Adquisición y transporte de hierro en plantas

TESIS DOCTORAL

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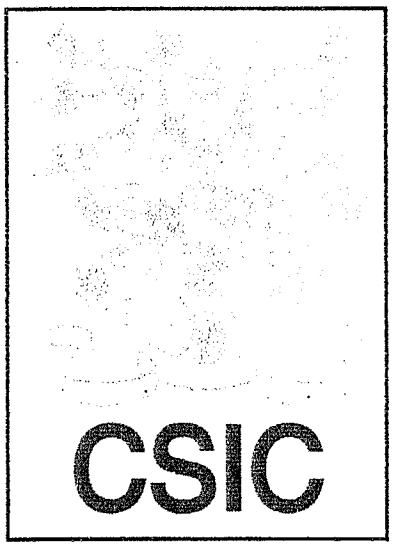

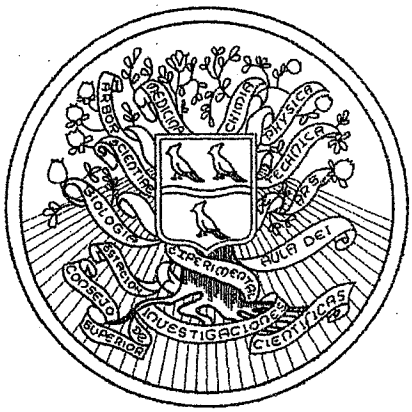

Tesis Doctoral

Adquisición y transporte de hierro en plantas

Memoria presentada por Dña. Ana Flor López Millán, Licenciada en Ciencias Químicas y en Ciencia y Tecnología de los Alimentos, para optar al grado de Doctor en Ciencias

Zaragoza, Febrero de 2000

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CERTIFICAN

Que la Tesis Doctoral titulada **"Adquisición y transporte de hierro en plantas"**, ha sido realizada por la Licenciada en Ciencias Químicas y en Ciencia y Tecnología de los Alimentos Dña ANA FLOR LÓPEZ MILLÁN, en el Departamento de Nutrición vegetal de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su dirección y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor en Ciencias

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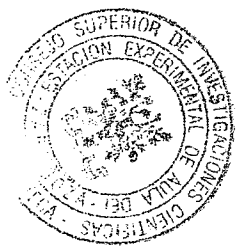

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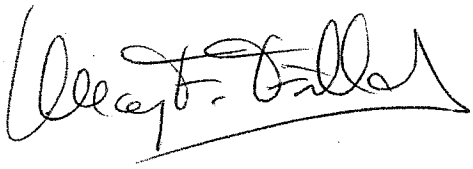

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ABREVIATURAS

2-D	dos dimensiones
5-CF	5-carboxifluoresceína
ABA	ácido abscísico
ACC oxidasa	aminoaciclopropano oxidasa
ADH	alcohol deshidrogenasa
AP	ascorbato peroxidasa
BPDS	ácido 4,7-difenil-1,10-batofenantrolin -disulfónico
BSA	seroalbúmina bovina
CA	anhidrasa carbónica
Chl	clorofila
CIC	capacidad de intercambio catiónico
CIT	citrato
CK	ciclo de Krebs
CS	citrato sintasa
DMA	ácido 2´-desoximugínico
DTT	ditiotreitol
E°	potencial de oxidoreducción estándar
EDDHA	ácido etilendiamino-di-(o-hidroxi)-pentaacético
EDTA	ácido etilen-diamino-tetraacético
ES(SE)	error estándar
FAD	dinucleótido de riboflavina y adenina
FC-R	reductasa férrica
FDH	formiato deshidrogenasa
FITC	isotiocianato de fluoresceína
FMN	riboflavina 3-monofosfato
Fru	fructosa
FW	peso fresco
G6PDH	glucosa 6 fosfato deshidrogenasa
G3PDH	gliceraldehido-3-fosfato deshidrogenasa
GABA	ácido γ amino butírico
Glc	glucosa
HEPES	ácido 2-hidroxietil piperazina-N´-2 etanosulfónico
HMA	ácido 3´-hidroximugínico
HPI	hexosa fosfato isomerasa

HPLC	cromatografía líquida de alta resolución
IAA	ácido indol-acético
ICDH	isocitrato deshidrogenasa
kDa	kilodalton
Km	constante de Michaelis-Menten
Ks	constante de estabilidad
LDH	lactato deshidrogenasa
MA	ácido mugínico
MAL	malato
MDH	malato deshidrogenasa
MES	ácido [2-(N-morfolino)-etanosulfónico]
MP	membrana plasmática
MS	espectroscopía de masas
MR	masa molecular aparente
NA	nicotianamina
OAA	oxalacetato
PAR	radiación fotosintética activa
PEP	fosfoenol piruvato
PEPC	fosfoenol piruvato carboxilasa
PPDF	densidad de flujo de fotones fotosintéticos
Q	quinona
PDC	piruvato descarboxilasa
pI	punto isoeléctrico
PK	piruvato quinasa
PS	peso seco
p/v	relación peso volumen
PVDF	fluoruro de polivinilideno
PVP	polivinil pirrolidona
Rbf	riboflavina
SI	sulfato 3´ de riboflavina
SII	sulfato 5´de riboflavina
ScoA	succinil coenzima A
SHAM	ácido hidroxisalicílico
SDS	sodio dodecil sulfato
SPAD	Soil-Plant Analysis Development
Suc	sacarosa
TCA	ciclo de los ácidos tricarboxílicos

TRIS	Tris-(hidroximetil)aminometano
v/v	relación volumen/volumen

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CAPÍTULO 1

INTRODUCCIÓN GENERAL

1. Introducción General

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1. 1. El Fe en las Plantas

El Fe es un nutriente esencial para prácticamente todo tipo de organismos. El Fe es un elemento de transición con gran facilidad para formar complejos octaédricos con diversos ligandos. El potencial par redox Fe(III) / Fe(II) es bastante elevado en medio ácido (E^0 0,77 V) y disminuye rápidamente al aumentar el pH, debido a la formación de hidróxido férrico. El potencial redox varía con el ligando y esta variabilidad le confiere una especial importancia en los sistemas redox biológicos (Smith, 1984; Marschner, 1995). El Fe es necesario para gran número de funciones biológicas puesto que forma parte de enzimas y otras moléculas de vital importancia. En las plantas, el Fe interviene en la síntesis de clorofila y es esencial para el mantenimiento de la función y estructura del cloroplasto (Abadía, 1992). También interviene en procesos como la respiración, la fijación de nitrógeno (Clark, 1983) y en la síntesis de ADN y hormonas (Briat y Lobréaux, 1997). Además, el Fe interviene en otros procesos, como la reducción de sulfato (Miller et al., 1984; Naik, 1984) y eliminación del peróxido de hidrógeno (Marschner et al., 1986; Guerinot y Yi, 1994).

1. 2. Localización del Fe en la Planta

En las plantas, el 80% del Fe está localizado en el cloroplasto (Terry y Low, 1982). Aproximadamente un 63% del Fe de la hoja está asociado a proteínas (Price, 1968). El tipo de proteínas en las que se incorpora el Fe, se puede clasificar en:

a) Proteínas con grupo hemo. Este grupo comprende todos los enzimas que contienen un grupo hemo, Fe-porfirina, como grupo prostético. Este conjunto lo integran proteínas como los citocromos, catalasas y peroxidasas, representando cerca del 10% del Fe en la hoja.

b) Proteínas Fe-S. En este grupo el Fe está coordinado con el grupo tiol de una cisteína. Este conjunto lo integran proteínas como la ferredoxina, superóxido dismutasa y la aconitasa. Representan aproximadamente un 20% del Fe en la hoja.

c) Fitoferritina. Se trata de una proteína de reserva de Fe, y representa cerca de un 35% del Fe en la hoja, llegando a contener hasta el 80% del Fe de los cloroplastos (Tiffin y Brown, 1972).

1. 3. Biodisponibilidad del Fe

La biodisponibilidad del Fe afecta a la distribución natural de las especies vegetales (Grime y Hutchinson, 1967; Snowden y Wheeler, 1993; Gries y Runge, 1995; Tyler y Falkengren-Grerup, 1998) y puede limitar el crecimiento de especies consideradas de gran importancia económica (Chen y Barak, 1982; Vost, 1982).

El Fe es el cuarto elemento en peso de la litosfera después del oxígeno, el silicio y el aluminio, siendo su concentración media de un 3,2% (Murad y Fisher, 1988). Sin embargo, en suelos alcalinos y calcáreos, el Fe se encuentra en forma de óxidos e hidróxidos de muy baja solubilidad. En estos suelos la concentración de Fe libre es de aproximadamente 10^{-10} M, mientras que los niveles necesarios para la planta son mucho mayores, cercanos a 10^{-7} M (Loeppert, 1986). Así, es frecuente que en este tipo de suelos el Fe sea un nutriente deficitario para las plantas.

Las condiciones que causan una baja solubilidad y/o movilidad del Fe en la solución del suelo son una de las causas principales de la poca disponibilidad de Fe para las plantas (Wallace, 1982). Un pH elevado, altas cantidades de arcillas y carbonatos (Kashirad y Marschner, 1974; Kolesch et al., 1987a,b), alta humedad, alta salinidad (Award et al; 1988) y altas cantidades de fosfatos (Lindsay y Schwab, 1982) pueden ser desencadenantes de la deficiencia de Fe.

Otros agentes que pueden ser responsables de la deficiencia de Fe son aquellos relacionados con la absorción y metabolismo del Fe (Romera y de la Guardia, 1991). Entre los factores que afectan a la absorción del Fe por la planta se encuentran la variabilidad genética, todos aquellos factores que inhiben el crecimiento de las raíces como bajas temperaturas, presencia de herbicidas, encharcamiento de los suelos, etc y factores que inhiben la absorción de este elemento como la presencia de metales pesados.

Por otro lado, el Fe en el interior de la planta puede estar formando parte de reservorios o acervos inactivos que no son utilizables, existiendo una deficiencia de Fe en la planta aunque las concentraciones de Fe en la misma puedan parecer adecuadas (Morales et al., 1998). Tanto altas cantidades de fosfato como un alto pH apoplástico podrían causar la precipitación del Fe en el interior de la planta en forma de fosfatos o hidróxidos férricos, compuestos no disponibles para la planta (Mengel, 1994).

Un factor importante que influye en la biodisponibilidad del Fe es la presencia de bicarbonato (Harley y Linder, 1945) debido al gran número de procesos en los que participa. Se ha propuesto que el bicarbonato puede afectar principalmente al crecimiento y metabolismo de las raíces (Yang et al., 1994), a la toma y transporte de Fe a la parte aérea (Alhendawi et al., 1997) y a la disponibilidad fisiológica del Fe en las hojas. La posible alcalinización del apoplasto y citoplasma, debida a la presencia de bicarbonato, podría causar una precipitación del Fe en dichos compartimentos (Romera et al., 1992; Mengel, 1994) y una disminución de la actividad de la reductasa férrica en la raíz (Susín et al., 1996).

1. 4. Síntomas de la Deficiencia de Fe

Las plantas deficientes en Fe muestran diversos síntomas, entre los que el más clásico es el amarilleamiento de las hojas jóvenes denominado clorosis (Terry y Abadía, 1986). En casos extremos, la hoja adquiere un color blanco y puede llegar a manifestar necrosis (Chaney, 1984). Cuando la clorosis es muy grave se produce incluso la muerte de la planta. El amarilleamiento proviene de un cambio en la composición pigmentaria de los cloroplastos de las hojas (Morales et al., 1990, 1994, Abadía y Abadía, 1993).

La deficiencia de Fe suele tener menos efecto en el crecimiento de la hoja y sólo en casos de deficiencia severa se produce una disminución en su crecimiento (Abbott, 1967). En hojas cloróticas se reduce el tamaño de los cloroplastos y su contenido proteico (Terry y Bonner, 1980). Los acúmulos de almidón disminuyen e incluso desaparecen. Cuando la deficiencia es severa, los cloroplastos presentan forma esférica o ligeramente alargada con muy pocas lamelas y un estroma muy grande sin prácticamente grana (Spiller y Terry, 1980).

Figura 1. 1. Hoja clorótica

La deficiencia de Fe se ha descrito en un amplio espectro de plantas de interés agronómico. En frutales tiene importantes connotaciones económicas, puesto que, si no se corrige, el crecimiento de los árboles se ve afectado, la floración es menor y los frutos son menos numerosos y más pequeños, pudiéndose llegar en casos extremos a la muerte prematura del árbol. Sólo en la cuenca del Ebro se ha estimado en 2.000 millones de pesetas por año el gasto que suponen los tratamientos correctores de la clorosis férrica (Sanz et al., 1992).

1. 5. Respuestas de las Raíces ante la Deficiencia de Fe

El principal sistema para mantener la homeostasis del Fe consiste en la adaptación de los mecanismos de adquisición de Fe a las condiciones externas. Ante la deficiencia de Fe las plantas pueden permanecer indiferentes (plantas no eficientes) o desarrollar mecanismos de adaptación para aumentar su capacidad de tomar Fe del suelo (plantas eficientes). Se han descrito dos estrategias distintas, que son utilizadas por las plantas eficientes, dependiendo de la forma de adquirir el Fe: la Estrategia II de las plantas gramíneas y la Estrategia I de las dicotiledóneas y monocotiledóneas no gramíneas. Las especies pertenecientes a estas estrategias se encuentran separadas filogenéticamente.

1. 5. 1. Estrategia II

Las raíces de las plantas gramíneas sintetizan y excretan compuestos de bajo peso molecular, llamados fitosideróforos. Estos compuestos quelan el Fe(III) presente en el suelo y posteriormente son absorbidos a través de receptores específicos en la membrana plasmática de la raíz, sin reducción previa. Todos los fitosideróforos identificados hasta la fecha son aminoácidos análogos al ácido mugínico y se sintetizan en las zonas subapicales de la raíz (Marschner et al., 1987; Mori y Nishizawa, 1987). La estructura química de estos compuestos se muestra en la figura 1.2.A.

En condiciones de deficiencia de Fe, las plantas gramíneas inducen una excreción de fitosideróforos, con aumentos de hasta 20 veces respecto a plantas control (Takagi et al., 1984), llegando a concentraciones en el suelo de 1 μM

(Romera y de la Guardia, 1991). En deficiencia de Fe también aumenta la velocidad de absorción de los complejos Fe(III)-fitosideróforo, lo que indica una mayor capacidad de transporte. Mori et al. (1991) propusieron un mecanismo de cotransporte de la excreción de fitosideróforos acoplado a protones o iones potasio. El transporte del complejo al citoplasma de la célula se produce a través de un transportador de alta especificidad y selectividad constitutivo de la membrana plasmática de las células de raíz (Marschner et al., 1989; Mori, 1998) (Figura 1.2.B). Una vez dentro de la planta se produce la liberación del Fe y el fitosideróforo se degrada o se excreta al exterior.

Se está trabajando activamente en el sistema de regulación de la síntesis de los fitosideróforos y de los mecanismos implicados en la misma (Mori, 1998). El primer paso de la síntesis de fitosideróforos requiere la combinación de tres moléculas de S-adenosil metionina para dar nicotianamina, este compuesto es un potencial transportador de Fe(II) en la planta (Stephan y Scholz, 1993). Esta reacción está catalizada por la nicotianamina sintasa (Shojima et al., 1990), enzima que aumenta su actividad en condiciones de deficiencia de Fe.

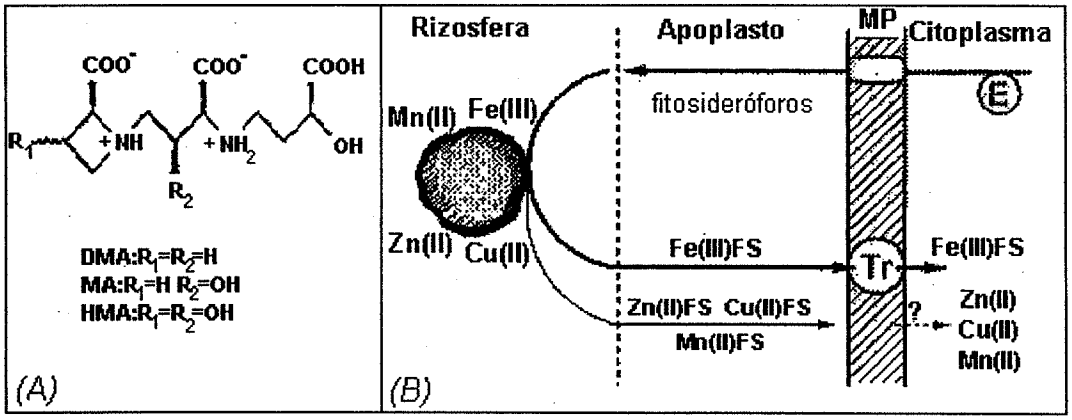

Figura 1. 2. (A) Estructura química de los fitosideróforos, MA, ácido mugínico; DMA, ácido 2´-desoximugínico; HMA, ácido 3´-hidroximugínico, (B) Adquisición de Fe por las raíces de las plantas de Estrategia II.

1. 5. 2. Estrategia I

Las plantas dicotiledóneas y monocotiledóneas no gramíneas desarrollan en condiciones de deficiencia de Fe una serie de respuestas que afectan a la estructura, morfología y fisiología de las raíces, todas ellas encaminadas a desarrollar mecanismos para aumentar la capacidad de absorción del Fe del suelo. Los fenómenos que afectan a la fisiología de las raíces son: un aumento de la capacidad de reducción de quelatos mediante la actividad de una reductasa férrica unida a la membrana plasmática (Chaney et al., 1972; Moog y Brüggemann, 1994), la acidificación de la rizosfera (Alcántara et al., 1991; Rabotti y Zocchi, 1994) y la producción y excreción de compuestos fenólicos, flavinas y ácidos orgánicos.

El modelo propuesto para la adquisición de Fe en plantas de Estrategia I se muestra en la figura 1.3. Los pasos serían los siguientes: a) adsorción de los quelatos de Fe(III) en la raíz, b) debilitamiento de los enlaces quelato-Fe(III), c) reducción del Fe(III) a Fe(II), d) disociación del quelato y e) transporte del Fe(II) a través de un transportador de membrana.

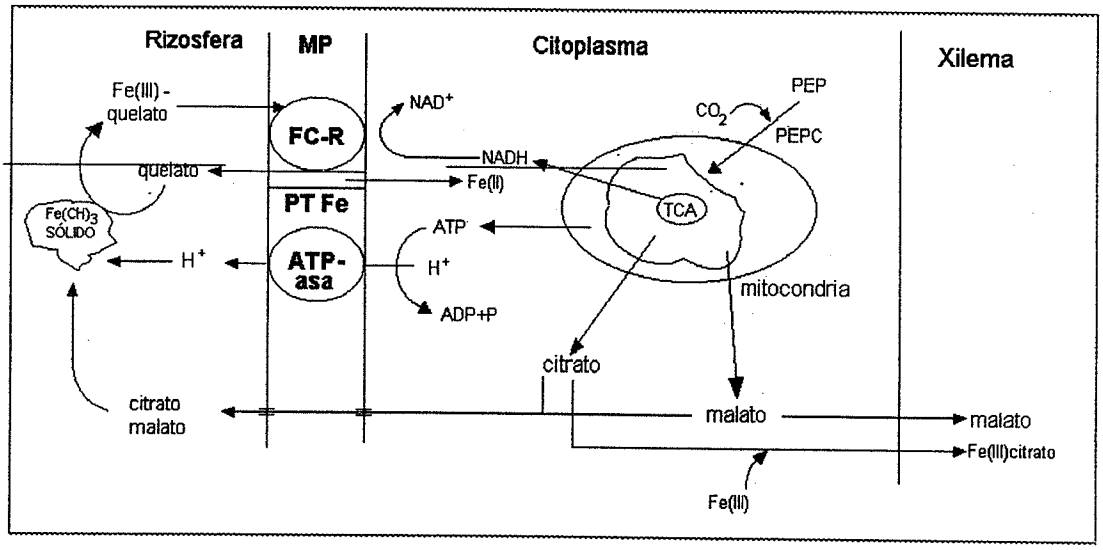

Figura 1. 3. Estrategia I

1. 5. 2. 1. Cambios Morfológicos en la Raíz Inducidos por la Deficiencia de Fe

En condiciones de deficiencia de Fe muchas especies presentan cambios macro y microscópicos en su estructura radicular. Los cambios macroscópicos incluyen una disminución en el crecimiento de la raíz primaria (Hutchinson, 1967; Brown y Ambler, 1974; Römheld y Marschner, 1981) acompañada por un aumento en el número de raíces laterales (Moog et al., 1995; Pinton et al., 1998). Estos cambios son evidentes después de un largo tiempo en deficiencia de Fe. Sin embargo, otros cambios como la formación de pelos radiculares y el engrosamiento de las zonas subapicales de la raíz (Welkie y Miller, 1993; Landsberg, 1996) ocurren antes de que se observe una disminución en el crecimiento de la raíz. El engrosamiento de las puntas de raíz no se observa en todas las especies (Wei et al., 1997). Este engrosamiento es debido al aumento del tamaño de las células corticales y a un incremento del número de células en la rizodermis e hipodermis (Landsberg, 1996). En general, estos cambios macroscópicos permiten aumentar la superficie de contacto entre las raíces y el suelo. El engrosamiento de las raíces coincide con aquellas zonas donde se produce la reducción de Fe (Bell et al., 1988) y la extrusión de protones (Alcántara et al., 1991). También se ha descrito la formación de agregados radiculares en condiciones de deficiencia de Fe (Gardner et al., 1982; White y Robson, 1989; Rosenfield et al., 1991). Estos agregados están formados por numerosas raíces laterales con múltiples ápices y un aumento de los pelos radiculares distribuidos en distintas secciones a lo largo de la raíz.

