity of the entire root system, number of seeds importing nutrients, and weekly change in total shoot Fe for Fe-grown pea plants throughout a complete life cycle. **A** Iron(III) reduction was measured using 100 μM Fe(III)-EDTA. All data are means ± SE. **B** Data are weekly increases in total shoot Fe content calculated from the weekly mean values reported in Fig. 2

(Landsberg 1984), may involve Fe-binding compounds (Maas et al. 1988; Stephan and Scholz 1993), or may be alterations in the level of recirculated Fe (Bienfait 1989). From our current results, we cannot speculate on the nature of this signal compound in Fe-grown pea, or whether it is the same signal presumed to upregulate root Fe(III) reductase activity in Fe-deficient plants.

Influence of seed Fe demand. Although pod removal promotes a decline in root Fe(III) reductase activity (Table 1), it does not appear that seed Fe requirements alone are responsible for the enhanced reductase activity observed on wks 6–7 (Fig. 1). The seed Fe requirement, per se, when viewed as number of importing seeds, is not directly correlated with whole-root system Fe(III)-reductase activity (Fig. 3) (seeds were considered to be actively importing nutrients if their age was between 8 and 29 DAF; see Grusak 1994). This suggests that the initial increases in reductase activity during wks 5–6 may have been due to a combined requirement for Fe from both the developing seeds and the continued growth of vegetative and pod wall tissues. At later time points (i.e., wks 8–9) when seed Fe requirements were still relatively high, the declining

Fe(III)-reductase rates can be explained in part by a termination of vegetative shoot growth, as well as the fact that some of the seed Fe requirements were now being met by Fe mobilization from non-seed tissues (Fig. 2).

Relationship between root Fe(III) reduction and Fe^{2+} influx. It is interesting to note that although whole-root system Fe(III)-reductase activity increased sixfold from wks 4–6, weekly Fe transport to the shoots was relatively unchanged during wks 4–7. Why does it appear that increased root reductase activity did not translate into increased whole-plant Fe absorption? It could be that much of the reduced Fe was reoxidized and adsorbed to root cell walls (Bienfait et al. 1985), or that the reduced Fe was absorbed, but was sequestered in a root intracellular pool. Our data do not support these possibilities, however, because neither the root apoplasmic or the root symplasmic pools (Table 3) can account for the presumed loss in Fe influx (e.g., by comparing wks 4 and 6, a sixfold increase in root reductase activity would presumably yield a weekly Fe gain of about 3000 μg; total root Fe content on wk 7 was only 195 μg).

It thus appears that, relative to root Fe(III)-reductase capacity, whole-root-system Fe influx was diminished or inhibited during mid to late stages of the plant's life cycle. One could explain this by suggesting that the transport efficiency/capacity of the Fe^{2+} transporter declined in older roots, or that there were fewer transporters per unit length of root. A more probable scenario may involve a lack of specificity of the Fe^{2+} transport protein. Kochian (1991) has speculated that a general divalent cation transporter may be responsible for the influx of a number of micronutrient ions; Co^{2+} has been shown to competitively inhibit Fe^{2+} influx in yeast (Lesuisse et al. 1987). Because the developing seeds are sinks not only for Fe, but other micronutrients as well, it is possible that an increased requirement by the plant for other ions (which utilize the same root-transport protein) might competitively inhibit the influx of Fe^{2+}. During certain stages of development, the plant may need to upregulate root Fe(III)-reductase activity (thereby generating higher activities of Fe^{2+} at the root surface), just to maintain a consistent net influx of Fe (i.e., about 500 $\mu g \cdot wk^{-1}$; Fig. 3).

Potential for increasing seed Fe content. Seed Fe import in pea occurs subsequent to the loading of Fe into the phloem pathway (Grusak 1994). The Fe available for phloem loading may come from vegetative storage sites or from recent root Fe absorption. Hocking and Pate (1977) analyzed Fe content of the uppermost leaflets and selected pods in pea (cv. Greenfest), and reported that these organs could mobilize 26–32% of their Fe for transport to the seeds. In the present study, based on an analysis of all plant tissues, we found that 50% of the non-seed shoot fraction could be mobilized to seeds (Fig. 2). However, relative to total seed Fe content at maturity (2244 μg), the non-seed shoot tissues provided only 35% of this amount. Clearly, there is insufficient Fe stored within the vegetative tissues to support maximal (or enhanced) seed Fe deposition; continued Fe influx throughout the period of seed development is therefore mandatory.

Recent studies have suggested that phloem Fe loading is regulated not only by the availability of Fe, but also by the synthesis/expression of an endogenous Fe chelator necessary for phloem loading (Grusak 1994). An overexpression of this chelator could provide a means of increasing seed Fe content, but only if the plant can also absorb additional amounts of Fe. Enhanced Fe influx would need to occur when seed storage sinks are available on the plant; excess, uncontrolled Fe accumulation during early growth, such as occurs in the *brz* pea mutant (Kneen et al. 1990), would lead to toxic conditions and severe tissue damage (Guinel and LaRue 1990). Our present results, demonstrating a coordination between shoot Fe utilization and root Fe(III)-reductase activity, suggest that if phloem Fe loading could be enhanced (i.e., perceived as an increase in seed Fe requirements), then root Fe acquisition processes (at least Fe[III] reduction) might be automatically upregulated at the appropriate stage of development. Iron-deficiency results (Table 1) reveal that root Fe(III)-reductase activity can be stimulated nearly fivefold over that of Fe-grown plants during the seed-fill period. Thus, assuming that Fe deficiency promotes a maximal Fe(III) reduction capacity, we would predict that seed Fe content could also potentially be augmented nearly fivefold. This would depend, however, on whether root Fe^{2+} influx will be similarly increased when additional Fe^{2+} is generated at the root surface. Studies are needed to determine the various factors which might limit the rate of Fe^{2+} membrane transport in pea and other legumes, especially during the seed-fill period.

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