Los cambios microscópicos incluyen la presencia de células de transferencia en la epidermis de la zona de engrosamiento, incrementando así la superficie de contacto entre la pared celular y el citoplasma (Welkie y Miller, 1993; Landsberg, 1994; Schmidt y Bartels, 1996). Las células de transferencia presentan protuberancias en la pared celular más próxima al exterior, citoplasma denso con numerosas mitocondrias y voluminosos protoplastidios, con multitud de invaginaciones (Landsberg, 1994). La función de estas células de transferencia aún no se ha determinado. La formación de células de transferencia coincide temporal y espacialmente con la extrusión de protones (Rosenfield et al., 1991), mientras que no se ha encontrado una relación tan evidente con la actividad reductora de las raíces (Smith y Bartels, 1996). Esto indicaría bien una regulación diferente para ambas respuestas (Moog et al., 1995) o bien que estos cambios no

forman parte de la respuesta específica a la deficiencia de Fe (Chaney et al., 1992). Por otro lado, la gran cantidad de mitocondrias existente en estas células pone de manifiesto la alta actividad respiratoria existente, proponiéndose como posible función el suministro de energía para el transporte iónico durante la extrusión de protones (Landsberg, 1994).

1. 5. 2. 2. Inducción de la Actividad Reductasa

La toma de Fe por las plantas de Estrategia I requiere la reducción previa de Fe(III) a Fe(II) (Chaney et al., 1972; Brown y Ambler, 1974). La capacidad de las raíces de reducir Fe(III) del medio aumenta en condiciones de deficiencia de Fe y puede llegar a ser superior en 10-20 veces a los valores control (Moog y Brüggemann, 1994; Susín et al., 1996; Sueyoshi et al., 1997). La actividad reductasa se localiza generalmente en la superficie de las partes subapicales de las raíces que muestran engrosamiento, así como en los pelos radiculares (Moog y Brüggemann, 1994).

La reducción del Fe ocurre a nivel de la membrana plasmática de la raíz, a través de un enzima específico (FC-R) capaz de reducir quelatos de Fe(III). Sin embargo, el mecanismo por el cual las raíces de las plantas eficientes pertenecientes a la Estrategia I inducen la reducción de Fe(III) dista de estar completamente dilucidado. El enzima FC-R podría ser inducido *de novo* bajo deficiencia de Fe, o ser una proteína constitutiva que se activara en condiciones de deficiencia de Fe. La actividad FC-R de preparaciones de membrana plasmática aisladas de plantas deficientes es solamente 2-3 veces superior a la de las plantas control (Rubinstein y Luster, 1993; Moog y Brüggemann, 1994; Susín et al., 1996) y muestra valores de afinidad por el sustrato (K_m) similares. La diferencia entre la magnitud de la inducción que se observa en las plantas intactas con respecto a la encontrada en membrana plasmática aislada se podría deber a la intervención de algún cofactor que se pudiese perder en el aislamiento de la membrana plasmática (Susín et al., 1996).

Recientemente se ha aislado en *Arabidopsis thaliana* el gen FRO2 que codifica para una FC-R y cuyos transcritos se acumulan en respuesta a la deficiencia de Fe (Robinson et al., 1999). Este gen pertenece a una familia de

flavocitocromos cuya función es el transporte de electrones a través de la membrana plasmática. La proteína que codifica consta de 725 aminoácidos, con un pI de 9,37 y una masa molecular relativa de 81,5, aunque la presencia de sitios de glicosilación sugiere que la masa relativa de la proteína nativa sea mayor. Este gen posee 6 dominios hidrofóbicos en el extremo amino terminal y 2 en el C-terminal, todos ellos formando α-hélices transmembrana (Fig. 1. 4). Existen sitios de unión a grupos hemo intramembrana y sitios de unión a FAD y a NADPH en el lado citoplasmático. El aumento de los transcritos de este gen en deficiencia de Fe corrobora los datos cinéticos, que indican que la inducción de la FC-R en deficiencia de Fe podría ser debida a la sobreexpresión de una FC-R constitutiva más que a una activación de dicho enzima (Brüggemann et al., 1991; Bagnaresi y Pupillo, 1995; Schmidt, 1999).

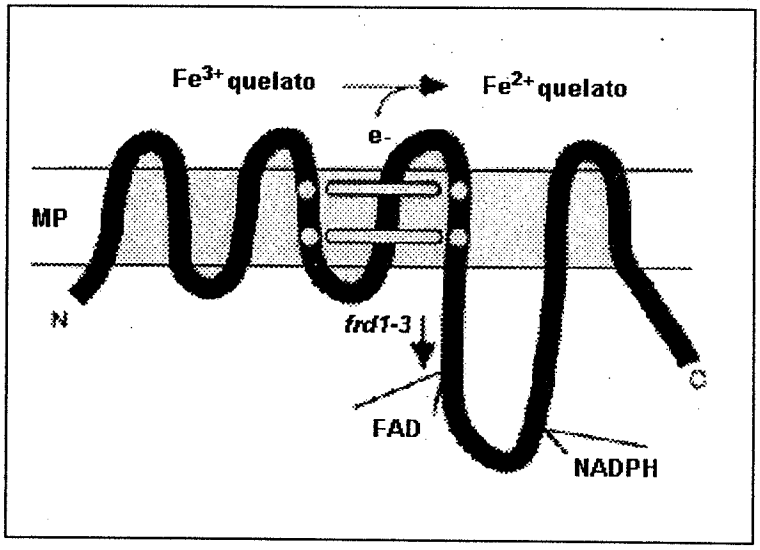

Figura 1. 4. Estructura molecular de la FC-R. (Robinson et al., 1999)

El donador electrónico acoplado a la FC-R todavía no se conoce. En un principio se propuso como donador electrónico al NADPH (Sijmons y Bienfait, 1984). Sin embargo, estudios con membrana plasmática aislada muestran que la FC-R siempre funciona mejor con NADH (Moog y Brüggemann, 1994). Schmidt y Schuck (1996) indican que la reducción de Fe(III) externo lleva a un descenso

momentáneo de NADPH y a un aumento de NADH, lo cual sugiere que el mecanismo es más complicado de lo inicialmente pensado.

Se ha descrito que para la inducción de la FC-R es necesaria la presencia de una cantidad mínima de Fe en el medio (Romera et al., 1996a,b; Zouari, 1996; Gogorcena et al., 2000). Esta dependencia de Fe podría ser debida a la relación de la actividad FC-R con el metabolismo del etileno (Romera y Alcántara, 1994; Romera et al., 1996a,b), una de cuyas enzimas, la ACC oxidasa, requiere Fe para su síntesis. Una especie en la que no ocurre esta dependencia es la remolacha, que induce un gran aumento en la actividad FC-R en condiciones en las que el Fe está totalmente ausente del medio (Susín et al., 1996).

1. 5. 2. 3. Acidificación de la Rizosfera

Las plantas pertenecientes a la Estrategia I suelen producir un descenso del pH de la solución en la que crecen (Welkie y Miller, 1993; Susín, 1994; Susín et al., 1994). La acidificación es debida a una bomba de protones dependiente de ATP situada en la membrana plasmática de la raíz (Poole, 1978; Rasi-Caldogno et al., 1981). Este tipo de ATPasa ha sido caracterizada bioquímicamente (Sze, 1985; Serrano, 1989). El pH óptimo *in vitro* es de 6,6 (Michelet y Boutry, 1995) y la eficiencia termodinámica H^+/ATPasa es igual a 1 (Rea y Sanders, 1987). Los cationes alcalinos (K^+, Rb^+, Na^+, Cs^+, Li^+) estimulan su actividad, mientras que los aniones no tienen ningún efecto. El tamaño molecular de la ATPasa es de 220-228 kDa, con una subunidad de 100 kDa que constituye la subunidad catalítica del enzima. Existen un total de 6 dominios funcionales en el péptido de 100 kDa (Serrano, 1989). El mecanismo de funcionamiento pasa por la creación de un intermediario fosforilado, estable a pH ácido, que requiere Mg para su funcionamiento (Briskin, 1990). No se sabe todavía si el aumento de la actividad de la ATPasa en deficiencia de Fe es debido a la expresión de una isoproteína específica, aunque existe una amplia familia de genes de H^+-ATPasa en *Arabidopsis thaliana*, al menos uno de los cuales, el aha_2, se regula en respuesta a la deficiencia de Fe (Fox y Guerinot, 1998).

La acidificación está localizada en las zonas subapicales de la raíz, diferenciándose de la acidificación generalizada a lo largo de toda la raíz debida a la

absorción preferente de cationes (Römheld et al., 1984). La estimulación de la acidificación en raíces deficientes en Fe varía de un 18% a un 100% (Buckhout et al., 1989; Valenti et al., 1991; Szabo-Nagi y Erdei, 1993; Rabotti y Zochi, 1994; Susín et al., 1996; Schmidt et al., 1997) y es capaz de acidificar el entorno hasta aproximadamente 2 mm de distancia de la raíz, incluso en suelos calcáreos (Schaller, 1987). Esta excreción de protones afecta a la velocidad de disolución de los óxidos de Fe, debilitando el enlace Fe-O y liberando posteriormente el metal (Schwertmann, 1981). De esta forma se aumenta la solubilidad de los compuestos de Fe en la zona próxima a los lugares de absorción. Otro papel de la acidificación podría ser la estimulación de la actividad reductasa férrica (Toulon et al., 1992; Susín et al., 1996).

1. 5. 2. 4. Excreción de Compuestos de Bajo Peso Molecular

Las raíces de las plantas son capaces de excretar activa o pasivamente gran variedad de compuestos orgánicos entre los que se encuentran azúcares reductores, aminoácidos y ácidos orgánicos. Las cantidades excretadas pueden ser hasta de un 20% del carbono asimilado. El tipo de exudados radiculares está determinado genéticamente aunque existen factores medioambientales que pueden causar una alteración en la composición de estos exudados. La tasa de exudación y su composición dependen del pH, temperatura y tipo de suelo, intensidad de la luz, así como de la edad y estado nutricional de la planta y de la presencia de microorganismos (Jones, 1998). La exudación de estos compuestos está estimulada en condiciones de deficiencia de Fe.

a) Fenoles

Las plantas deficientes en Fe producen y excretan diferentes compuestos de naturaleza fenólica (Brown y Ambler, 1974). Entre los compuestos identificados se encuentra el ácido cafeico (Olsen et al., 1981), que puede llegar a representar el 85% de los compuestos fenólicos excretados, el ácido fenólico, el ácido clorogénico (Hether et al., 1984) y el ácido p-cumárico. En un principio se pensó que estos compuestos eran una buena fuente de electrones para la reducción del Fe(III). Sin

embargo, varios estudios han mostrado que las cantidades excretadas no son suficientes para explicar la tasa de reducción encontrada en plantas deficientes (Chaney et al., 1972; Barret-Lennard et al., 1983; Bienfait et al., 1983). Dependiendo de las condiciones de los ensayos y del estado nutricional del Fe en la planta, la reducción de Fe debida a los compuestos excretados supondría sólo un 6% de la tasa total de reducción de las raíces (Grusak et al., 1990a,b; Grusak, 1995; Grusak y Pezeshgi, 1996; Susín et al., 1996; Wei et al., 1997), aunque se han llegado a encontrar valores de hasta un 37% (Egilla et al., 1994). Además, el hecho de que los orto-difenoles inhiban la actividad reductasa de la raíz no apoyaría que los fenoles participaran en la reducción de Fe (Römheld y Marschner, 1983).

Probablemente el papel principal de los fenoles consista en la inhibición de la degradación de los ácidos orgánicos, cuyo papel en la nutrición férrica como quelantes de Fe es muy importante (Schmidt, 1999). Una función alternativa fue propuesta por Sijmons et al. (1985) al relacionar la secreción de estos compuestos con la pérdida de suberina, recubrimiento protector de las células corticales de la raíz, que presentan las raíces deficientes en Fe.

b) Flavinas

En condiciones de deficiencia de Fe, algunas especies acumulan y excretan flavinas (Welkie y Miller, 1993). Las flavinas mayoritarias son la riboflavina (Kannan y Seshandri, 1988; Shinmachi et al., 1994, 1995; Welkie, 1996) y, en algunas plantas como la remolacha y espinaca, los sulfatos de riboflavina (Susín et al., 1993, 1994a,b). A pH alto las flavinas se acumulan en la zona subapical de la raíz (Susín et al., 1993) siendo excretadas al medio al disminuir el pH (Susín et al., 1994a,b).

Las plantas que producen flavinas como remolacha (Welkie y Miller, 1988; Susín et al., 1993, 1994a,b), tabaco (Welkie y Miller, 1988), lechuga (Welkie y Miller, 1992), melón (Shinmachi et al., 1995), pepino (Shinmachi et al., 1992), alfalfa y pimiento (Shinmachi et al., 1995) son generalmente muy eficientes en la toma de Fe (Welkie y Miller, 1993). El papel de las flavinas en la deficiencia de Fe es todavía desconocido. Sin embargo, el hecho de que la acumulación de flavinas tenga lugar simultáneamente al incremento en la actividad reductasa sugiere que

estos compuestos podrían ser parte integral de los sistemas de reductasa férrica de las raíces (Cakmak et al., 1987; Susín et al., 1993). Esta afirmación se ve reforzada por el hecho de que recientemente se ha aislado el gen FRO2 (Robinson et al., 1999) que codifica para una FC-R y se expresa en condiciones de deficiencia de Fe en las raíces de *Arabidopsis thaliana*. Este gen pertenece a la familia de los flavocitocromos y contiene un sitio de unión para FAD.

Por otro lado, los sulfatos de riboflavina pueden ejercer, una vez excretados, una acción antimicrobiana en las proximidades de la raíz, disminuyendo la posibilidad de que los microorganismos del entorno compitan con la planta por la adquisición de Fe (Susín et al., 1993).

c) Ácidos orgánicos.

Los ácidos orgánicos pueden llegar a ser el componente más abundante entre los compuestos de bajo peso molecular excretados por las raíces deficientes en Fe (Kraffczyk et al., 1984). La forma de excreción de los ácidos y su localización exacta en las raíces de plantas deficientes no se conoce con exactitud. Dependiendo de sus propiedades de disociación y del número de grupos carboxílicos, los ácidos orgánicos pueden variar su carga negativa y por tanto quelar distintos metales de la solución del suelo. Por este motivo, los ácidos están implicados en un gran número de procesos en el suelo como la movilización y toma de nutrientes (por ejemplo Fe y P) por las plantas y microorganismos, la detoxificación de metales por las plantas (Al), la proliferación de microorganismos en la rizosfera y la disolución de minerales del suelo (Jones, 1998).

La excreción de estos ácidos orgánicos tiene lugar probablemente por difusión pasiva gracias al gradiente de potencial electroquímico de la membrana plasmática (Jones y Darrah, 1995). Sin embargo, también se ha sugerido la posible existencia de un canal o transportador acoplado con la ATPasa de la membrana plasmática (Dinkelaker et al., 1995; Fox et al., 1996).

En condiciones de deficiencia de Fe la función más probable de estos ácidos consiste en la solubilización de Fe(III) (Fig. 1. 5). Cuando el pH del suelo es bajo, tanto citrato como malato forman complejos muy estables con el Fe(III)

favoreciendo su disolución en la solución del suelo (Jones et al., 1996). En suelos calcáreos, con pH alto, la movilización del Fe por malato y citrato es lenta, puesto que los complejos que se forman son inestables y se degradan rápidamente (Jones et al., 1996; Gerke, 1997). Sin embargo, en estas condiciones la combinación del poder de acidificación de la ATPasa de la raíz con el poder quelante del citrato (que aumenta conforme disminuye el pH) podrían constituir un mecanismo viable para movilizar el Fe de la rizosfera (Jones, 1998). Usando un modelo de simulación por ordenador Jones et al. (1996) estimaron que la concentración de Fe(III)-citrato en la interfase raíz-suelo podía llegar a ser de 0,1-50 μM. Por lo tanto, la concentración de Fe unida a ácidos orgánicos sería adecuada para satisfacer las necesidades de las plantas.

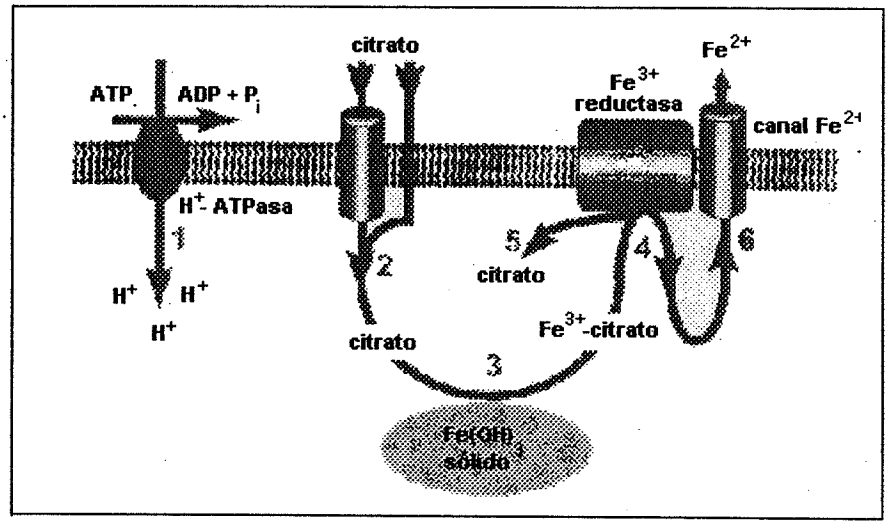

Figura 1. 5. Papel de los ácidos en la solubilización del Fe en la rizosfera

1. 5. 2. 5. Cambios en el Patrón Polipeptídico

Distintos trabajos realizados mediante electroforesis de dos dimensiones y por translación *in vitro* de mRNA de raíces (Bienfait, 1988b; Herbik et al., 1996; Schmidt y Buckhout, 1997) han demostrado que se producen cambios en la síntesis proteica de distintos polipéptidos en condiciones de deficiencia de Fe. No se conoce todavía si estos cambios están relacionados directamente con la adquisición de Fe. En puntas de raíz de plantas de tomate deficientes en Fe se han encontrado aumentos en las concentraciones de G3PDH, FDH y AP (Herbik et al.,

1996). Recientemente se ha determinado la secuencia de un DNAc que codifica para una lisil-t-RNA-sintetasa, cuya expresión aumenta en raíces deficientes sugiriendo que tiene un papel en la deficiencia de Fe, aparte de su función en la síntesis proteica (Giritch et al., 1997). También en tomate, Schmidt y Buckhout (1997) han encontrado, mediante técnicas de traducción *in vitro* de mRNA, siete polipéptidos cuya expresión aumenta con la deficiencia de Fe (masas moleculares aparentes de 62, 54, 41, 39, 38, 38 y 36 kDa, con pI 6,1, 6,2, 5,8, 7,3, 6,5, 6,4 y 6,7, respectivamente). En raíces de remolacha deficientes en Fe (González-Vallejo et al., 1998) se ha encontrado que cuatro polipéptidos aparecen *de novo* (masas moleculares aparentes de 46,8, 42,0, 34,6, y 17,3 kDa, con pI de 5,9, 5,5, 4,8 y 6,0, respectivamente), mientras que otros ocho cambian sus niveles de expresión. Entre estos últimos se encuentran la FDH y la PEPC (42,7 y 64,6 kDa, respectivamente). La FDH es una enzima relacionada con el metabolismo anaeróbico y la inducción de su expresión en deficiencia de Fe ha sido confirmada también en cebada (Suzuki, 1998). Asimismo, se han descrito aumentos en la actividad de la PEPC en raíces de plantas deficientes en Fe (Miller et al., 1990; Rabotti et al., 1995), enzima relacionada con el metabolismo del carbono. En condiciones de deficiencia de Fe también se han encontrado aumentos en la actividad de distintos enzimas relacionados con la glicólisis como la G3PDH (Rabotti et al., 1995).

Experimentos realizados con inhibidores de síntesis proteica y agentes modificadores de proteínas han demostrado que la síntesis proteica es necesaria para la actividad FC-R (Schmidt, 1993; Sueyoshi et al., 1997; Robinson et al., 1999). La inhibición con cicloheximida es más efectiva en plantas deficientes en Fe, lo cual indica que una o más proteínas son sintetizadas en cantidades más altas en deficiencia de Fe. La actividad de la FC-R de la membrana plasmática no está afectada por el tratamiento con cicloheximida, indicando que los efectos no son en la reductasa en sí misma sino en alguna subunidad o cofactor esencial o en alguna proteína reguladora. La extrusión de protones se inhibe por la cicloheximida y otros inhibidores de la síntesis proteica (Bienfait, 1989). Esto puede indicar bien un efecto secundario en la reductasa por inhibición de la H^+-ATPasa, o una disminución en la síntesis de un enzima implicado en la regulación de ambas respuestas.

1. 5. 2. 6. Acumulación de Ácidos Orgánicos

Probablemente, la característica más generalizada de las plantas deficientes en Fe es un aumento en la concentración de ácidos orgánicos, principalmente malato y citrato, en todas las partes de la planta (Brown, 1966; Landsberg, 1981; Alhendawi et al., 1997). La concentración de citrato se correlaciona negativamente con la concentración de Fe en la raíz (Pich y Scholz, 1991). La acumulación de citrato en las raíces deficientes en Fe no es debida a que disminuya la actividad de la aconitasa, enzima que contiene Fe, ya que se ha descrito que dicha actividad no cambia (de Vos et al., 1986) o incluso aumenta (Pich y Schölz, 1993) en raíces deficientes en Fe. No se conoce con exactitud el mecanismo por el que se acumulan los ácidos orgánicos.

Hasta el momento se han propuesto dos hipótesis para explicar el aumento en la concentración de ácidos orgánicos con la deficiencia de Fe. La primera (Landsberg, 1981) sugiere que la acumulación de ácidos sea consecuencia de la alcalinización del citoplasma debida a la extrusión de protones y la consiguiente inducción del mecanismo de regulación del pH citoplasmático (teoría del "pH-stat"; Davies, 1973). En esta ruta metabólica, el aumento del pH citoplasmático conduciría a una activación de la PEPC. Esta enzima cataliza la carboxilación del PEP para dar oxalacetato y éste, vía malato deshidrogenasa, conduce al aumento en la concentración de ácidos orgánicos. El aumento en la concentración de ácidos mantendría el balance iónico en el citoplasma, neutralizando el efecto alcalinizante producido por la extrusión de protones. Sin embargo, la acumulación de ácidos orgánicos en condiciones de deficiencia de Fe no está necesariamente acoplada a la extrusión de protones (Landsberg, 1981; Bienfait, 1989), lo que hace dudar sobre la validez de esta hipótesis.

La segunda hipótesis sugiere que la síntesis elevada de ácidos orgánicos en condiciones de deficiencia de Fe puede ser debida a un aumento en la glicólisis (de Vos et al., 1986). En esta hipótesis, la fosfofructoquinasa en condiciones de deficiencia de Fe se haría insensible a la regulación por citrato, debido a un aumento en los niveles endógenos de amonio, como ocurre en algunos microorganismos (Habison et al., 1979). La actividad piruvato quinasa, que convierte PEP a piruvato en la glicólisis, se inhibe por citrato (Goodwin y Mercer,

1983). Estos dos fenómenos conducirían a un aumento en la concentración de PEP que, a través de la actividad de la PEPC, produciría un aumento en la concentración de ácidos orgánicos. En consonancia con esta hipótesis se ha medido un incremento en la actividad de la G3PDH, enzima de la ruta glicolítica, en raíces deficientes de pepino (Rabotti et al., 1995) y judía (Bienfait et al., 1983). En esta segunda hipótesis se sugiere que la acumulación de ácidos orgánicos sería el efecto primario de la deficiencia de Fe. Este aumento produciría una acidificación del citoplasma. La extrusión de protones podría ocurrir o no, dependiendo de otros factores tales como el balance entre la absorción de cationes y aniones, la actividad de la nitrato reductasa y otros.

Independientemente de la causa de la acumulación de ácidos, en ambas hipótesis tal acumulación pasa por un aumento en la actividad de la PEPC. Confirmando este hecho, se ha descrito que tanto la actividad PEPC como la fijación de CO_2 en raíz están estimuladas en condiciones de deficiencia de Fe (Rhoads y Wallace, 1960; Landsberg, 1986; Bienfait, 1989; Rabotti et al., 1995). El aumento de fijación de CO_2 reduciría la demanda de CO_2 fijado en la fotosíntesis (Vance et al., 1994) que se encuentra fuertemente afectada por la deficiencia de Fe (Abadía, 1992). La acumulación de ácidos orgánicos no está restringida a la deficiencia de Fe, sino que también se ha descrito una acumulación de ácidos en deficiencia de P. El citrato exudado en condiciones de deficiencia de P viene en parte de la fijación no fotosintética de carbono vía PEPC (Johnson et al., 1996a). La actividad de la PEPC está regulada a nivel transcripcional y post translacional (Johnson et al., 1996a,b).

Las posibles funciones de los ácidos orgánicos son muy diversas. Sus características como agentes quelantes les permite intervenir en la movilización de Fe de la rizosfera. El citrato tiene un papel muy importante en la translocación y el transporte de Fe a larga distancia (Tiffin, 1966a,b; Pich et al., 1995). Independientemente de qué proceso sea la causa y cual la consecuencia, se ha encontrado una correlación entre la acumulación de ácidos orgánicos y la extrusión de protones (Landsberg, 1986; Fournier et al., 1992). Por otro lado, Bienfait (1996) propuso que la acumulación de ácidos está implicada en la síntesis de NADH y NADPH, a través de las actividades enzimáticas de la ICDH y G6PDH. Estos nucleótidos podrían actuar como fuente de poder reductor para la FC-R de raíz (Fig. 1. 6). Sin embargo, se ha encontrado alta actividad reductasa en variedades

de girasol que no acumulan citrato (Fournier et al., 1992), por lo que la relación entre la acumulación de ácidos y la actividad reductasa no resulta tan evidente.

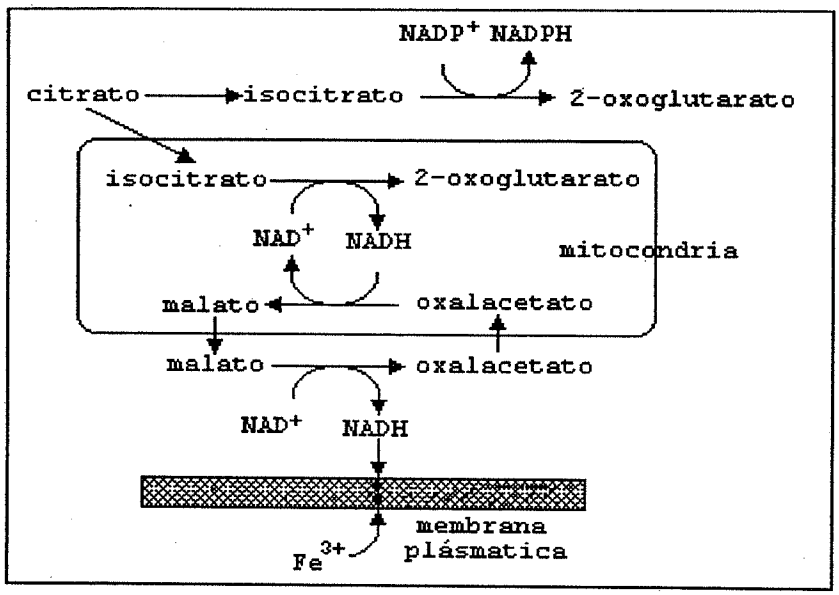

Figura 1. 6. Papel de los ácidos en la obtención de poder reductor para la FC-R de raíz propuesto por Bienfait (1996)

1. 6. Transporte de Fe

1. 6. 1. Transporte en la Raíz

Una vez que el Fe ha sido reducido se transporta a través de la membrana plasmática como Fe(II) mediante transportadores específicos (Fox y Guerinot, 1998). La capacidad de adquisición y translocación de Fe aumenta en condiciones de deficiencia de Fe (Römheld y Kramer, 1983; Yi y Guerinot, 1996). A pesar de que los cambios en la absorción de Fe se correlacionan con los cambios en la actividad reductasa, todavía no se conoce si el aumento en la adquisición es debido a una mayor actividad de la reductasa (Grusak et al., 1990a), del transportador (Young y Terry, 1982) o de ambos. Recientemente Fox et al. (1996) usando soluciones con Fe(II) quelado demostraron un aumento del transporte de Fe(II) inducido por deficiencia de Fe, sugiriendo un aumento en la síntesis o bien un aumento en la actividad del transportador. A pesar de que este experimento permite desacoplar el

transporte de Fe de la reducción, no puede excluirse que la actividad reductasa tenga efectos en el transportador. Asimismo, debería clarificarse si el Fe(II) generado por la reductasa y el que se encuentra libre en solución entran en la célula por la misma ruta.

Se ha caracterizado en *Arabidopsis* un gen, *IRT1* (*iron-regulated transporter*) que codifica un transportador de Fe(II) (Eide et al., 1996). Este gen sólo se expresa en raíces en condiciones de deficiencia de Fe. El *IRT1* es un transportador específico para Fe, aunque muestra una fuerte inhibición con Cd^{2+} y Mn^{2+}. El análisis de su secuencia sugiere que es una proteína integral de membrana con 339 aminoácidos y con 8 dominios transmembrana. Entre los dominios 3 y 4 posee 4 repeticiones histidina-glicina, que constituyen lugares potenciales de unión a metales.

Los iones de Fe(II) libres son muy reactivos y potencialmente tóxicos, debido a que pueden catalizar la transferencia de electrones a especies de oxígeno, para producir finalmente radicales hidroxilo (Guerinot y Yi, 1994). Esto hace suponer que el Fe(II) en el citoplasma está unido a algún tipo de compuesto que evite estos efectos. Distintos autores (Schölz et al., 1988; Pich et al., 1997) han propuesto que la nicotianamina es capaz de quelar el Fe(II) en el citoplasma permitiendo su transporte al lugar de translocación al xilema. Una vez allí sería oxidado a Fe(III) de forma todavía desconocida y transportado a larga distancia en el xilema.

1. 6. 2. Transporte en el Xilema

Aunque en principio se propuso el malato como transportador de Fe(III) en el xilema (Tiffin y Brown, 1972), trabajos posteriores sugirieron que el Fe(III) se transporta unido a citrato (Tiffin 1966a,b; Chaney, 1989). Sin embargo, también se ha propuesto que el Fe(III) esté en el exudado de xilema en forma de complejos con otros ácidos orgánicos, como malato y malonato (Brown, 1963; Chaney et al., 1989), e incluso con otro tipo de sustancias orgánicas (Cataldo et al., 1988). El transporte de Fe(III) formando un complejo con el malato se vería favorecido cuando los niveles de citrato fueran bajos (Chaney et al., 1989). El tipo de complejo en el que se encuentra el Fe(III) podría variar con la edad de las plantas y también

en situaciones de estrés, ya que la composición y pH de la savia del xilema podría variar en dichos casos.

1. 6. 3. Transporte al Interior de las Hojas

Una de las características más llamativas de las hojas cloróticas de especies cultivadas en el campo es que la concentración de Fe total puede ser relativamente alta (Morales et al., 1998). Esta característica se ha denominado recientemente la "paradoja de la clorosis" (Römheld, 1997; Morales et al., 1998). Este hecho sugiere la existencia de un depósito de Fe foliar que sería fisiológicamente inactivo. Para explicar estos datos se ha propuesto que la deficiencia de Fe produce un aumento en el pH apoplástico de la hoja y la consiguiente inactivación del Fe (Mengel, 1995). Sin embargo, esta hipótesis no ha sido todavía comprobada.

Una vez que el Fe llega a las hojas debe ser reducido a Fe(II) para entrar en las células (Mengel y Geurtzen, 1986; Jolley et al., 1987; Kolesch et al., 1987b). Para la reducción del Fe(III) en la hoja se ha propuesto la existencia de un enzima FC-R en la membrana plasmática de las células del mesófilo de la hoja, que podría ser similar al presente en la membrana plasmática de la raíz (Brüggemann et al., 1993; de la Guardia y Alcántara, 1996; Rombolà et al., 1998; González-Vallejo et al., 1999a,b, Larbi, 1999). Esta reductasa podría funcionar con citrato férrico u otro quelato como fuente de Fe. Existen discrepancias sobre si la FC-R en hoja aumenta su actividad en condiciones de deficiencia de Fe (Lass et al., 1986; Brüggemann et al., 1993; de la Guardia y Alcántara, 1996; Rómbolà et al., 1997; González-Vallejo et al., 1999). Por otro lado, se ha propuesto que el citrato de Fe(III) podría ser fotoreducido *in vivo* en la hoja por luz ultravioleta y/o azul (Jolley et al., 1987), dado que la citada reacción se puede producir *in vitro* (Bienfait y Scheffers, 1992). En todo caso, la reducción de quelatos férricos en hoja está claramente estimulada por luz (Brüggemann et al., 1993; González-Vallejo et al., 2000).

1. 6. 4. Transporte al Cloroplasto

El cloroplasto es el destino final de una gran parte del Fe de la planta (Terry y Abadía, 1986). Recientemente se ha comenzado la caracterización del transporte de Fe hacia el interior del cloroplasto a través de la envoltura del mismo (Bughio et al., 1997a,b; Mori, 1998). Los datos disponibles hasta el momento indican que es un transporte activo y dependiente de luz. Estudios con cloroplastos aislados de plantas de cebada control sugieren que el transporte está mediado, entre otros mecanismos, por una reductasa de Fe(III) no dependiente ni de ATP ni de un flujo de H^+ (Bughio et al., 1997b). Este transporte es dependiente de pH, con un máximo a pH 8 y, a diferencia de lo que ocurre en raíz, no se inhibe por metales pesados en el medio.

Una vez que el Fe alcanza su destino final, si no va a ser utilizado, es necesario que sea acumulado en forma no tóxica y soluble y que posteriormente pueda ser liberado según las necesidades metabólicas de la planta. Esta función la realiza una proteína multimérica denominada ferritina (Briat, 1996; Briat y Lobréaux, 1997).

1. 6. 5. Transporte en el Floema

El Fe libre tiene una movilidad intermedia en el floema (Marschner, 1995), debido principalmente a su alta afinidad por el fosfato, el anión inorgánico más abundante en él y con el que forma fosfato férrico, altamente insoluble (Hill, 1980). El transporte de Fe en el floema se realiza en forma de uno o varios complejos de Fe (Grusak, 1995). Se ha propuesto que el Fe se transporte unido a un péptido de bajo peso molecular (2,4 kDa) (Maas et al., 1988; Marentes y Grusak, 1998), pero hasta el momento no se ha identificado dicho compuesto. Por otro lado, diversos autores (Stephan y Schölz, 1993; Hider et al., 1997) han propuesto que el Fe en el floema se transporte en forma de Fe(II) formando un complejo con nicotianamina.

1. 7. Regulación de las Respuestas ante la Deficiencia de Fe a Nivel de Planta

Las raíces por sí mismas son capaces de inducir las respuestas a la deficiencia de Fe (Bienfait, 1987; Kneen et al., 1990; Welch y La Rue, 1990). Recientemente se ha demostrado que la cantidad de Fe en el citoplasma de la raíz controla la síntesis o la actividad de distintos enzimas implicados en las respuestas fisiológicas a la deficiencia de Fe, así como en los cambios morfológicos (Wei et al., 1997). Sin embargo, experimentos de naturaleza muy diversa han demostrado que las respuestas a la deficiencia de Fe están también reguladas por señales que provienen de la parte aérea, a través del floema (Landsberg, 1982; Ao et al., 1985; Sijmons y Bienfait, 1986; Maas et al., 1988; Romera et al., 1992; Grusak, 1995; Briat y Lobréaux, 1997). No se conoce la naturaleza de esta señal o señales. Se ha propuesto que sea una proteína reguladora sensible a los niveles de Fe en la planta (Grusak y Pezeshgi, 1996), un complejo nicotianamina-Fe-proteína reguladora (Schölz et al., 1992), azúcares (Maas et al., 1988) y/o hormonas, como la auxina (Landsberg, 1982; Römheld y Marschner, 1986; Muday y Haworth, 1994) o el etileno (Romera y Alcántara, 1994; Romera et al., 1996a,b).

Por otro lado, tanto experimentos de aporte de Fe en hoja (Moog et al., 1995; Grusak y Pezeshgi, 1996), como la falta de correlación espacial y temporal encontrada en algunas especies en las respuestas de las raíces ante la deficiencia de Fe (Römheld y Kramer, 1983; Chaney y Bell, 1987; Grusak et al., 1989; Chaney et al., 1992; Cohen et al., 1997) han puesto de manifiesto la complejidad del sistema regulador, así como la posible existencia de distintos mecanismos reguladores para las respuestas de las raíces inducidas por deficiencia de Fe.

1. 8. Xilema

1. 8. 1. Estructura del Xilema

El xilema es el tejido a través del cual la savia asciende por el tallo en plantas superiores. En angiospermas, la savia se mueve primordialmente a través de vasos, elementos que tras su maduración pierden el citoplasma, desarrollan

paredes perforadas y se fusionan en hileras para formar conductos largos. Estos vasos pueden tener muchos metros de longitud y oscilan, dependiendo de la especie, entre 20 y 700 µm de diámetro. En gimnospermas, el elemento conductor es la traqueida. Las traqueidas son más cortas (<5 mm) y estrechas (< 30 µm) que los vasos, poseen paredes terminales adelgazadas que se superponen con las células contiguas y punteaduras reforzadas, tanto en las paredes laterales como en las terminales, a través de las cuales fluye el agua. Debido a que el citoplasma desaparece en ambos tipos de células, no están vivas y poseen paredes muy lignificadas y relativamente rígidas. Existen conexiones frecuentes entre las hileras adyacentes de las células a través de punteaduras en las paredes laterales, que permiten considerar al xilema como un sistema de conductos interrumpidos a intervalos frecuentes. Los vasos y las traqueidas se extienden desde las raíces hasta las hojas donde se ramifican repetidamente.

1. 8. 2. Transporte en el Xilema

Para explicar la ascensión de la savia a través del xilema está ampliamente aceptada la teoría de la cohesión, propuesta por Dixon en 1914. De acuerdo con esta teoría, cuyos aspectos más importantes se recogen en la Fig. 1. 7, la transpiración en las células del mesófilo de la hoja crea un gradiente de potencial hídrico negativo generando un movimiento de agua desde la raíz hasta la hoja, vía xilema. La transpiración provoca que el agua desaparezca de los extremos de los nervios foliares reduciendo el potencial hídrico en las células inmediatas a los vasos y provocando la salida de agua de éstos. Los vasos y las traqueidas del xilema, de diámetro estrecho, encierran columnas de agua continuas, en forma de hilos frecuentemente entrelazados, que se extienden desde los nervios foliares hasta el xilema de las raíces más pequeñas. La pérdida de agua en la parte superior de estos vasos crea una tensión en la columna de agua, cuya magnitud depende de la intensidad de transpiración, que se transmite a lo largo de la columna. Esta tensión provoca en el xilema, un movimiento de agua ascendente a lo largo del gradiente de presión, hacia la hoja y en última instancia hasta la atmósfera exterior. La presión negativa creada en la base del xilema en la raíz es de magnitud suficiente para provocar un flujo de agua desde el suelo hacia el interior de la raíz, a través del cortex. El ascenso de agua en el xilema no es exclusivamente un fenómeno de

capilaridad, que impondría una resistencia al movimiento incompatible con las velocidades y el flujo existentes en las plantas. El acoplamiento de un sistema capilar de evaporación de reducido espesor (mesófilo) a un sistema conductor de mayor diámetro (vasos y traqueidas) permite combinar los bajos potenciales hídricos generados en el mesófilo con una vía de transporte de baja resistencia. El funcionamiento de este mecanismo de transporte depende del desarrollo de potenciales hídricos lo suficientemente bajos en las hojas para provocar el ascenso de agua y de la estabilidad de las columnas de agua sometidas a tensión.

Las tensiones a que está sometida el agua en el xilema llegan a ser hasta de 2 MPa. Las fuerzas de cohesión entre las moléculas de agua son, sin embargo, suficientemente elevadas para soportar estas tensiones, siempre que se encuentren confinadas en el interior de tubos capilares. Las columnas sometidas a tensión son inestables ya que en estas condiciones el agua tiende a pasar a vapor formándose burbujas (embolia gaseosa), y estas burbujas pueden interrumpir la cohesión del agua en la columna impidiendo su transporte. Las fuerzas cohesivas en el xilema son operativas tanto vertical como horizontalmente. De este modo, si alguno de los elementos xilemáticos se bloquease por burbujas de aire o por corte en la continuidad vertical, el agua se movería lateralmente alrededor del obstáculo y continuaría a través de los elementos no lesionados. Las dos necesidades básicas de la planta, que son la existencia de una vía de transporte de agua de alta capacidad y baja resistencia, y la protección contra la interrupción del transporte provocada por el embolismo, están perfectamente conjugadas en el xilema.

El flujo a través de los vasos del xilema se puede analizar utilizando la ecuación de Hagen-Poiseville, que establece que el volumen del flujo de agua a través de un conducto es proporcional al cuadrado del radio:

$$J_v = r^2 (8\eta)^{-1} (\Delta\Psi)$$

siendo J_v el volumen del flujo de agua ($m^3\ m^{-2}\ s^{-1}$), η la viscosidad (Pa s), r el radio y $\Delta\Psi$ el gradiente de presión hidrostática (Pa). Se han encontrado valores próximos a los predichos por la ley de Hagen-Poiseville en vid y en árboles de leño poroso en anillo, como el roble, especies que poseen vasos de gran diámetro y continuos. Sin embargo, en plantas herbáceas y en árboles con leño poroso difuso, de diámetro de vasos marcadamente menor y de corta longitud, el paso de agua de un vaso al siguiente supone una resistencia adicional que hace que el movimiento del agua se

6. Changes Induced by Iron Deficiency in the Composition of the Leaf Apoplastic Fluid from Field-Grown Pear (*Pyrus communis* L.) Trees.

6. 1. Abstract

Experiments have been carried out with field-grown, pear leaves to investigate the effect of iron chlorosis on leaf apoplastic composition. Iron deficiency caused an increase in the apoplastic pH of pear leaves from the control values of 5.5-5.9 to 6.5-6.6, as judged from direct pH measurements in apoplastic fluid obtained by centrifugation and by fluorescence of leaves incubated with 5-carboxy fluorescein. The major organic acids found in leaf apoplastic fluid of iron-deficient and iron-sufficient pear leaves were malate, citrate and ascorbate. The total concentration of organic acids was 2.9 mM and increased to 5.5 mM in Fe-deficient leaves. The total apoplastic concentration of inorganic cations (Ca, K and Mg) increased from 15 to 20 mM. The total apoplastic concentration of inorganic anions (Cl^-, NO_3^-, SO_4^{2-} and HPO_4^{2-}) did not change with Fe deficiency. Iron concentrations in the apoplastic fluid decreased from 4 to 1.6 μM with Fe deficiency.

The major Fe species predicted by the chemical speciation software MinteqA2 for the apoplastic ionic environment was $FeCitOH^-$ in both Fe-sufficient and deficient apoplastic samples, indicating that citrate plays a major role in the long distance Fe transport. Organic acids in whole leaf homogenates increased from 20 to 40 nmol m^{-2} with Fe deficiency. The activities of several enzymes involved in organic acid metabolism (fumarase, isocitrate-dehydrogenase, aconitase and citrate synthase) increased in total leaf extracts with Fe deficiency, whereas the activity of phosphoenol pyruvate carboxylase did not change.

6. 1. 1. Abbreviations: CS, citrate synthase; DTNB, 5-5´dithio-bis-2-nitrobenzoic acid; FC-R, ferric-chelate reductase; FDH, formate dehydrogenase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MS, mass spectroscopy; PDC, pyruvate decarboxylase; PEPC, phosphoenol pyruvate carboxylase; PM, plasma membrane; PVDF, polyvinyl fluoride; SPAD, portable Chl meter; 5-CF, 5-carboxy fluorescein.

6. 2. Introduction

Iron deficiency is one of the major abiotic stresses affecting fruit tree crops in the Mediterranean area. One of the most obvious characteristics of the plants affected by Fe deficiency is that their leaves become chlorotic (Terry and Abadía, 1986; Abadía and Abadía, 1993). In a geographical region of great agricultural importance in northeastern Spain, such as the Ebro river basin, many fruit tree crops are affected by Fe chlorosis. Crops affected include peach, pear, apple, cherry, citrus, grape and plum (Sanz et al., 1992). Pear is one of the fruit tree species most affected by Fe chlorosis, that causes decreases in yield and shortens the productive lifetime of the orchards by 5-6 years (Sanz et al., 1992). In the Ebro river basin, approximately 67% of pear orchards (over 13200 ha) are currently being treated with Fe compounds to correct Fe chlorosis.

Iron chlorosis is caused by low Fe availability in calcareous, high pH soils (Lindsay and Schwab, 1982). Iron deficiency produces physiological and biochemical responses at the plant root level in dicotyledoneous plants. The

physiological responses include higher proton extrusion which decreases the pH of the rhizosphere (Brown, 1978), a release of reducing and/or chelating substances such phenolics and flavins (Welkie and Miller, 1960; Susín et al., 1996) and a two-step mechanism for Fe uptake in which Fe is first reduced by a plasma membrane (PM)-bound ferric-chelate reductase (FC-R) (Moog and Brüggemann, 1994; Susín et al., 1996; Robinson et al., 1999) and subsequently absorbed as Fe(II) (Chaney et al., 1972; Eide et al., 1996). Among the biochemical responses, dicotyledoneous plants accumulate organic acids, mainly citrate and malate, both in leaves (Iljin, 1951; Palmer et al., 1963; Landsberg, 1981) and roots (de Kock and Morrison, 1958; Brown, 1966; de Vos et al., 1986).

In the xylem, Fe is transported to the leaves as Fe(III), probably chelated by citrate (Tiffin, 1966; Brown and Chaney, 1971; White et al., 1981, Schmidt, 1999). The way Fe enters the leaf cell has been much less studied than the corresponding processes in the roots. Once in the leaf apoplast, Fe(III) is reduced before uptake by the leaf cell. Reduction of Fe-chelates are mediated by a PM-bound FC-R enzyme (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; González-Vallejo et al., 2000). Therefore, the composition of the apoplast is of crucial importance to understand the processes involved in the mechanism of Fe uptake by leaves. For instance, apoplastic pH could be important for the mobility of Fe and the activity of the leaf PM FC-R (Mengel, 1995; Schmidt, 1999; Kösegarten et al., 1999). No report has been made so far, to our knowledge, on the effects of Fe deficiency on the composition of the leaf apoplast. Fe trafficking in the apoplast is also mandatory for Fe uptake by the root cells (Longnecker and Welch, 1990; Zhang et al., 1991).

The leaf apoplast is involved in transmission of signals (Hartung et al., 1992), transport and storage of mineral nutrients (Starrach and Mayer, 1989; Wolf et al., 1990; Zhang et al., 1991), trace gas exchange with the atmosphere (Kesselmeier, 1998; Gabriel et al., 1999) and different responses to plant environmental stresses (Polle et al., 1990; Pfanz et al., 1991; Dietz et al, 1997). Studies have been made on the composition of the apoplast under different environmental conditions (Clarkson, 1984; Blatt, 1985; Bowling, 1987; Speer and Kaiser, 1991; Grignon and Sentenac, 1991; Tetlow and Farrar, 1993; Canny, 1995). The apoplast contains significant concentrations of inorganic and organic

anions (Petersen and Böttger,1991; Hedrich et al., 1994; Schurr and Schulze, 1995; Dietz et al., 1997).

The aim of this work was to investigate the effects of Fe deficiency on the composition of the leaf apoplast in pear trees grown in the field, in order to understand the role of this compartment in the transport and acquisition of Fe by leaf cells. We have investigated the changes induced by Fe deficiency on the concentrations of different inorganic and organic ions in pear leaf apoplast. Chemical speciation has been carried out with the MinteqA2 software. The activities of different enzymes, including PEPC and several TCA-cycle enzymes, and the concentrations of pyridine nucleotides and organic anions have been measured in leaf extracts of Fe-deficient and control leaves.

6. 3. Materials and Methods

6. 3. 1. Plant Material

Leaves were sampled from pear trees (*Pyrus communis* L.) growing in calcareous soils and affected by iron chlorosis. The orchard was located in El Temple (Huesca) in the Ebro basin in north-eastern Spain. The soil has a clay-loamy texture, with 32% total calcium carbonate, 12.6% active lime, 1.89% organic matter and pH in water 8.4. The pear cultivar was 'Blanquilla' grafted on quince 'BA29'. The trees were 17 years old, trained as palmette, with a frame of 3x4 m. The orchard had not been treated with Fe-chelates in the last two or three years. Under these conditions chlorosis became more marked in the orchards studied every year. In 1997 growing season there were trees with marked chlorosis symptoms (average leaf chlorophyll 200 μmol m^{-2}) and other trees that still remained green (average leaf chlorophyll 600 μmol m^{-2}). Samplings were conducted during the summer of 1997. Young, fully-expanded leaves, showing no interveinal chlorosis and almost homogeneous colour throughout the leaf, were chosen for all measurements.

6. 3. 2. Chlorophyll Determination

Chlorophyll concentration was estimated non-destructively with a SPAD-502 device (Minolta, Osaka, Japan). For calibration, leaf disks with different degrees of Fe deficiency were first measured with the SPAD, then extracted with 100% acetone in presence of Na ascorbate and chlorophyll measured spectrophotometrically (Abadía and Abadía, 1993).

6. 3. 3. Apoplastic Fluid Isolation

Apoplastic fluid was obtained from whole pear leaves by direct centrifugation as in Dannel et al. (1995) with some modifications. Leaves were excised at the base of the leaf lamina with a razor blade in the field and transported to the laboratory with the petiole immersed in de-ionized water. Once in the laboratory the petiole was excised under water. Five leaves were then rolled and placed into a plastic syringe barrel, with the petiole side at the narrow end of the syringe. Leaf-filled syringes were centrifuged at 4°C. A small volume of apoplastic fluid was obtained from the bottom of the centrifuge tube. A first centrifugation was made at low speed (2500*g*, 15 min) to remove the xylem sap of the main vein. Apoplastic fluid was obtained after a second centrifugation for 15 min at 4000*g* and 4°C.

Malate dehydrogenase (c-mdh; EC 1.1.1.37) was used as a cytosolic contamination marker for apoplastic fluid. The activity of c-mdh was determined using oxalacetate as substrate and measuring the decrease in A_{340} due to the enzymatic oxidation of NADH. The final reaction mixture (pH 9.5) was 46.5 mM Tris, 0.1 mM NADH and 0.4 mM oxalacetate (Dannel et al., 1995). The activity of the marker in leaf apoplast was related to the total activity in whole leaf homogenates. To measure enzymatic activities in total leaf homogenates 3 leaf disks of 0.95 cm^{-2} were homogenised with 2 mL of buffer (pH 8.0) containing 100 mM Hepes, 30 mM sorbitol, 2 mM DTT, 1 mM $CaCl_2$, 1% BSA and 1% PVP. The supernatant was collected and analysed immediately after 10 min centrifugation at 10000*g*.

6. 3. 4. pH Measurements

The pH of the apoplastic fluid was measured directly in the fluid obtained by centrifugation with a microelectrode (Physitemp, USA). Apoplastic pH was also measured *in vivo* by fluorescence according to Hoffman et al. (1992) with 5-carboxyfluorescein (5-CF). Fluorescence emitted by 5-CF at 540 nm is pH-dependent when excited at 490 nm but nearly pH-independent when excited at 450 nm. Therefore, the ratio of fluorescence intensities obtained with excitation at 490 and 450 nm is an estimate of the pH of the compartment where the dye is located. Leaves were excised and the cut end of the petiole was exposed to incubation medium containing 5 µM 5-CF, 1 mM KCl, 0.1 mM NaCl and 0.1 mM $CaCl_2$ at pH 5.5. The incubation was carried out for 5 h at room ambient light and temperature (15-25 µmol photons m^{-2} s^{-1}, 20-25°C). The level of auto-fluorescence was subtracted from total fluorescence. Three leaves per Chl level were taken and four measurements were carried out in different areas of each leaf.

6. 3. 5. Inorganic Ion Determination

Ca and Mg were determined by atomic absorption spectrophotometry and K by emission spectrophotometry. Fe was determined in the apoplastic fluid by atomic absorption spectrometry with a graphite furnace (Varian SpectrAA with Zeeman correction). Each sample was analysed in triplicate.

Inorganic anions (NO_3^-, SO_4^{2-}, Cl^- and HPO_4^{2-}) were quantified by HPLC with a 4.6 x 75 mm IC-Pak A HR ion-exchange column (Waters, Milford, MA, USA) in a HPLC Waters system, including a 600E pump, a 432 conductivity detector and Millennium 2010 software. Samples were injected with a Rheodyne injector (50 µL loop). Mobile phase (11 mM borate-gluconate) was pumped with a 1.0 mL min^{-1} flow rate. Quantification was made with known amounts of each anion using peak areas.

6. 3. 6. Organic Ion Analysis

Leaf samples (3 leaf disks of 0.95 cm^2 taken with a calibrated cork borer) were frozen in liquid N_2 and ground in a mortar with 8 mM sulphuric acid. Homogenates were boiled for 30 min, centrifuged 10 min at 10000*g*, filtered with a 0.2 µm PVDF filter (LIDA, Kenosha, WI, USA), taken to a final volume of 2 mL with 8 mM sulphuric acid and kept at -80°C until analysis. Apoplast samples were filtered (0.45 µm PVDF, LIDA) before HPLC analysis.

Organic anions were analysed by HPLC with a 300 x 7.8 mm Aminex ion-exchange column (HPX-87H from Bio-Rad, Hercules, CA, USA) with a HPLC system (Waters, Milford, MA, USA), including a 600E pump, a 996 photodiode array detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (8 mM sulphuric acid) was pumped with a 0.6 mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, citrate, 2-oxoglutarate, ascorbate, malate, shikimate and fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma (Fig. 6.1). The peak with retention time of 12 min was composed of succinate and an unidentified compound with maxima at 210 and 262 nm. Because of this reason, succinate was not quantified. Another peak with a retention time of approximately 14 min, with absorption maxima at 219 and 281 nm, was tentatively identified as p-coumaric acid. The identity of some peaks was further confirmed by UV-VIS and MS. Quantification was made with known amounts of each anion using peak areas.

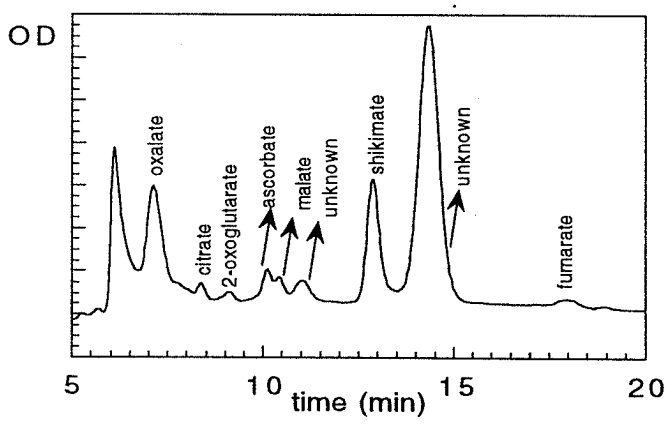

Figure 6. 1. Separation of organic anions by ion exchange high pressure liquid chromatography. Organic anions were detected at 210 nm.

6. 3. 7. Sugar Analysis

Sugars (glucose, fructose and sucrose) were analysed by HPLC with a 300 x 4 mm Spherisorb-NH_2 column (Waters) and a HPLC system (Waters), including a 590 pump, a differential refractometer R401 detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (acetonitrile: water 860:140) was pumped with a 3.5 mL min^{-1} flow rate. Peaks corresponding to glucose, fructose and sucrose were identified by comparison of their retention times with those of known standards from Sigma. Quantification was related to peak areas of known amounts of the standards.

6. 3. 8. Enzyme Assays

Extracts for measuring enzyme activities were made by grinding 3 leaf disks of 0.95 cm^2 each in a mortar with 2 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10000*g* and 4°C, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 1 mL final volume, of the media indicated below.

Malate dehydrogenase (MDH; EC 1.1.1.37) was determined as described above. Citrate synthase (CS; EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1967) by monitoring the reduction of acetyl CoA to CoA with 5-5'-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. The reaction was carried out with 50 µL of extract in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalacetate and 100 mM Tris-HCl, pH 8.1. Aconitase (EC 4.2.1.3) was measured from the formation of cis-aconitate, monitored at 240 nm (Bacon et al., 1961) with 60 µL of extract in 500 mM sucrose, 50 mM isocitrate and 100 mM Tris-HCl (pH 8.5). Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was determined with 50 µL of extract by monitoring the reduction of $NADP^+$ at 340 nm in a reaction mixture containing 3.5 mM $MgCl_2$, 0.41 mM $NADP^+$, 0.55 mM isocitrate and 88 mM imidazole at pH 8.0 (Bergmeyer et al., 1974). Fumarase (EC 4.2.1.2) was assayed with 50 µL of extract following the increase in optical density at 240 nm due to the formation of fumarate (Bergmeyer et al., 1974). The reaction buffer was 50 mM malate and 100 mM phosphate, pH 7.4.

Phosphoenol pyruvate carboxilase (PEPC; EC 4.1.1.31) was measured in a coupled enzymatic assay with MDH according to Vance et al. (1983) with 75 µL of extract in 2 mM phosphoenol pyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH, and 100 mM bicine-HCl, pH 8.5. For the determination of lactate dehydrogenase (LDH; EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1) the oxidation of NADH was monitored at 340 nm with 50 µL of extract. LDH was assayed in a reaction buffer containing 94.5 mM phosphate buffer (pH 9.5), 0.77 mM pyruvate and 0.2 mM NADH. PDC was determined in 190 mM citrate-KOH buffer (pH 6.0), 30 mM pyruvate, 0.32 mM NADH and 33 µg mL^{-1} alcohol dehydrogenase.

6. 3. 9. Nucleotide Analysis

Pyridine nucleotides were extracted from liquid N_2-frozen leaf disks (0.95 cm^2) in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA (for $NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12000 g for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 100 mM bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method of Matsumura and Miyachi (1980).

6. 3. 10. Chemical Speciation

Concentrations of the different Fe-chelate species were estimated with the software MinteqA2 (Allison et al., 1991) by using the ionic environment of the apoplastic fluid. Chelate formation constants used for citrate and malate were derived from those given by Holden et al. (1991) and Cline et al. (1982), respectively. At an ionic strength of 0 M, the $\log_{10}$ of the chelate formation constants used for the Fe-citrate species $[FeCit]^0$, $[FeCitH]^{+1}$, $[FeCitOH]^{-1}$, $[FeCit_2]^{-3}$ and $[Fe_2Cit_2(OH)_2]^{-2}$ were 13.13, 14.43, 10.11, 20.13 and 24.51, respectively. The $\log_{10}$ of the chelate formation constants for the Fe-malate species $[FeMal]^{+1}$, $[Fe_2(MalOH)_2]^0$, $[Fe_2Mal(MalOH)_2]^{-2}$ and $[Fe_3Mal_5OH_4]^{-5}$ were 8.39, 15.32, 20.33 and 27.75, respectively.

1. 9. 3. Sistemas de Aislamiento

Una de las principales razones del escaso conocimiento de los procesos que tienen lugar en el apoplasto es la limitación en los métodos disponibles para su aislamiento. Tradicionalmente, las muestras de apoplasto se han obtenido utilizando dos métodos. El primero consiste en colocar trozos pequeños de hojas o fragmentos más grandes sin epidermis (Pfanz y Dietz, 1987) en una solución. El análisis posterior de esta solución permite conocer la composición del apoplasto. El principal problema de este método es la gran contaminación con material citoplasmático debida a la rotura celular y la dilución de los componentes del apoplasto en la solución, que en muchas ocasiones hace difícil su cuantificación. El segundo método es una modificación de la cámara de presión de Schölander (Jachetta et al., 1986; Hartung et al., 1992). La aplicación de pequeñas presiones a los tejidos permite el aislamiento de fluido apoplástico puro, pero el volumen obtenido es muy pequeño para realizar cualquier análisis y en función de la presión aplicada puede presentar gran contaminación citoplasmática.

En la actualidad, los métodos más ampliamente utilizados son los de infiltración de los tejidos con agua o una solución tamponada y posterior centrifugación de los mismos (Terry y Bonner, 1980; Rohringer et al., 1983; Pfanz y Oppmann, 1991; Pinedo et al., 1993; Husted y Schjoerring, 1995). Estos métodos permiten la obtención de volúmenes mayores, pero el uso de sales y tampones en las soluciones de infiltración alteran las condiciones del apoplasto, variando a veces hasta el pH. Una modificación de estos métodos consiste en la centrifugación directa de los tejidos (Dannel et al., 1995). Así, se llega a un aislamiento rápido y a la obtención de suficiente volumen para realizar los análisis necesarios, sin el problema de los efectos de dilución.

En los últimos años se vienen desarrollando métodos no destructivos que nos permiten conocer la composición del apoplasto como, el microanálisis con rayos X (Pichakashi-Maunsbach y Harvey, 1992), la utilización de electrodos selectivos (Blatt, 1985) y de colorantes selectivos a iones (Bright et al., 1989; Hoffmann y Kosegarten, 1995; Mühling et al., 1995; Mühling y Sattelmacher, 1997; Mühling et al., 1998). El uso de microelectrodos selectivos requiere el uso de micromanipuladores muy complicados y además sólo permite el acceso al apoplasto de zonas muy específicas (Felle, 1993). Los colorantes selectivos tienen

gran resolución temporal y espacial y además alteran muy poco las condiciones del apoplasto. La gran ventaja de estos métodos es que permiten conocer los gradientes de concentración que existen en el apoplasto.

1. 9. 4. pH Apoplástico

El pH apoplástico tiene gran importancia en la síntesis de la pared celular (Jacobs y Ray, 1976), en la regulación de la absorción de carbohidratos y su transporte al floema (Giaquinta, 1977; Tetlow y Farrar, 1993). Asimismo, el papel del pH apoplástico en condiciones de deficiencia de Fe se pone de manifiesto en experimentos en los que se aplican ácidos diluídos en las hojas o reactivos que estimulan la bomba de protones (Mengel y Geurtzen, 1988) y que consiguèn un re-enverdecimiento de las hojas.

Los valores de pH apoplástico de raíz se han obtenido por medio de indicadores en agar o con microelectrodos localizados a pocos µm de la epidermis. En órganos aéreos se han utilizado principalmente 4 tipos de métodos: 1) Medida directa del pH en los fluidos obtenidos por presión con cámara de Schlölander, por centrifugación del tejido o en soluciones que bañan las hojas una vez eliminada la epidermis; 2) Mediante electrodos selectivos colocados en pequeños agujeros en los tejidos o en la superficie; en algunos casos los microelectrodos se pueden colocar directamente en el apoplasto de algunas células; 3) Midiendo los cambios espectrales de sondas fluorescentes infiltradas en el apoplasto e incapaces de cruzar la membrana plasmática (Pfanz y Dietz, 1987; Hoffmann et al., 1992); 4) Incubando los tejidos en soluciones con distinto pH y determinado el pH de equilibrio en el que no hay flujo neto de protones.

Los valores más bajos descritos se encuentran alrededor de 4 y los más altos sobre 7, pero la mayoría se encuentran entre 5 y 6,5. En general los valores son mayores en dicotiledóneas que en monocotiledóneas, y mayores en angiospermas que en gimnospermas (Pfanz y Dietz, 1987).

Las paredes celulares tienen gran capacidad para amortiguar los cambios de pH. Sin embargo, el pH apoplástico puede variar dependiendo de muchos factores. Por ejemplo, la actividad fotosintética estimula la extrusión de protones

al apoplasto causando una acidificación del mismo. Asimismo, los tejidos en crecimiento tienen pH más bajos. Entre los factores que causan una alcalinización del apoplasto se encuentran el estrés hídrico y el tipo de nutrición de N. Se ha comprobado que el pH apoplástico aumenta si la forma de nitrógeno aplicada es nitrato (Kosegarten y Englisch, 1994; Mengel et al., 1994). Finalmente, existen grandes diferencias entre los valores de pH descritos dependiendo del tipo de tejido y del tipo de célula.

1. 9. 5. Solutos en el Apoplasto

Las concentraciones en el apoplasto de raíz vienen determinadas principalmente por el medio en el que se encuentran y por las características electrostáticas de las cargas de la pared celular, así como por las características del transporte de la membrana plasmática. En el apoplasto de órganos aéreos, además de los factores anteriores, las concentraciones de solutos están determinadas por el balance entre la entrada vía xilema, absorción por la célula y la salida al floema.

El contenido en agua del apoplasto medido por centrifugación varía de 1 a 6 mL g^{-1} PS, aunque la mayoría de valores se encuentran entre 1-2 mL g^{-1} PS. En general la concentración de solutos es menor en el apoplasto que en la savia. Los electrolitos inorgánicos suponen el 25% de los solutos apoplásticos, la mayoría son sales de K^+. Los aniones y cationes predominantes en el apoplasto son K^+, Ca^{2+}, Mg^{2+}, Cl^-, NO^{3-} y PO_4^{3-}. Existe gran variabilidad en la literatura respecto a las concentraciones iónicas en el apoplasto. Por ejemplo, los valores de K^+ descritos en la bibliografía se encuentran entre 50 µM (Blatt, 1985) y 100 mM (Long y Widders, 1990). Esta variabilidad es debida principalmente a la existencia de gradientes de concentración espaciales y temporales (Wilson et al., 1991; Mühling y Sattelmacher, 1997), a las diferentes especies usadas, al método de extracción y diferente edad y estado fisiológico de las plantas. En general la concentración apoplástica de K^+ es un reflejo de la absorción de K^+ por las raíces (Mühling y Sattelmacher, 1997), mientras que la de Ca^{2+} es más estable (De Silva et al., 1998).

En el apoplasto también se han descrito altas concentraciones de metabolitos orgánicos como aminoácidos, péptidos (Clark et al., 1986), ácidos orgánicos como citrato, malato y ascorbato (Mullins et al., 1986; Polle et al., 1990; Luwe et al., 1993), azúcares (Tetlow y Farrar, 1993) y ácidos volátiles como acético y fórmico. La concentración apoplástica de ácidos volátiles está determinada por el intercambio de gases con la atmósfera, y la de ácidos orgánicos oscila entre 0,5 y 2 mM. Los azúcares mayoritarios en apoplasto son sacarosa, glucosa y sorbitol (Moing et al., 1997) con concentraciones entre 25 y 45 mM.

CAPÍTULO 2

OBJETIVOS

2. Objetivos

1- Caracterizar los cambios bioquímicos que se producen en condiciones de deficiencia de Fe en las puntas de raíz de las plantas pertenecientes a la estrategia I, utilizando como especie modelo remolacha (*Beta vulgaris* L.).

2- Estudiar los efectos de la deficiencia de Fe sobre el transporte de Fe a larga distancia en la planta modelo remolacha (*Beta vulgaris* L.) y en peral (*Pyrus communis* L.): cambios en la composición de la savia y del fluido apoplástico de hoja con la deficiencia de Fe.

3- Determinar los cambios bioquímicos producidos por la deficiencia de Fe en las hojas de las especies de plantas pertenecientes a la estrategia I: remolacha (*Beta vulgaris* L.), tomate (*Lycopersicon esculentum L.*) y peral (*Pyrus communis* L.).

CAPÍTULO 3

MECANISMOS PROTECTORES EN LAS RAÍCES DE REMOLACHA DEFICIENTE EN Fe. CAMBIOS EN LA ASIMILACIÓN DE CARBONO Y EN EL USO DE OXÍGENO

3. Protective Mechanisms in Roots of Fe Deficient Sugar Beet: Changes in Carbon Assimilation and Oxygen Use

3. Protective Mechanisms in Roots of Iron Deficient Sugar Beet: Changes in Carbon Assimilation and Oxygen Use

3. 1. Abstract

Different root parts with or without increased Fe-reducing activities have been studied in Fe-deficient and Fe-sufficient control sugar beet plants. The distal root parts of the Fe-deficient plants, 0 to 5 mm from the root apex, were capable to reduce Fe(III)-chelates and contained concentrations of flavin sulfates near 700 µM, two characteristics that were absent in the 5-10 mm root sections of Fe-deficient plants and the whole root of Fe-sufficient plants. These flavin-containing root tips had large pools of carboxylic acids and high activities of different enzymes involved in organic acid metabolism. The redox state of the mitochondrial quinone pool was slightly more reduced in the Fe-deficient roots than in the controls. The nucleotide pool of these roots was, however, largely oxidised in Fe-deficient root tips when compared to the controls. We conclude that in Fe-deficient yellow root tips there is a large increase in carbon fixation, mediated by an increase in phosphoenol pyruvate carboxylase activity. Part of this carbon was used, through an increase

in mitochondrial activity, to increase the capacity to produce reducing power, whereas another part was exported to the shoot via xylem. Root respiration was markedly increased by iron deficiency. Based on the experimental results we propose a new metabolic model for the functioning of sugar beet roots under Fe deficiency. In sugar beet Fe-deficient roots flavin sulfates would provide a suitable link between the increased capacity to produce reduced nucleotides and the plasma membrane associated ferric chelate reductase enzyme(s). The Fe-deficient sugar beet root distal parts had a large residual oxygen consumption in the presence of cyanide and hydroxi-salycilic acid, suggesting that the ferric chelate reductase enzyme would be able to reduce external oxygen in the absence of Fe(III)-chelates.

3. 1. 1. Abbreviations: ADH, alcohol dehydrogenase; BPDS, bathophenantroline disulfonate; BSA, bovine serum albumin; CA, carbonic anydrase; CS, citrate synthase; DTNB, 5-5´-dithio-bis-2-nitrobenzoic acid; FC-R, ferric-chelate reductase; FDH, formate dehydrogenase; FMN, riboflavin 3-phosphate; G3PDH, glyceraldehide 3 phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MS, mass spectroscopy; PDC, pyruvate decarboxylase; PEP, phosphoenol pyruvate; PEPC, phosphoenol pyruvate carboxylase; PK, pyruvate kinase; PM, plasma membrane; PVDF, polyvinyl fluoride; PVP, polyvinylpirrolydone; Q, quinone; Rbf, riboflavin; SI, riboflavin 3'-sulphate; SII, riboflavin 5'-sulphate; SHAM, hydroxi salicilyc acid; SPAD, portable Chl meter.

3. 2. Introduction

Iron deficiency is a widespread agricultural problem in many crops grown in alkaline, calcareous soils. Iron in these soils, although abundant, is often not soluble and therefore is unavailable for the roots (Lindsay and Schwab, 1982). Based on the mechanisms of Fe uptake and on the physiological response to Fe deficiency, plants can be classified into two groups: (i) Strategy I plants, which include dicotyledonous and non-*Graminaceae* monocotyledonous species and (ii)

Strategy II plants, which include *Graminaceae* species (Marschner et al., 1986). When grown under a limited Fe supply both Strategy I and II plants increase their capacity for Fe uptake. Strategy II plants respond by an increased synthesis and secretion of phytosiderophores to the rhizosphere (Marschner and Römheld, 1994). Strategy I plants develop morphological and biochemical changes. Among the morphological changes there is an increased formation of lateral roots, root hairs and transfer cells. All these changes increase the root surface available for Fe uptake (Kramer et al., 1980; Landsberg, 1982). Strategy I also includes a higher proton extrusion capacity, which decreases the pH of the rhizosphere (Brown, 1978), a release of reducing and/or chelating substances such as phenolics and flavins (Welkie and Miller, 1960; Susín et al., 1994) and the development of a two step mechanism for Fe uptake. In this mechanism Fe is first reduced by a plasma membrane-bound ferric-chelate reductase enzyme (Moog and Brüggemann, 1994; Susín et al., 1996; Robinson et al., 1999; Schmidt, 1999) and subsequently absorbed as Fe(II) (Chaney et al., 1972) by a specialised transporter (Eide et al., 1996).

When grown under Fe deficiency, Strategy I plants accumulate organic acids, mainly citrate and malate, both in leaves (Iljin, 1951; de Kock and Morrison, 1958; Palmer et al., 1963; Landsberg, 1981) and roots (Brown, 1966; de Vos et al., 1986; Venkat-Raju et al., 1972; van Egmond and Atkas., 1977). The role of these organic acids in the Fe deficiency responses is not well established (Schmidt, 1999), although is commonly accepted that citrate could play an important role in the translocation of Fe in roots (Tiffin, 1966; White et al., 1981a) and in Fe transport via xylem to the mesophyll cells (Brown, 1966; Brown et al., 1971).

Two hypotheses have been put forward so far to explain the accumulation of organic acids in Fe-deficient roots. Landsberg (1986) reported that organic acid increases coincided with the enhanced proton extrusion found in Fe-deficient roots. This may occur through cytoplasm alkalinization associated to proton efflux, which could activate phosphoenol pyruvate carboxilase (PEPC) activity (Rabotti et al., 1995, and references therein). The second hypothesis (de Vos et al., 1986) suggested that Fe deficiency causes an alteration in the glycolytic pathway, as reported in fungi (Habison et al., 1979). Under Fe deficiency, phosphofructokinase would lose its regulation by citrate and pyruvate kinase would be inhibited by citrate, causing an accumulation of PEP that in turn, via PEPC activity, would

cause increases in organic acid contents. The cytoplasm acidification produced by the increases in organic acids would be responsible for H^+ extrusion. An increased PEPC activity may lead to organic acid accumulation to maintain the ionic balance of the root cell cytoplasm (pH-stat theory; Davies, 1973). Organic acid concentrations, however, also increased in Fe-deficient plants in which proton extrusion was not increased (Landsberg, 1981). Miller et al. (1990) reported increases in CO_2 fixation, organic acid contents and PEPC activity in roots of Fe-deficient plants, and suggested that the increased PEPC activity may feed the TCA cycle via malate, thus bypassing the key control point at pyruvate kinase (PK) (Lance and Rustin, 1984).

The aim of this work was to investigate the organic acid metabolism in Fe-deficient plants to further understand the biochemical responses of plants to Fe deficiency. We have measured organic acid concentrations and the enzymatic activities of PEPC, CA, G6PDH, several enzymes involved in organic acid metabolism and LDH and PDC, two enzymes related to anaerobic metabolism, in root tips of sugar beets affected by Fe deficiency. The redox poise of the pyridine nucleotide and mitochondrial quinone pools and the ATP levels were also determined. The possible relationships between the accumulation of organic acids and root tip O_2 consumption were also investigated. Based on the experimental results found we propose a new metabolic model that provides a comprehensive scheme of the functioning of sugar beet root cells under Fe deficiency.

3. 3. Materials and Methods

3. 3. 1. Plant Material

Sugar beet (*Beta vulgaris* L. Monohil hybrid from Hilleshög, Landskröna, Sweden) was grown in a growth chamber with a PPFD of 350 µmol m^{-2} s^{-1} PAR at a temperature of 25°C, 80% relative humidity and a photoperiod of 16 h light/ 8 h dark. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for two more weeks in half-strength Hoagland nutrient solution with 45 µM Fe and then transplanted to 20 L plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution (Terry, 1980) with either 0 or

45 μM Fe. The pH of the Fe-free nutrient solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$. Root tips from plants grown for ten days in the presence or absence of Fe were used in all experiments.

Root sections were taken approximately 0-5 and 5-10 mm from the root apex with a surgical blade. The 0-5 mm section from the zero-Fe treatment had root hairs (Fig. 3.1B) and was yellow due to the presence of flavins (Susín et al., 1993), whereas the 5-10 mm section in the zero-Fe treatment (Fig. 3.1A) and both the 0-5 and 5-10 mm sections in the control treatments (Fig. 3.1D,C) had practically no root hairs and were white.

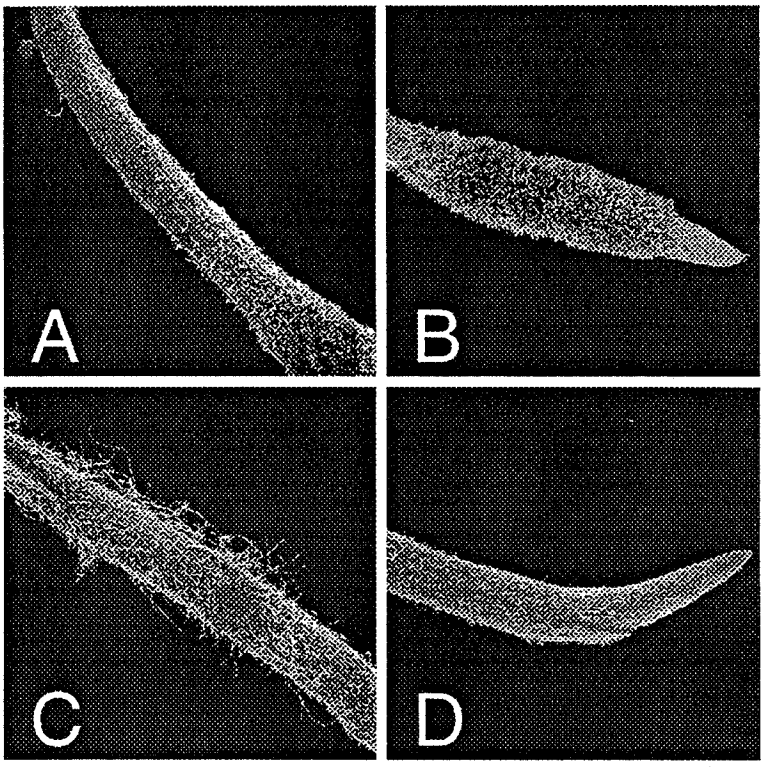

Figure 3. 1. Scanning electron micrography showing the root distal segments of Fe-deficient plants 5 to 10 mm from the apex (A), 0 to 5 mm from the apex (B) and Fe-sufficient sugar beet plants (C) 5 to 10 mm from the apex and (D) 0 to 5 mm from the apex.

3. 3. 2. Organic Anion Analysis

Root material (100 mg FW) was frozen in liquid N_2 and ground in a mortar with 8 mM sulphuric acid. Homogenates were boiled for 30 min, filtered with a 0.2 µM PVDF filter (LIDA, Kenosha, WI, USA), taken to a final volume of 2 mL with 8 mM sulphuric acid and kept at -80°C until analysis.

Organic anions were analysed by HPLC with a 300 x 7.8 mm Aminex ion-exchange column (HPX-87H from Bio-Rad, Hercules, CA, USA) with a HPLC Waters system, including a 600E multisolvent delivery system, a 996 photodiode array detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (8 mM sulphuric acid) was pumped with a 0.6 mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, cis-aconitate, citrate, 2-oxoglutarate, ascorbate, malate, succinate and fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma (Fig. 3.2A). Identification was confirmed by the UV peak spectra and/or HPLC-MS (Waters) (in this latter case formic acid was used as mobile phase instead of sulphuric acid). Quantification was made with known amounts of each organic anion using peak areas

3. 3. 3. Enzyme Assays

Extracts for measuring enzyme activities were made by grinding 100 mg FW of root material in a mortar with 1 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10000 g and 4°C, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 1 mL (final volume) of the media indicated below.

Malate dehydrogenase (MDH; EC 1.1.1.37) was determined with oxalacetate as substrate (Dannel et al., 1995) by measuring the decrease in A_{340} nm due to the enzymatic oxidation of NADH. The reaction was carried out with 5 µL of extract in 0.1 mM NADH, 0.4 mM oxalacetate and 46.5 mM Tris-HCl, pH 9.5. Citrate synthase (CS; EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1967) by monitoring the reduction of acetyl CoA to CoA with 5-

5'-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. The reaction was carried out with 50 µL of extract in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalacetate and 100 mM Tris-HCl, pH 8.1. Aconitase (EC 4.2.1.3) was measured from the formation of cis-aconitate, monitored at 240 nm (Bacon et al., 1961) with 60 µL of extract in 500 mM sucrose, 50 mM isocitrate and 100 mM Tris-HCl (pH 8.5). Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was determined with 50 µL of extract by monitoring the reduction of $NADP^+$ at 340 nm in a reaction mixture containing 3.5 mM $MgCl_2$, 0.41 mM $NADP^+$, 0.55 mM isocitrate and 88 mM imidazole, pH 8.0 (Bergmeyer et al., 1974). Fumarase (EC 4.2.1.2) was assayed with 50 µL of extract following the increase in A_{240} due to the formation of fumarate (Bergmeyer et al., 1974). The reaction buffer was 50 mM malate and 100 mM phosphate, pH 7.4.

Phosphoenol pyruvate carboxilase (PEPC; EC 4.1.1.31) was measured in a coupled enzymatic assay with MDH according to Vance et al. (1983) with 75 µL of extract in 2 mM phosphoenol-pyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH and 100 mM bicine-HCl, pH 8.5. The effect of malate on the PEPC activity in root extracts was assayed in 1 mL of a reaction mixture containing 75 µL of extract in 2 mM PEP, 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH, 50 mM HEPES, pH 7.3 and concentrations of malate from 50 µM to 5 mM. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was determined with D-glucose-6-phosphate as substrate (Bergmeyer et al., 1974) by measuring the increase in A_{340} due to the enzymatic reduction of $NADP^+$. The reaction was carried out with 50 µL of extract in 138 mM $MgCl_2$, 20 mM glucose-6-phosphate, 7.8 mM $NADP^+$ and 100 mM HEPES, pH 7.6. For the determination of lactate dehydrogenase (LDH; EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1) the oxidation of NADH was monitored at 340 nm with 50 µL of extract. LDH was assayed in a reaction buffer containing 94.5 mM phosphate buffer (pH 9.5), 0.77 mM pyruvate and 0.2 mM NADH. PDC was determined in 190 mM citrate-KOH buffer (pH 6.0), 30 mM pyruvate, 0.32 mM NADH and 33 µg mL^{-1} alcohol dehydrogenase.

Extracts for measuring carbonic anhydrase (CA; EC 4.2.11) were made by grinding 200 mg FW of root material in 1.5 mL of 100 mM TRIS, 10 mM mercaptoethanol and 1 mM EDTA, pH 8.3. The extract was stirred for 15 min at room temperature, centrifuged at 1000*g* for 5 min and the supernatant stored on ice until assayed. The Wilbur-Anderson electrometric method was used to assay

CA activity (Wilbur and Anderson, 1948). One mL of extract was added to 3 mL of 25 mM veronal (barbitone, 5-5-diethyl barbituric acid), pH 8.2. Four mL of CO_2-saturated water were added and the time taken for the pH to change from 8.2 to 7.0 was measured. Blanks were run using 1 mL of extract buffer.

3. 3. 4. Nucleotide Analysis

Pyridine nucleotides were extracted from liquid N_2-frozen root material (approximately 30 mg FW) in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA (for $NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12000*g* for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 100 mM bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method of Matsumura and Miyachi (1980).

ATP was extracted by grinding 100 mg FW of root material in a mortar with 1 mL of 2 mM EDTA and 100 mM Tris-acetate buffer, pH 7.75. The extract was centrifuged at 10000*g* for 15 min at 4°C. The supernatant was mixed with dimethyl sulfoxide (1:9, v:v), and ATP was measured with a luminometer (Labsystems Luminoskan, Life Sciences International, Finland) using an ATP monitoring kit (Bio Orbit Oy, Finland).

3. 3. 5. Analysis of Q-pool Redox Poise

The extraction of quinones (Q) from root tips was conducted according to Millar et al. (1998). Root material (approximately 1 g FW) was immersed in liquid N_2 and crushed to a fine powder with mortar and pestle. The powder was freeze-dried to remove the root aqueous phase and decrease the possibility of Q oxidation during organic extraction. Dried samples were vortexed for 3 min in a mixture of 1.5 mL of methanol (containing 200 mM perchloric acid) and 1.5 mL of petroleum ether (35-50°C boiling point, d 0.64). After centrifugation for 3 min at 1000*g* to separate the two phases, the upper phase was collected. Additional petroleum ether was added to the lower phase, and the procedure was repeated. The two upper phases were combined, dried under a stream of N_2 and dissolved in 100 µL of methanol purged with N_2 and containing 1 mM HCl.

The total pool and redox poise of mitochondrial quinones in sugar beet roots were determined after Q organic extraction by reverse-phase HPLC (Fig. 3.2B). A 100 x 8 mm Novapak C_{18} radial compression column (Waters) was used, with an isocratic mobile phase (ethanol:methanol 7:3, v:v, purged with N_2) and a flow rate of 1 mL min^{-1}. The oxidised and reduced forms of Q_9 and Q_{10} were differentiated by retention time and extinction coefficients at 275 and 290 nm (Millar et al., 1998; Millenaar et al., 1998). Retention times for reduced Q_9, reduced Q_{10}, oxidised Q_9 and oxidised Q_{10} were 8.2, 10.0, 12.5 and 16.3 min, respectively. Both Q_9 and Q_{10} were reduced with dithionite to their respective QH_2 (Rich, 1978). Quantification of the quinone-type compounds was made from the peak areas obtained with known amounts of Q_9 and Q_{10} standards from Sigma.

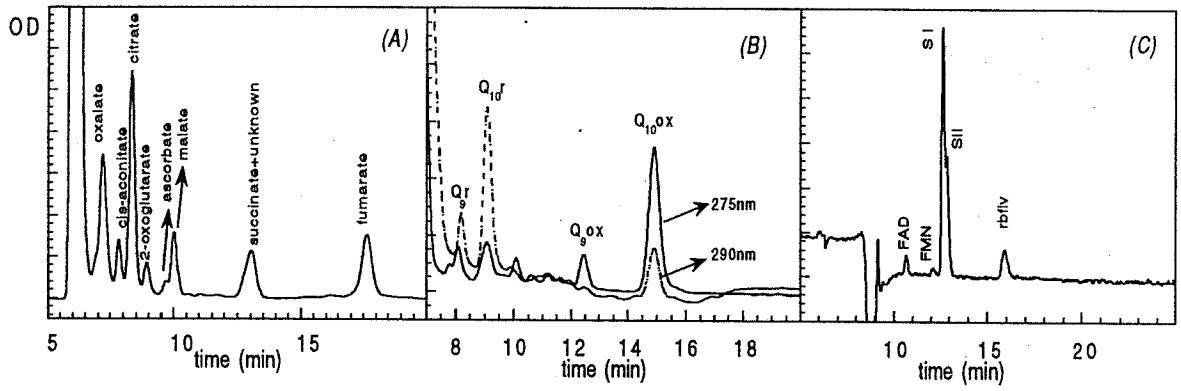

Figure 3. 2. Separation of organic acids (A), quinones (B) and flavins (C) by high performance liquid chromatography. Organic acids (detected at 210 nm) were oxalate, cis-aconitate, citrate, 2- oxoglutarate, ascorbate, malate, succinate and fumarate. Flavins (detected at 445 nm) were FAD, FMN, riboflavin 3'-sulphate, riboflavin 5'-sulphate and riboflavin. Reduced Q_9 and Q_{10} were detected at 290 nm and oxidised Q_9 and Q_{10} at 275 nm.

3. 3. 6. O_2 Consumption

Roots were excised under water at room temperature from plants illuminated for several h. Root O_2 consumption rates were measured from the decrease in O_2 concentration in an aqueous phase with a Clark-type O_2 electrode (Hansatech, Kings Lynn, UK). Calibration was made from the difference in signal between air and N_2-saturated water (Walker, 1987). The effects of the respiration

inhibitors KCN and hydroxi-salycilic acid (SHAM) were studied at different concentrations. Sequential additions of KCN were made directly to roots in the measurement cuvette. Roots were pre-incubated with different concentrations of SHAM for 30 min prior to measurement. A new batch of root material was used for each SHAM concentration. Oxygen consumption rates were also determined in the presence of 2 mM Fe(III)-EDTA alone or in combination with KCN, SHAM or KCN plus SHAM.

3. 3. 7. Iron-Reducing Activity

The FC-R activity of root tips was followed by measuring the formation of the Fe(II)-BPDS complex from Fe(III)-EDTA at 535 nm (Bienfait et al., 1983). Three root tips were added to 1 mL of nutrient solution, pH 6.0, supplemented with 400 µM BPDS and 500 µM Fe(III)-EDTA. The reaction was performed in the dark for 7 min. A 0.8 mL aliquot was taken, centrifuged at 10000*g* for 3 min and absorbance measured at 535 nm.

3. 3. 8. Flavin Determination

FAD, FMN, rivoflavin, riboflavin 3´-sulphate and riboflavin 5´-sulphate were extracted from root tips and separated by reverse-phase HPLC according to Susín et al. (1993) (Fig. 3.2C).

3. 3. 9. Electron Microscopy

Electron microscopy was performed with root tips fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature, 20 h at 4°C and then washed in the same buffer. After dehydration in acetone series, samples were critical-point dried, gold-palladium coated and viewed at 10 kV in a LEO 430 (LEO Ltd., Cambridge, UK) scanning electron microscope.

3. 4. Results

3. 4. 1. Changes in Flavin Concentrations and Ferric Chelate Reductase Activity in Roots with Iron Deficiency

The distal roots from Fe-deficient plants were divided in two parts, the yellow zone (approximately 0 to 5 mm from the apex) which has root hairs (Fig. 3.1B), is enriched in flavins and has increased Fe(III)-reductase activity (Table 3.1) (Susín et al., 1993, 1996), and a white adjacent zone (approximately 5 to 10 mm from the apex), which does not have root hairs (Fig. 3.1A) and has much lower flavin content and Fe(III)-reducing activities (Table 3.1). In the controls (Fig. 3.1C, D) flavin concentrations and Fe(III)-reductase activities were low, both in the 0-5 and 5-10 mm root sections (Table 3.1).

Riboflavin 3'-sulphate and riboflavin 5'-sulphate concentrations increased in the yellow zones of Fe-deficient roots 102- and 49-fold, respectively, when compared to the controls (Table 3.1). The concentration of FAD was also increased 7-fold in the yellow zones when compared to the controls (Table 3.1). In the white zones of the Fe-deficient roots the riboflavin 3'-sulphate, riboflavin 5'-sulphate and FAD increased 23-, 15- and 5-fold when compared to the controls (Table 3.1).

		-Fe			
	+Fe (0-10 mm)	Yellow zone (YZ)	White zone (WZ)	-Fe YZ/+Fe	-Fe WZ/+Fe
FC-R activity	13±2	138±5	39±5	11	3
rflv 3'-sulphate	4±1	406±79	91±30	102	23
rflv 5'-sulphate	2±1	98±12	30±4	49	15
FAD	10±2	67±3	49±9	7	5
riboflavin	n.d.	13±2	6±1	-	-

Table 3. 1. Ferric chelate reductase activities (nmol min^{-1} g^{-1} FW) and flavin concentrations (nmol g^{-1} FW) in root tips from Fe-sufficient and Fe-deficient sugar beet. Data are means ± SE of 5 replicates.

3. 4. 2. Changes in Root Organic Anion Concentrations with Iron Deficiency

A typical chromatographic separation of the organic anions present in roots is shown in Figure 3.2A. Retention times for oxalate, cis-aconitate, citrate, 2-oxoglutarate, ascorbate, malate and fumarate were 6.5, 7.5, 8.2, 8.6, 10.0, 10.2 and 17.4 min, respectively. Other peak with a retention time of 13.0 min contained succinate and an unidentified component with absorption maxima at 210 and 261 nm. In all root zones the major organic anions (more than 95% of the total organic anion contents) were oxalate, citrate, malate and ascorbate. In the control roots no differences were found between the organic anion concentrations in the 0-5 mm and 5-10 mm fractions (data not shown). Because of this reason, only mean values corresponding to the root 0-10 mm section from the apex are given (Table 3.2).

	+Fe (0-10 mm)	-Fe Yellow zone (YZ)	-Fe White zone (WZ)	-Fe YZ/+Fe	-Fe WZ/+Fe
malate	0.37±0.05	5.95±0.78	4.32±0.80	16.1	11.7
oxalacetate	< 0.001	< 0.001	< 0.001	-	-
citrate	0.10±0.02	2.60±0.54	2.70±0.65	26.0	27.0
cis-aconitate	0.030±0.002	0.403±0.140	0.040±0.001	13.4	1.3
isocitrate	< 0.001	< 0.001	< 0.001	-	-
2-oxoglutarate	0.020±0.006	0.065±0.016	0.020±0.006	3.2	1.0
succinate*	0.047	0.123	0.048	2.6	1.0
fumarate	0.006±0.001	0.800±0.300	0.298±0.040	133.3	49.7
oxalate	7.71±1.54	18.40±4.50	19.60±8.20	2.4	2.5
ascorbate	0.23±0.07	2.40±0.37	2.55±0.50	10.4	11.1
pyruvate	< 0.001	< 0.001	< 0.001	-	-
total	8.47	30.62	29.53	3.6	3.5

Table 3. 2. Concentrations of organic anions (μmol g^{-1} FW) in root tips from Fe-sufficient and Fe-deficient sugar beet. Data are means ± SE of 10 replicates. *Succinate co-eluted with an unidentified compound.

In the yellow zones of Fe-deficient sugar beet root tips there was a 3-fold increase in total organic anion concentration when compared to Fe-sufficient control roots. This was associated to 26-fold increases in citrate, 16-fold increases in malate and 10-fold increases in ascorbate (Table 3.2). Oxalate, however, was increased only by 2-fold with Fe deficiency. The minor organic anions cis-aconitate, 2-oxoglutarate, succinate and fumarate (less than 5% of the total organic anion concentration in all cases) increased 13-, 3-, 3- and 133-fold in the yellow parts of the Fe-deficient root tips when compared to the Fe-sufficient controls (Table 3.2).

The concentrations of the major organic anions oxalate, citrate, malate and ascorbate were similar in the yellow and white zones of the Fe-deficient sugar beet root tips (Table 3.2). However, the concentrations of cis-aconitate, succinate and 2-oxoglutarate in the white parts of the Fe-deficient roots were much lower than those present in the yellow, flavin-enriched areas, and similar to those found in Fe-sufficient root tips (Table 3.2). The fumarate concentration in the white parts of the Fe-deficient roots was intermediate between those found in Fe-sufficient roots and in the yellow areas of the Fe-deficient roots (Table 3.2).

3. 4. 3. Changes in Root Extract Enzymatic Activities with Fe Deficiency

We measured five enzymatic activities involved in organic acid metabolism. All these enzymatic activities were markedly increased in the extracts from the yellow parts of Fe-deficient roots when compared to those obtained from Fe-sufficient roots (Table 3.3). Increases were 16-fold for MDH, 9-fold for fumarase, 14-fold for ICDH, 5-fold for aconitase and 45-fold for CS.

In the Fe-deficient root extracts the activities of the five measured enzymes were markedly lower in the white than in the yellow zone (Table 3.3). Activities in the white zone of Fe-deficient roots were generally intermediate between the activities found in the yellow zone and those found in the Fe-sufficient controls. The increases over the control values were approximately 4-, 2-, 2- and 4-fold for MDH, fumarase, ICDH and CS, respectively. The activity of aconitase was similar to the control values.

The PEPC activity in extracts of the yellow zone of Fe-deficient roots was 60 times higher than in Fe-sufficient root tips at pH 8.5 and 40 times at pH 7.3. In the white zone of the Fe-deficient roots, however, the PEPC activity was only 2-fold of that found in the Fe-sufficient controls (Table 3.3). The inhibition of the PEPC activity (measured at pH 7.3) by 500 µM malate was approximately 59 and 42% in Fe-sufficient and Fe-deficient yellow root tip extracts.

	+Fe (0-10 mm)	-Fe Yellow zone (YZ)	-Fe White zone (WZ)	-Fe YZ/+Fe	-Fe WZ/+Fe
MDH	6.84±0.48	112.1±24.6	25.3±2.0	16.4	3.7
CS	0.036±0.021	1.611±0.360	0.133±0.010	44.8	3.7
Aconitase	282.0±64.8	1560±133	380±140	5.5	1.3
ICDH	0.168±0.048	2.39±0.34	0.356±0.03	14.2	2.1
Fumarase	381±99	3340±1100	594±112	8.7	1.6
PEPC	0.222±0.066	13.32±2.52	0.542±0.04	60.0	2.4
CA	13.92±3.22	11.69±2.30	21.88±5.41	0.8	1.6
G6PDH	0.589±0.070	2.270±0.573	0.785±0.127	3.8	1.3
LDH	0.016±0.006	0.216±0.059	0.087±0.024	13.5	5.4
PDC	0.048±0.024	0.288±0.120	0.097±0.026	6.0	2.0

Table 3. 3. Enzymatic activities (nmol g^{-1} FW min^{-1}) in root tip homogenates from Fe-sufficient and Fe-deficient sugar beet. Data are means ± SE of 5 replicates.

The CA activity in the extracts of the yellow zone of Fe-deficient roots was similar to that found in the controls, whereas in the white zone of the same roots CA activity was slightly increased (Table 3.3). G6PDH activity increased 4-fold in the yellow zones of Fe-deficient roots respect to the controls, whereas the white zones had similar values to the controls. LDH and PDC activities were also 14- and 6-fold higher in the yellow parts of the Fe-deficient roots when compared to the controls. The activities of LDH and PDC in the white zone of the Fe-deficient root tips were between the activities found in the yellow zone and those found in the Fe-sufficient controls (Table 3.3).

3. 4. 4. Changes in Root Nucleotide Concentrations with Fe Deficiency

The pool of pyridine nucleotides increased 3.3-fold in the yellow root tips of Fe-deficient plants when compared to the controls (Table 3.4). Iron deficiency increased the concentrations of both reduced and oxidised nucleotide forms. The largest increase was 8-fold for NAD^+, followed by 4.5-fold for $NADP^+$, 2.2-fold for NADPH and 1.5-fold for NADH. As a result of these changes, the NADH/NAD^+ and NADPH/$NADP^+$ ratios decreased by 82 and 50%, respectively, in the yellow tips of Fe-deficient roots when compared to the controls.

	+Fe (0-10 mm)	-Fe Yellow zone (YZ)	-Fe White zone (WZ)	-Fe YZ/+Fe	-Fe WZ/+Fe
NAD^+	0.80±0.47	6.42±0.17	1.91±0.01	8.0	2.4
$NADP^+$	1.41±0.92	6.34±0.34	2.13±0.28	4.5	1.5
NADH	1.87±0.27	2.75±0.51	3.23±0.31	1.5	1.7
NADPH	1.70±0.09	3.73±1.18	2.58±0.44	2.2	1.5
Total pool	5.78	19.24	9.85	3.3	1.7
NADH/NAD^+	2.3	0.4	1.7	0.2	0.7
NADPH/$NADP^+$	1.2	0.6	1.2	0.5	1.0
ATP (x 10^{-3})	0.25±0.03	1.26±0.37	0.64±0.13	5.0	2.5

Table 3. 4. Concentrations of pyridine nucleotides and ATP (nmol g^{-1} FW) in root tips from Fe-sufficient and Fe-deficient sugar beet. Data are means ± SE of 7 replications.

The white zone of Fe-deficient root tips had pyridine nucleotide concentrations only 1.7-fold higher than those found in Fe-sufficient root tips. The concentrations of reduced forms in these white parts were not significantly different to those found in the yellow parts (Table 3.4). However, the concentrations of the oxidised forms were intermediate between those found in the yellow parts of the same root and the control, Fe-sufficient values. In these white

parts the NADH/NAD^+ and NADPH/$NADP^+$ ratios were similar to those found in Fe-sufficient root tips (Table 3.4).

The ATP concentration in the yellow part of the Fe-deficient root tips was 5-fold higher than that found in Fe-sufficient root tips (Table 3.4). The white parts of Fe-deficient roots had ATP concentrations still 2.5-fold higher than those found in the controls.

3. 4. 5. Changes in Root Tip Oxygen Consumption Rates Induced by Iron Deficiency

The yellow parts of the roots from Fe-deficient plants had increased O_2 consumption rates when compared to the control roots. The yellow and white parts of the Fe-deficient roots consumed approximately 627 (Table 3.5) and 137 nmol O_2 min^{-1} g^{-1} FW, whereas control roots consumed 164 nmol O_2 min^{-1} g^{-1} FW (Table 3.5).

	+Fe	-Fe Yellow zone
initial rate	164±23	627±69
2 mM KCN	83.4±6 (49%)	326±23 (48%)
2 mM SHAM	113±26 (31%)	499±23 (20%)
2 mM KCN+2 mM SHAM	0 (100%)	108±31 (82%)
2 mM KCN+2 mM SHAM+2 mM Fe(III)-EDTA	-	0 (100%)
2 mM KCN+2 mM Fe(III)-EDTA	-	263±11 (58%)
2 mM SHAM+2 mM Fe(III)-EDTA	-	422±20 (33%)
2 mM Fe(III)-EDTA	164±23 (0%)	514±7 (18%)

Table 3. 5. O_2 consumption rates (nmol O_2 g^{-1} FW min^{-1}) in root tips from Fe-sufficient and Fe-deficient sugar beet. Data are means ± SE of 10 replications. Data in brackets are the percentage of inhibition of O_2 consumption respect to the initial rate.

Cyanide resistant O_2 consumption was approximately 50% of the total consumption in both Fe-sufficient and deficient roots (Table 3.5, Fig. 3.3). In Fe-deficient roots the presence of SHAM decreased root O_2 consumption by 20% at 4

mM (Fig. 3.3A) and by 80% at 20 mM. In the controls 4 mM SHAM decreased O_2 consumption by 40% (Fig. 3.3B), whereas 20 mM SHAM inhibited completely O_2 consumption. Residual O_2 consumption, the fraction of oxygen uptake that is resistant to the combination of KCN and SHAM (Ribas-Carbó et al., 1997), was approximately 20% of the maximal rates in Fe-deficient roots and practically zero in the controls.

The addition of 2 mM Fe(III)-EDTA decreased the O_2 consumption rate in Fe-deficient root tips by approximately 20%, whereas in Fe-sufficient roots the rate was unaffected (Table 3.5). The inhibition of root O_2 consumption rates by 2 mM Fe(III)-EDTA reached values of 58, 33 and 100% in Fe-deficient roots treated with KCN, SHAM or KCN plus SHAM, respectively (Table 3.5).

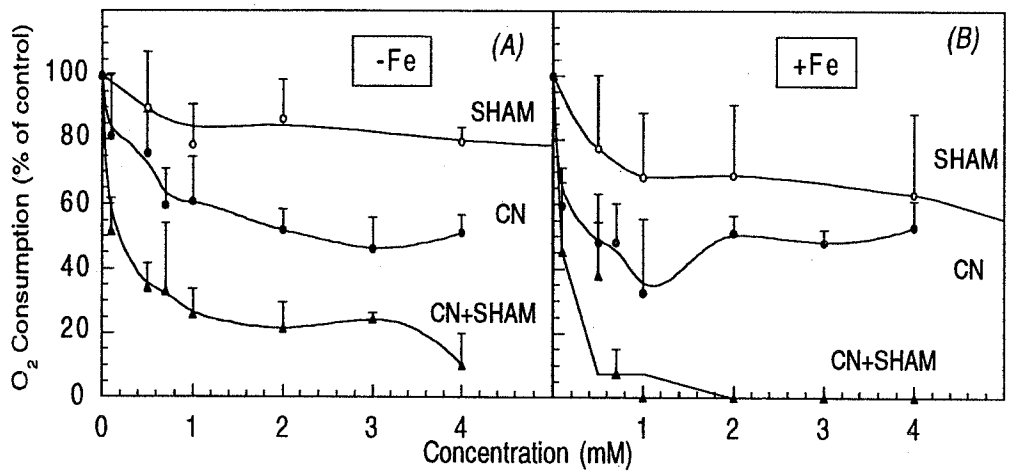

Figure 3. 3. Changes in O_2 consumption rates in yellow, Fe-deficient (A) and Fe-sufficient root tips (B) with different concentrations of SHAM (open circles), CN^- (solid circles) and CN^- + SHAM (solid triangles). Data are means ± SE of three different replications. Actual O_2 consumption rates are shown in Table 3.5.

3. 4. 6. Redox Poise of the Q Pool

The Q_{10} homolog was the predominant quinone form present in sugar beet roots. The total pool of Q_{10} increased in the yellow and white parts of Fe-deficient roots by 3- and 2.5-fold, respectively, when compared to the controls (Table 3.6).

The Q_{10} pools were 42% reduced in Fe-sufficient roots and 52 and 27% reduced in the yellow and white zones of Fe-deficient roots, respectively (Table 3.6). The total amount of Q_9 was always less than 5% of that of Q_{10}.

	+Fe (0-10 mm)	-Fe Yellow zone (YZ)	-Fe White zone (WZ)	-Fe YZ/+Fe	-Fe WZ/+Fe
Qr (Q_{10})	1.73±0.74	6.48±2.80	2.79±0.48	3.7	1.6
Qo (Q_{10})	2.34±0.62	6.01±3.07	7.54±2.80	2.5	3.2
Qt (Q_{10})	4.07	12.49	10.3	3.0	2.5
Qr/Qt (Q_{10})	42±2 %	52±4 %	27±4 %	1.23	0.64

Table 3. 6. Concentrations of mitochondrial quinones (in nmol g^{-1} FW) in root tips from Fe-sufficient and Fe-deficient sugar beet. Data are means ± SE of 5 replications.

3. 5. Discussion

Most studies on Fe-deficient roots have focused on very specific aspects of their physiology or biochemistry, resulting in very fragmentary information (Welkie and Miller, 1993; Schmidt, 1999). In the present work we have made a comprehensive study of two zones of the distal part of the root of Fe-deficient sugar beet, having or not increased Fe-reducing activities. Measurements included Fe(III)-reducing activities, flavin concentrations, O_2 consumption rates, concentrations of organic anions, enzymatic activities and redox poises of the nucleotide and mitochondrial quinone pools. The results found provide support for the view that Fe deficiency leads to a large increase in C fixation (approximately 50-fold) together with a significant, but smaller increase in O_2 utilisation by roots (approximately 5-fold).

Fe-deficient root tips had a markedly enhanced capacity to fix C from bicarbonate. The main features of the metabolic pathway leading to C fixation in roots of Fe-deficient plants have been included in Fig. 3.4. Carbon fixation was associated to large (40 to 60-fold) increases in PEPC activity over the control values.

PEPC catalyses the carboxylation of PEP to oxalacetate, which could be subsequently reduced to malate via cytosolic MDH. Malate could then be transported to the mitochondria via the malate-oxalacetate shuttle and converted to citrate by CS. The increase in PEPC activity in the yellow Fe-deficient roots was also accompanied by large increases in MDH and CS enzymatic activities, supporting the significance of C fixation by PEPC. Thermodynamically, malate production from PEP, via PEPC and MDH is more favourable energetically than from pyruvate via pyruvate kinase (Lance and Rustin, 1984). Both CO_2 fixation (Rhoads and Wallace, 1960; van Egmond and Atkas, 1977; Landsberg, 1986; Bienfait, 1988, 1989; Rabotti et al., 1995) and PEPC activity (Hiatt, 1966; Landsberg, 1986; Fournier et al., 1992; Rabotti et al., 1995; Alhendawi et al., 1997) have been reported to be stimulated by Fe deficiency. Miller et al. (1990) also reported an increase in incorporation of labelled CO_2 into organic acids in the roots of Fe-deficient tomato, and suggested that PEPC activity may feed the TCA cycle (Krebs Cycle) (see also Welkie and Miller, 1993). PEP needed to maintain PEPC activity could possibly come from sugars, via glycolysis. In fact, sugar concentrations have been reported recently to increase in Fe-deficient roots, probably associated to an enhanced expression of several genes related to carbohydrate biosynthesis (Thoiron and Briat, 1999). Moreover, the activity of G3PDH, an enzyme involved in the glycolitic pathway, also increases in Fe-deficient roots (Rabotti et al., 1995).

The large increase in PEPC activity in Fe-deficient roots is likely to be regulated at several levels. First, Fe deficiency was associated to an increase in the amount of PEPC. For instance, a 6-fold increase in the amount of a 56 kD fragment of PEPC was measured in 2-D gels with antibodies against PEPC (González-Vallejo, 1999). Secondly, increases of PEPC activity could be also mediated by post-transcriptional regulation through phosphorilation, as it occurs in the leaves of C4 and CAM species and in proteid roots of P-stressed plants (see Chollet et al., 1996, for a review). This is supported by the fact that the PEPC sensitivity to malate was lower in extracts of Fe-deficient roots than in those of controls, with 41 and 58% of the initial activity (at pH 7.3) remaining in the presence of 500 µM malate.

The increase in PEPC activity does not appear to be due to the level of bicarbonate in the nutrient solution, since root tips of plants grown without Fe in the absence of $CaCO_3$ also had PEPC activities 40-fold higher (at pH 8.5) than the controls. Also, CA activities did not increase with Fe deficiency.

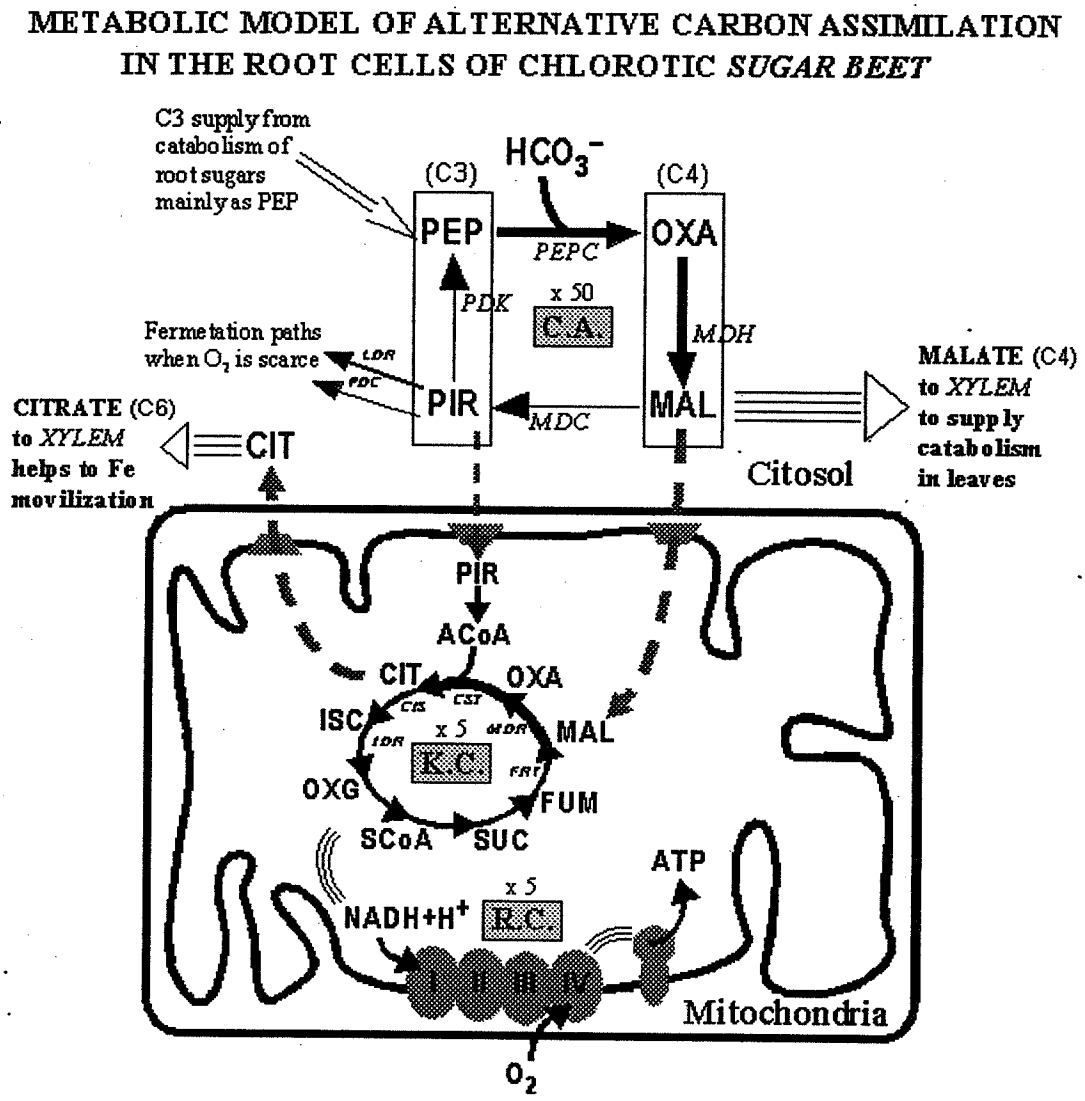

Figure 3. 4. Metabolic model for carbon assimilation in sugar beet roots under Fe deficiency. Iron deficiency would cause an approximately 50-fold enhancement in carbon assimilation (C.A.) in the cytosol through the increase in PEPC activity, and approximately 5-fold increases in the Krebs cycle (K.C.) and respiratory chain (R.C.) in the mitochondria. Part of the malate and the citrate would be exported via xylem, thus providing respiratory substrates to the shoot.

The yellow, Fe(III)-reducing tips of the Fe-deficient roots (Fig. 3.1B) had signs of enhanced mitochondrial activity. These root parts had 4-fold increases in total O_2 consumption rates, 3-fold increases in total nucleotide pools, 2-fold increases in total mitochondrial quinone pools and 5-fold increases in ATP concentrations over the controls. An enhanced mitochondrial activity is in agreement with the presence in these root tips of a large number of transfer cells containing mitochondria (Landsberg, 1994). Furthermore, both the pools of organic anions and enzymatic activities involved in organic acid metabolism were increased in the yellow, Fe-deficient root tips when compared to the controls. For instance, the root concentrations of citrate and malate increased 26- and 16-fold, and the activities of CS, MDH and fumarase increased 45-, 16- and 9-fold when compared to the controls.

As indicated above, Fe deficiency increases more C fixation than mitochondrial respiratory processes. This is supported by the differential increases caused by Fe deficiency in C fixation processes (40 to 60-fold for PEPC and CS) and respiration processes (2 to 5-fold). This may suggest that one of the major functions of the yellow Fe-deficient root tips is to provide malate to be exported via xylem to the leaves, that are chlorotic, have very low photosynthetic rates and consequently need an external C supply to maintain respiration. This enhancement of C assimilation in the root is linked to a relatively smaller increase of basal catabolism, i.e. respiration. Such an activation of the TCA cycle and respiratory chain is needed to provide enough ATP to the cell. In these conditions the oxygen supply for the root could be limiting. Such a limitation is supported by the fact that the mitochondrial quinone pool was more reduced in the yellow root tips of the Fe-deficient plants than in the controls. The possible shortcoming of O_2 for respiration could be enhanced by the substantial rate of residual O_2 uptake, insensitive to the presence of KCN and SHAM, in the yellow zones of the Fe-deficient sugar beet roots. This residual O_2 uptake rate was approximately 130 nmol O_2 g^{-1} FW min^{-1}, of the same order than the rates of reduction of Fe(III)-chelates in the same roots (Susín et al., 1996). When Fe(III)-EDTA was added to KCN plus SHAM-treated roots they ceased to consume O_2. Since Fe(III)-EDTA cannot cross the plasma membrane, these data support that a FC-R enzyme induced by Fe deficiency in the sugar beet root PM could use O_2 when ferric chelates are not present. That O_2 could be an acceptor for the PM FC-R enzyme

had been suggested previously from the increases caused by anaerobiosis on the Fe(III)-reducing activity of purified plasma membranes (González-Vallejo et al., 1998, 1999).

The activities of two enzymes typical of anaerobic metabolism, LDH and PDC, increase markedly in activity (6 to 14-fold) in yellow root tips of Fe-deficient plants when compared to the controls. These enzymes use pyruvate as substrate and form lactate and ethanol, respectively. This again suggests that Fe-deficient roots may suffer a certain degree of hypoxia, as a consequence of the markedly increased O_2 consumption rates found in these roots. Another enzyme of the anaerobic metabolism, FDH, has been shown to increase in activity with Fe deficiency in roots of barley (Suzuki et al., 1998). The increased activities of LDH and PDC could account for part of the NADH consumption in Fe-deficient yellow root tips, but their contribution to the energy requirements of Fe-deficient plants is still unclear.

The pyridine nucleotide pool was more oxidised in the yellow parts of the Fe-deficient sugar beet roots than in the controls, conversely to what has been reported previously in other species (Sijmons et al., 1984; Schmidt and Schuck, 1996). This total pool increased 3-fold in the yellow parts of the Fe-deficient roots when compared to the controls, similarly to what has been reported previously in roots of bean and *Plantago lanceolata* (Sijmons et al., 1984; Schmidt and Schuck, 1996). The shift towards oxidation occurred in spite of the increased potential for the production of reducing power associated to the large pools of organic acids and increased activities of several NADH and NADPH producing enzymes. This could be explained by the high concentrations in the cytosol of Fe-deficient sugar beet roots of oxidised riboflavin sulphates (approximately 700 µM; Susín et al., 1993), since NADH and NADPH can reduce easily both flavin sulphates and riboflavin (González-Vallejo et al., 1998). These flavin compounds are not present in roots where the nucleotide pool is more reduced with Fe deficiency. Also, the white parts of Fe-deficient sugar beet roots do not have significant amounts of flavins and had NADPH/$NADP^+$ ratios similar to the control roots. The increased activities of some NADH consuming enzymes, such as cytosolic LDH and PDC, could also contribute to oxidise the nucleotide pool. It should be mentioned that the shift to a more oxidised state with Fe deficiency observed in the pyridine nucleotide pool does

not reflect a generalised oxidation, since the pool of mitochondrial quinones was more reduced than in the controls.

Our data support that flavins could be crucial in a metabolic link in Fe-deficient sugar beet roots, involving a redox chain between organic acids and FC-R activity (Fig. 3.5). A link between organic acids and FC-R was suggested previously by Bienfait (1996). The major increase in PEPC activity in yellow Fe-deficient roots would lead to C fixation, accumulation of organic acids and in turn to increased activities of several NADPH-producing enzymes such as cytosolic ICDH, MDH and G6PDH. Reduced pyridine nucleotides would then act as electron donors for flavins, including riboflavin sulphates and riboflavin, which are mainly oxidised and could reach root concentrations 35-fold higher than that of the nucleotide pool (700 and 20 μM, respectively). Electrons can pass from one flavin molecule to the next forming a "redox bridge" (Rawn, 1989), to reach different final acceptors at the level of the cell plasma membrane, including membrane flavoproteins, Fe(III)-chelates or directly oxygen. Indeed, the marked increases in FAD concentrations in the yellow zones of Fe-deficient roots (7-fold over the control values) may suggest that the increases in FC-R activities (11-fold increases over the control values) could be associated to increases in a FC-R enzyme similar to the FAD-enzyme recently characterised in *Arabidopsis thaliana* (Robinson et al., 1999).

The increases found in the organic anion pools with Fe deficiency confirm data obtained previously for roots of different plant species (Iljin, 1951; de Kock and Morrison, 1958; Brown, 1966; Venkat-Raju et al., 1972; de Vos et al., 1986; Sijmons and Bienfait, 1986). Our data and those reported in the literature (de Kock and Morrison, 1958; Brown, 1966; Landsberg, 1981; de Vos et al., 1986; Alhendawi et al., 1997) indicate that, among the organic anions, citrate increases most in Fe-deficient roots. Aconitase activity was markedly increased in the yellow Fe-deficient sugar beet root tips when compared to the controls. The absence of decreases in aconitase activity with Fe deficiency has been also observed in bean (Landsberg, 1981), *Capsicum annuum* (de Vos et al., 1986) and tomato (Pich and Scholz, 1993). Therefore, the accumulation of citrate and other organic acids are likely be caused by stimulation of the anabolic pathways involved in their formation, and not by the inhibition of any particular enzyme of the cycle.

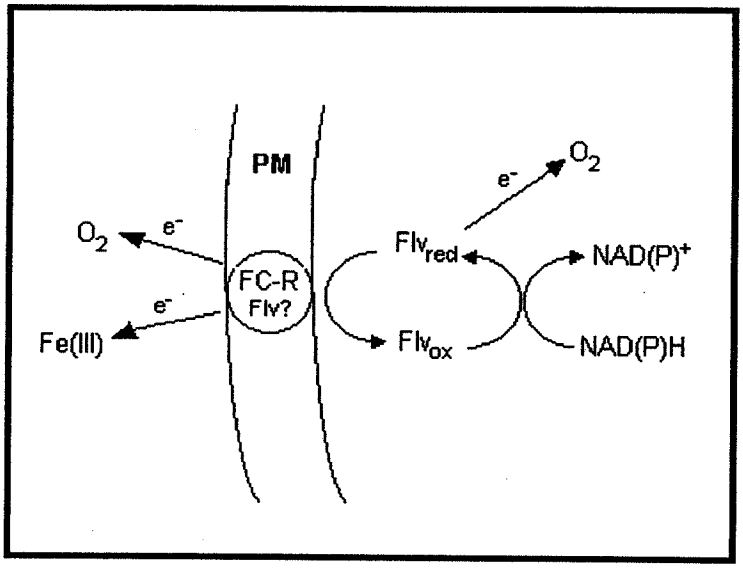

Figure 3. 5. Proposed electron transport pathways in Fe deficient sugar beet roots. Reduced pyridine nucleotides would reduce flavins (Flv) which are oxidised and in large amounts in the cytosol (up to 700 µM). Flavins would finally provide electrons to the ferric chelate reductase enzyme(s) of the plasma membrane (FC-R). This enzyme would be able to reduce not only Fe(III)-chelates but also oxygen when Fe(III)-chelates are absent.

The white parts of the Fe-deficient roots, which do not contain flavin sulphates and do not have increased Fe(III)-reducing activities, had large pools of organic anions, similar to those of the yellow distal root parts. However, the respiration rates of these root zones were similar to the controls, and increases of the TCA cycle enzymatic activities, although still significant, were less marked than in the yellow tips. Also, the increase in PEPC activity over the controls was much smaller than that found in the yellow tips. These data clearly show that accumulation of organic acids *per se* is not sufficient to increase Fe(III)-reducing activities.

Two of the major findings documented in this work, the utilisation of C and O_2 by the root zones expressing the biochemical responses typical of strategy I plant species, may both constitute a metabolic protective mechanism which implies ecologically significant advantages. The increased PEPC activity of Fe-deficient roots and other enzyme and metabolite changes indicate the existence of a non-autotrophic, anaplerotic carbon fixation by roots, similar to that reported

previously in P-stressed *Lupinus* (Johnson et al., 1994). It should be mentioned that Fe deficiency occurs mostly in nature in soils with high carbonate content, where bicarbonate is in large supply. In general, CO_2 uptake by roots is considered negligible (Farmer and Adams, 1991) although it may be of great importance for the carbon balance of roots (Vuorinen et al., 1992) and N fixing root nodules (Vance et al., 1994). C fixed anaplerotically in roots of Fe-deficient plants can be exported via xylem (Bialzyk and Lechowski, 1992; López-Millán, chapter 5) and then used for basic maintenance processes in leaves with drastically reduced photosynthetic rates (Terry, 1980). The possibility that the PM FC-R enzyme may donate electrons to O_2 under physiological conditions in the absence of Fe(III)-chelates would also provide a significant advantage for the Fe-deficient roots, since, in the absence of ferric chelates, O_2 usually available in the rhizosphere of aerated soils would permit the dissipation of reducing power, that would otherwise accumulate in the cell and lead to adverse consequences.

In summary, in Fe-deficient yellow root tips there is a large increase in C fixation, mediated by an increase in PEPC activity. Part of this C is used, through an increase in mitochondrial activity, to increase the capacity to produce reducing power, whereas the main part is exported as malate to the shoot via xylem. In sugar beet Fe-deficient roots, flavin sulphates provide a suitable link between the increased capacity to produce reduced nucleotides and the plasma-membrane associated FC-R enzyme(s). Finally, in sugar beet roots the FC-R enzyme is able to reduce external O_2 in the absence of Fe(III)-chelates, thus permitting to maintain the whole system active through an optimal redox poise.

CAPÍTULO 4

DESACTIVACIÓN TRAS EL APORTE DE Fe DE LAS RESPUESTAS INDUCIDAS POR LA DEFICIENCIA DE Fe EN LAS RAÍCES DE REMOLACHA

4. Iron Resupply-Mediated Deactivation of the Root Responses to Iron Deficiency in Sugar Beet

4. Iron Resupply-Mediated Deactivation of the Root Responses to Iron Deficiency in Sugar Beet

4. 1. Abstract

Different root parts with or without increased iron-reducing activities have been studied in iron-deficient sugar beet plants after iron resupply to the nutrient solution. The distal root parts of the iron-deficient plants decreased by 20 and 80% their capacity to reduce ferric-chelates after 24 and 96 h of iron resupply, respectively. These iron-deficient distal root parts fix carbon through an increase in the activity of phosphoenol pyruvate carboxylase and other enzymes related to carboxylic acid metabolism. Iron resupply caused 50 and 90% decreases in phosphoenol pyruvate carboxilase activity after 24 and 96 h, respectively, and general decreases in other enzyme activities involved in carboxylic acid metabolism. Iron-deficient distal root parts have large total pools of carboxylic acids, which decreased by 10 and 40% after 24 and 96 h of iron resupply, respectively. The activities of PDC and LDH, two enzymes related to

anaerobic metabolism, decreased by approximately 70% within 24 h after Fe resupply to the Fe-deficient roots. The redox state of the mitochondrial quinone and pyridine nucleotide pools became more oxidised in the Fe-deficient root tips after resupply. Iron resupply caused 70% decreases in root oxygen consumption rates. Results indicate that the deactivation of the responses of sugar beet roots to iron deficiency upon iron resupply is relatively slow, occurring in a progressive manner in a time scale of several days.

4. 1. 1. Abbreviations: ADH, alcohol dehydrogenase; BPDS, bathophenantroline disulfonate; BSA, bovine serum albumin; CS, citrate synthase; DTNB, 5-5´dithio-bis-2-nitrobenzoic acid; FC-R, ferric-chelate reductase; FMN, riboflavin 3 phosphate; G3PDH, glyceraldehide-3 phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MS, mass spectroscopy; PDC, pyruvate decarboxylase; PEP, phosphoenol pyruvate; PEPC, phosphoenol pyruvate carboxylase; PM, plasma membrane; PVDF, polyvinyl fluoride; PVP, polyvinylpirrolydone; Q, quinone; Rbf, riboflavin; SI, riboflavin 3'-sulphate; SII, riboflavin 5'-sulphate; SHAM, hydroxi salicilyc acid; SPAD, portable Chl meter.

4. 2. Introduction

Iron deficiency is a widespread agricultural problem in many crops, especially in calcareous soils where although abundant, Fe is not available for plant roots (Lindsay y Schwab, 1982). The adaptive response of Strategy I plants, which include dicotyledoneous and non-*Graminaceae* monocotyledoneous species, involves morphological and physiological changes (Marschner et al., 1986). Among the morphological changes there is an increased formation of lateral roots, root hairs and transfer cells. All these changes increase the root surface available for Fe uptake (Kramer et al., 1980; Landsberg, 1982). The physiological responses of Strategy I plants include a higher proton extrusion which decreases the pH of the rhizosphere (Brown, 1978), a release of reducing and/or chelating substances such as phenolics and flavins (Welkie and Miller, 1960; Susín et al.,

1996) and a two step mechanism for Fe uptake in which Fe is first reduced by a plasma membrane-bound ferric-chelate reductase (FC-R) (Moog and Brüggemann, 1994; Susín et al., 1996) and subsequently absorbed as Fe(II) (Chaney et al., 1972; Robinson et al., 1999).

Root metabolism in Strategy I plants is highly affected by Fe deficiency. Several changes at the metabolic level have been reported to occur in Fe-deficient roots, including an accumulation of organic acids, mainly malate and citrate (de Kock and Morrison, 1958; Brown, 1966; de Vos et al., 1986) a shift in the redox state of the cytoplasm (Schmidt and Schuck 1996, Sijmons et al., 1984; Schmidt, 1999) and an increase in the activities of PEPC and several TCA cycle enzymes (Hiatt, 1966; Landsberg, 1986; Miller et al., 1990; Pich and Scholz, 1993). Both, the physiological interplay and regulation of enzymes and metabolic concentrations of metabolites remain controversial (Schmidt, 1999). The organic acid accumulation induced by Fe deficiency in swollen root tips coincided with enhanced proton extrusion and reductase activity in different species (Landsberg, 1986; Marschner et al., 1987; Rabotti and Zocchi, 1994; Brancadoro et al., 1995). It has been proposed that cytoplasm alkalinization associated to proton efflux could activate PEPC activity (Yang et al., 1994; Rabotti et al., 1995) which can feed the TCA cycle leading to an organic acid accumulation (Landsberg, 1986; Miller et al., 1990, López-Millán, chapter 3). These organic acids could maintain the ionic balance of the root cell cytoplasm (pH-stat theory; Davies, 1973). The relationship between organic acid accumulation and reductase activity seems to be less clear. Fournier et al. (1992) showed that amounts of citrate were correlated with the capacity for rhizosphere acidification in two sunflower lines but no correlation was found between the organic acid accumulation and the reduction activity. Under Fe deficiency, citric acid accumulates in *Plantago*, which does not show proton extrusion, but does show increased root FC-R (Schmidt, 1999). Bienfait (1996) proposed that citrate accumulation may contribute via cytosolic isocitrate dehydrogenase activity to high NADPH levels and thus increase Fe reduction capacity.

Iron deficiency causes a decrease in the chlorophyll content of the leaves, which accounts for drastically reduced photosynthetic rates (Terry and Abadía, 1986; Abadía and Abadía, 1993). Iron resupply to Fe-deficient sugar beet plants

causes restoration of chlorophyll levels and photosynthetic activity (Nishio and Terry, 1983; Nishio et al., 1985) and an increase in the iron content in roots and leaves of maize (Lobréaux et al., 1992, 1993). Although Fe must be taken by the roots before reaching the leaves, little is known about the responses of Fe-deficient roots to Fe resupply.

In the present work we have investigated the kinetics of the deactivation caused by Fe resupply on the responses induced by Fe deficiency in sugar beet roots. This has been done in root parts with or without increased Fe-reducing activities. Measurements included mineral composition, Fe(III)-reducing activities, acidification of the external medium, oxygen consumption rates, concentrations of organic anions and flavins, different enzymatic activities and the redox poises of the nucleotide and mitochondrial quinone pools.

4. 3. Materials and Methods

4. 3. 1. Plant Material

Sugar beet (*Beta vulgaris* L. Monohil hybrid from Hilleshög, Landskröna, Sweden) was grown in a growth chamber with a PPFD of 350 µmol m^{-2} s^{-1} PAR at a temperature of 25°C, 80% relative humidity and a photoperiod of 16 h light/8 h dark. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for two more weeks in half-strength Hoagland nutrient solution with 45 µM Fe and then transplanted to 20 L plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution (Terry, 1980) with either 0 or 45 µM Fe. The pH of the Fe-free nutrient solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$.

In the Fe resupply experiment, plants grown for ten days in absence of Fe were transferred to 20 L plastic buckets containing half-strength Hoagland nutrient solution pH 5.5 with 45 µM Fe and without $CaCO_3$. Samples were taken at 0 (before adding Fe), 24 and 96 h after the Fe addition.

4. 3. 2. Mineral Content Analysis

The mineral composition of roots was determined as described previously (Abadía et al., 1985). Potassium was measured by emission spectrophotometry and Ca, Mg, Fe, Mn, Cu and Zn by atomic absorption spectrometry.

4. 3. 3. Flavin Determination

FAD, FMN, riboflavin, riboflavin 3´-sulphate and riboflavin 5´-sulphate were extracted from root tips and separated by reverse-phase HPLC according to Susín et al. (1993).

4. 3. 4. Fe-Reducing Activity

The FC-R activity of root tips was followed by measuring the formation of the Fe(II)-BPDS complex from Fe(III)-EDTA at 535 nm (Bienfait et al., 1983). Three root tips were added to 1 mL of nutrient solution, pH 6.0, supplemented with 400 µM BPDS and 500 µM Fe(III)-EDTA. The reaction was performed in the dark for 7 min. A 0.8 mL aliquot was taken, centrifuged at 10000*g* for 3 min and absorbance measured at 535 nm.

4. 3. 5. External Acidification of the Medium

The acidification of the medium induced by Fe deficiency was determined qualitatively and quantitatively. Qualitative determination was done according to Marschner et al. (1982). Root tips (0-10 mm) were excised with a surgical blade and placed onto agar plates with 0.6% agar, 50 mg L^{-1} bromcresol purple and nutrient solution without Fe, pH 5.8. Variations in the medium pH induced changes in the agar colour due to the bromcresol purple, which changes from purple to yellow colour under pH 4.8. For the quantitative determination of H^+ extrusion, root tips (20 mg FW) were excised with a surgical blade and placed in 5 mL of nutrient solution (pH 6.8) without Fe. The decrease in pH was recorded

for 1 h with a pH electrode. The rate of H^+ extrusion was calculated between 5 and 30 min.

4. 3. 6. Organic Anion Analysis

Root material (100 mg FW) was frozen in liquid N_2 and ground in a mortar with 8 mM sulphuric acid. Homogenates were boiled for 30 min, filtered with a 0.2 µM PVDF filter (LIDA, Kenosha, WI, USA), taken to a final volume of 2 mL with 8 mM sulphuric acid and kept at -80°C until analysis.

Organic anions were analysed by HPLC with a 300 x 7.8 mm Aminex ion-exchange column (HPX-87H from Bio-Rad, Hercules, CA, USA) with a HPLC Waters system, including a 600E pump, a 996 photodiode array detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (8 mM sulphuric acid) was pumped with a 0.6 mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, cis-aconitate, citrate, 2-oxoglutarate, ascorbate, malate, succinate and fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma. Succinate coeluted with an unidentified compound with absorption maxima at 210 and 261 nm. Identification was confirmed by their peaks in the UV peak spectra and/or HPLC-MS (Waters) (in this latter case formic acid was used as mobile phase instead of sulphuric acid). Quantification was made with known amounts of each organic anion using peak areas.

4. 3. 7. Enzyme Assays

Extracts for measuring enzyme activities were made by grinding 100 mg FW of root material in a mortar with 1 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10000g and 4°C, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 1 mL (final volume) of the media indicated below.

Malate dehydrogenase (MDH; EC 1.1.1.37) was determined with oxalacetate as substrate (Dannel et al., 1995) by measuring the decrease in A_{340} due to the enzymatic oxidation of NADH. The reaction was carried out with 5 µL of extract in 0.1 mM NADH, 0.4 mM oxalacetate and 46.5 mM Tris-HCl, pH 9.5. Citrate synthase (CS, EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1967) by monitoring the reduction of acetyl CoA to CoA with 5-5'-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. The reaction was carried out with 50 µL of extract in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalacetate and 100 mM Tris-HCl, pH 8.1. Aconitase (EC 4.2.1.3) was measured from the formation of cis-aconitate, monitored at 240 nm (Bacon et al., 1961) with 60 µL of extract in 500 mM sucrose, 50 mM isocitrate and 100 mM Tris-HCl (pH 8.5). Isocitrate dehydrogenase (ICDH, EC 1.1.1.42) activity was determined with 50 µL of extract by monitoring the reduction of $NADP^+$ at 340 nm in a reaction mixture containing 3.5 mM $MgCl_2$, 0.41 mM $NADP^+$, 0.55 mM isocitrate and 88 mM imidazole, pH 8.0 (Bergmeyer et al., 1974). Fumarase (EC 4.2.1.2) activity was assayed with 50 µL of extract following the increase in A_{240} due to the formation of fumarate (Bergmeyer et al., 1974). The reaction buffer was 50 mM malate and 100 mM phosphate, pH 7.4.

Phosphoenol pyruvate carboxilase (PEPC; EC 4.1.1.31) was measured in a coupled enzymatic assay with MDH according to Vance et al. (1983). The reaction was made with 75 µL of extract in 2 mM phosphoenol pyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH and 100 mM bicine-HCl, pH 8.5. For the determination of lactate dehydrogenase (LDH; EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1) the oxidation of NADH was monitored at 340 nm with 50 µL of extract. LDH was assayed in a reaction buffer containing 94.5 mM phosphate buffer (pH 9.5), 0.77 mM pyruvate and 0.2 mM NADH. PDC was determined in 190 mM citrate-KOH buffer (pH 6.0), 30 mM pyruvate, 0.32 mM NADH and 33 µg mL^{-1} alcohol dehydrogenase (ADH).

4. 3. 8. Nucleotide Analysis

Pyridine nucleotides were extracted from liquid N_2-frozen root material (approximately 30 mg FW) in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA

(for $NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12000 g for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 100 mM bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method of Matsumura and Miyachi (1980).

ATP was extracted by grinding 100 mg FW of root material in a mortar with 1 mL of 2 mM EDTA and 100 mM Tris-acetate buffer, pH 7.75. The extract was centrifuged at 10000*g* for 15 min at 4°C. The supernatant was mixed with dimethyl sulfoxide (1:9, v:v), and ATP was measured with a luminometer (Labsystems Luminoskan, Life Sciences International, Finland) using an ATP monitoring kit (Bio Orbit Oy, Finland).

4. 3. 9. Root O_2 Consumption

Roots were excised under water at room temperature from plants illuminated for several h. Root O_2 consumption rates were measured from the decrease in O_2 concentration in an aqueous phase with a Clark-type O_2 electrode (Hansatech, Kings Lynn, UK). Calibration was made from the difference in signal between air and N_2-saturated water (Walker, 1987).

4. 3. 10. Analysis of Q-Pool Redox Poise

The extraction of quinones (Q) from root tips was conducted according to Millar et al. (1998). Root material (approximately 1 g FW) was immersed in liquid N_2 and crushed to a fine powder with mortar and pestle. The powder was freeze-dried to remove the root aqueous phase and decrease the possibility of Q oxidation during organic extraction. Dried samples were vortexed for 3 min in a mixture of 1.5 mL of methanol (containing 200 mM perchloric acid) and 1.5 mL of petroleum ether (35-50°C boiling point, d 0.64, Panreac, Spain). After centrifugation for 3 min at 1000*g* to separate the two phases, the upper phase was collected. Additional petroleum ether was added to the lower phase, and the procedure was repeated. The two upper phases were combined, dried under a

stream of N_2 and dissolved in 100 µL of methanol purged with N_2 and containing 1 mM HCl.

The total pool and redox poise of mitochondrial quinones in sugar beet roots were determined after Q organic extraction by reverse-phase HPLC. A 100 x 8 mm Novapak C_{18} radial compression column (Waters) was used, with an isocratic mobile phase (ethanol:methanol 7:3, v:v, purged with N_2) and a flow rate of 1 mL min^{-1}. The oxidised and reduced forms of Q_9 and Q_{10} were differentiated by retention time and extinction coefficients at 275 and 290 nm (Millar et al., 1998; Millenaar et al., 1998). Retention times for reduced Q_9, reduced Q_{10}, oxidised Q_9 and oxidised Q_{10} were 8.2, 10.0, 12.5 and 16.3 min, respectively. Both Q_9 and Q_{10} were reduced with dithionite to their respective QH_2 (Rich, 1978). Quantification of the quinone-type compounds was made from the peak areas obtained with known amounts of Q_9 and Q_{10} standards from Sigma.

4. 4. Results

4. 4. 1. Mineral Composition

The mineral composition of whole roots taken from Fe-sufficient, Fe-deficient and Fe-deficient plants resupplied with Fe is shown in Table 4.1. Iron deficiency decreased by approximately 90% the concentrations of Mn and Fe, by approximately 30% the concentrations of P and K and increased almost 3-fold the concentration of Cu when compared to the values found in control, Fe-sufficient roots. Ca, Mg, and Zn concentrations did not change significantly with Fe deficiency.

Iron resupply caused significant changes in the mineral composition of roots (Table 4.1). Upon Fe resupply Fe increased 5.1-fold at 24 h and remained at similar values at 96 h, whereas Mn increased 4-fold at 24 h and reached values similar to those of Fe-sufficient roots at 96 h. The concentration of Cu decreased by approximately 30% and 40% at 24 and 96 h after Fe resupply, respectively.

The concentrations of K and Mg increased by 50 and 57% within 24 h respectively, and at 96 h both decreased to values similar to Fe-deficient roots.

	Fe-sufficient	Fe-deficient	-Fe resupplied	
	+Fe	-Fe	24 h	96 h
P	0.8±0.1	0.5±0.0	0.6±0.0	0.6±0.0
K	2.3±0.9	1.6±0.2	3.4±0.1	2.0±0.2
Ca	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.01
Mg	0.3±0.05	0.40±0.02	0.63±0.01	0.43±0.04
Fe	1141±159	142±22	730±45	706±35
Mn	164±49	10±4	40±7	168±70
Cu	25±9	73±16	52±13	46±9
Zn	39±16	33±10	37±8	40±0.3

Table 4. 1. Mineral composition of Fe-sufficient (+Fe) and Fe-deficient (-Fe) sugar beet roots at 0, 24 and 96 hours after Fe resupply. K, P and Mg are in g/100g DW and Fe, Mn, Cu and Zn in μg g^{-1} DW. Data are means ± SE of 5 replications.

4. 4. 2. Root Morphology

The root distal parts from sugar beet plants grown for ten days in absence of Fe (0 h) were divided in two parts, the yellow tip (approximately 0 to 5 mm from the root apex) and the white adjacent part (5 to 10 mm from the root apex). Samples were also taken 24 and 96 h after Fe resupply to these plants. Root tips from Fe-deficient plants grown for 24 h in the presence of Fe had only a minor (<1 mm) enlargement of the root tip, yellow root sections were still swollen and showed root hairs as Fe-deficient roots before adding Fe. Therefore, at 24 h the root distal parts were divided in the same two sections than those selected at 0 h, the yellow root tip and the white adjacent part. Ninety-six h after Fe resupply root length increased approximately by 5 mm and new root tips were similar to those of controls. The yellow part enriched in flavins was still there, but located approximately 5 mm from the apex. Therefore, at 96 h samples were taken from

two parts, the newly developed white root tip (approximately 0 to 5 mm from the root apex) and the swollen yellow part (approximately 5 to 10 mm from the root apex).

4. 4. 3. Changes in the Flavin Concentration Induced by Fe Resupply

The total flavin concentration in the root yellow parts from Fe-deficient plants showed a tendency to increase with Fe resupply, although this increase was not statistically significant due to large SE of the data (Table 4.2). Average FAD, SI and SII increases were 15-27% and 30-40% at 24 and 96 h, respectively. Flavin mononucleotide phosphate was not detected in the samples (Table 4.2). In the white root parts the total flavin content decreased by 34% 24 h after Fe resupply, due to decreases in FAD, SI, SII and riboflavin (Table 4.2). The new white tips sampled 96 h after resupply had the lowest flavin concentrations, similar to those of plants grown with sufficient Fe. Flavin mononucleotide phosphate was only detected in the newly developed white root parts 96 h after Fe resupply.

	0 h		24 h		96 h	
	0-5 mm yellow tip	5-10 mm white part	0-5 mm yellow tip	5-10 mm white part	0-5 mm new white tip	5-10 mm yellow part
FAD	67.0±3.0	48.5±8.6	77.3±17.1	34.6±7.4	27.2±8.9	88.0±7.6
SI	405.7±78.5	91.0±30.4	505.4±61.2	65.2±11.8	8.9±2.7	559.0±192.0
SII	97.5±12.2	30.2±4.1	123.8±24.0	11.9±1.6	8.7±2.9	136.0±57.5
Rbf	13.1±1.8	6.1±0.9	33.1±17.0	5.0±0.5	2.9±0.2	18.8±4.0
FMN	<0.01	<0.01	<0.01	<0.01	16.5±5.4	<0.01
Total	583.3	175.8	739.6	116.7	64.2	801.8

Table 4. 2. Flavin concentrations (nmol g^{-1} FW) in root tips from Fe-deficient sugar beet plants at 0, 24 and 96 h after Fe resupply. (S I- Riboflavin 3´-sulphate and S II-Riboflavin 5´-sulphate) Data are means ± SE of 5 replications.

4. 4. 4. Changes in the Fe(III)-Chelate Reductase and Proton Extrusion Activities and Oxygen Consumption Rates with Fe Resupply

The FC-R activity decreased with Fe resupply both in the yellow and white parts of Fe-deficient roots (Table 4.3). The FC-R activity of the yellow parts decreased by approximately 20 and 80% within 24 and 96 h, respectively. The FC-R activity in the white parts decreased by 24% 24 h after Fe resupply. The newly developed white root parts had the lowest FC-R activities.

The acidification capacity was fully restricted to the yellow parts of Fe-deficient roots according to results obtained with agar plates (Fig 4.1). Quantification of the acidification was therefore determined only in the yellow root parts. Proton extrusion decreased by 30 and 71% within 24 and 96 h after Fe resupply, respectively, when compared to Fe-deficient yellow roots (Table 4.3).

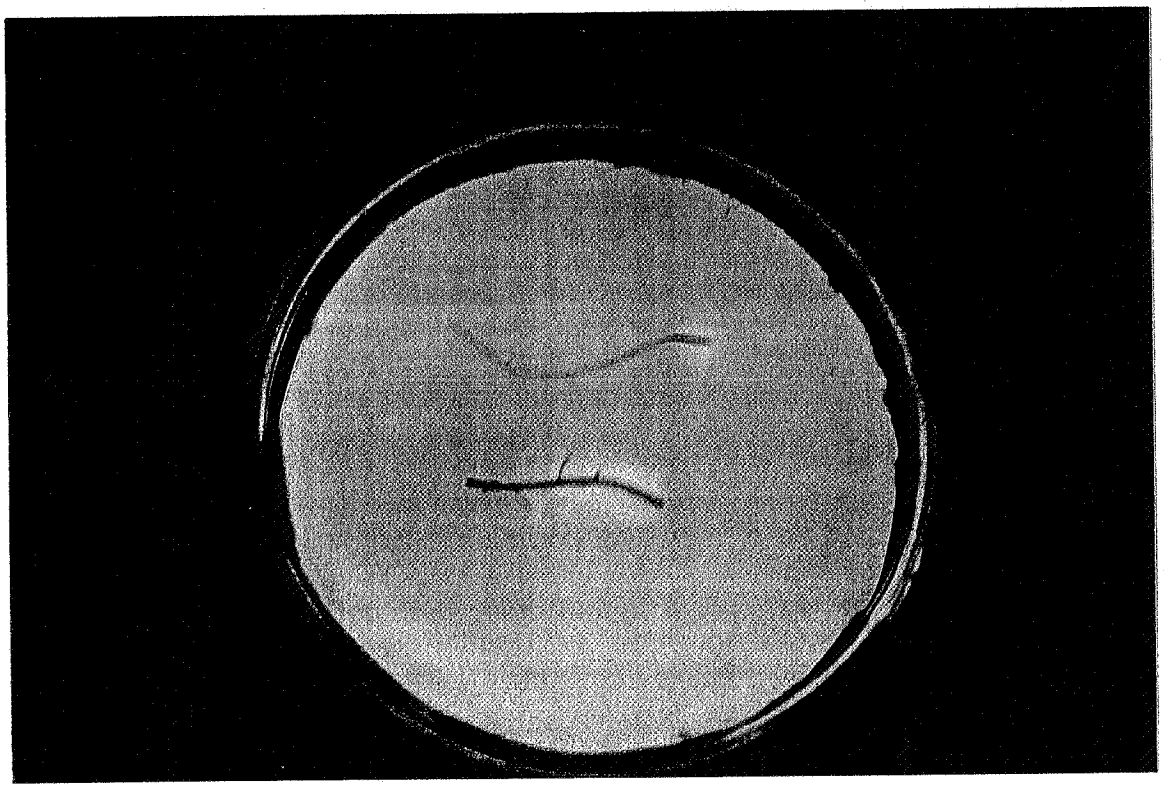

Figure 4. 1. Proton extrusion by Fe-deficient root tips in agar plates

Both the yellow and the white parts of the roots from Fe-deficient plants had decreased O_2 consumption rates after Fe resupply (Table 4. 3). Root yellow parts had 15 and 70% decreases in O_2 consumption rates within 24 and 96 h of Fe resupply, respectively. The O_2 consumption rates of the white parts decreased by 25% within 24 h of Fe resupply. The newly developed white root parts had the lowest O_2 consumption rates (Table 4.3), similar to those of plants grown with sufficient Fe.

	0 h		24 h		96 h	
	0-5 mm yellow tip	**5-10 mm white part**	**0-5 mm yellow tip**	**5-10 mm white part**	**0-5 mm new white tip**	**5-10 mm yellow part**
FC-R activity	137.9±9.2	39.3±4.7	112.1±9.6	30.0±5.8	16.7±3.0	25.0±4.0
ATPase activity	1.37±0.24	nm	0.96±0.04	nm	nm	0.40±0.14
O_2-consumption	627±69	137±17	536±46	103±6	75±5	190±10

Table 4. 3. Activities of FC-R (in nmol min^{-1} g^{-1} FW) and ATPase (in nmol H^+ min^{-1} g^{-1} FW), and oxygen consumption rates (in nmol O_2 min^{-1} g^{-1} FW) in Fe-deficient roots (0-5 and 5-10 mm from the root apex) 0, 24 and 96 hours after Fe resupply. Data are means ± SE of 4-10 replications. nm: not measured

4. 4. 5. Changes in Root Organic Anion Concentrations with Fe Resupply

The major organic anions in Fe-deficient roots (more than 95% of the total organic anion contents) were oxalate, malate, citrate and ascorbate. In the yellow parts of Fe-deficient sugar beet roots the total organic acid concentration decreased by 10 and 40% within 24 and 96 h of Fe resupply, respectively, on a FW basis (Table 4.4). Within 24 h citrate and malate decreased approximately by 20 and 35%, respectively, whereas at 96 h the decreases were 70 and 50%, respectively (Table 4.4). Ascorbate could not be detected after resupply in any of the root samples. Oxalate increased by 18% 24 h after Fe resupply and within 96h decreased to values similar to those found in Fe-deficient root tips. The minor organic anions cis-aconitate, succinate and fumarate decreased with Fe resupply whereas 2-oxoglutarate increased (Table 4.4).

The white root parts of Fe-deficient plants had 56% increases in total organic anion concentration 24 h after Fe resupply. This increase was caused by a 2.2-fold increase in oxalate concentration, since citrate, malate and ascorbate decreased by 50, 35 and 100%. The newly produced white root tips had 96 h after Fe resupply the lowest organic acid concentrations except for the minor acid 2-oxoglutarate, that was larger than that of the white parts of the Fe-deficient roots before resupply (Table 4.4).

	0 h		24 h		96 h	
	0-5 mm yellow tip	5-10 mm white part	0-5 mm yellow tip	5-10 mm white part	0-5 mm new white tip	5-10 mm yellow part
malate	5.95±0.78	4.32±0.80	3.81±0.46	2.79±0.44	2.14±0.35	2.87±0.51
citrate	2.60±0.54	2.70±0.65	2.15±0.48	1.37±0.28	0.37±0.08	0.80±0.40
cis-aconitate	0.40±0.14	0.04±0.00	<0.01	<0.01	<0.01	<0.01
2-oxoglutarate	0.06±0.01	0.02±0.01	0.12±0.04	0.03±0.01	0.06±0.02	0.28±0.10
fumarate	0.80±0.30	0.30±0.04	0.03±0.00	0.01±0.01	0.01±0.01	0.01±0.01
succinate*	0.123	0.048	0.053	0.008	0.007	0.018
oxalate	18.40±4.50	19.60±8.20	21.69±3.51	42.04±4.60	13.45±0.77	14.03±1.73
ascorbate	2.40±0.37	2.55±0.50	<0.01	<0.01	<0.01	<0.01
total	30.61	29.53	27.8	46.24	16.03	17.99

Table 4. 4. Concentrations of organic anions (μmol g^{-1} FW) in root tips from Fe-deficient sugar beet plants 0, 24 and 96 after Fe resupply. Data are means ± SE of 10 replications. * succinate co-eluted with an unidentified compound

4. 4. 6. Changes in Root Enzymatic Activities with Fe Resupply

We measured five enzymatic activities related to the TCA cycle (Table 4.5). The activities of MDH, fumarase and aconitase in extracts of the root yellow parts increased by 45, 19 and 8%, respectively, within 24 h of Fe resupply. After 96 h these activities decreased by 47, 50 and 65%, respectively, respect to the values found before resupply (Table 4.5). The activities of CS and ICDH decreased by 18 and 49%, respectively, within 24 h after Fe resupply, and by approximately 80% after 96 h.

Iron resupply caused within 24 h an increase in MDH, CS, aconitase and fumarase activities in the extracts of the root white parts with respect to the

values found before resupply (Table 4.5). Increases were 2.5-fold for MDH, 2.2-fold for fumarase, 2.5-fold for aconitase and 2.8-fold for CS. In the new root section developed after resupply activities were 82 (MDH), 200 (fumarase), 52 (aconitase) and 123% (CS) of those found in Fe-deficient white root tips. ICDH activity did not change significantly with Fe resupply.

The PEPC activity in the extracts of the yellow parts of Fe-deficient roots decreased by 52 and 91% within 24 and 96 h after Fe resupply, respectively (Table 4.5). In the extracts of the white root parts, PEPC activity increased by 50% after 24 h of resupply, whereas the newly developed root tips had 96 h after resupply values similar to those found in the extracts of the white parts of Fe-deficient roots.

The activities of LDH and PDC in the extracts of the root yellow tips decreased by 64 and 72%, respectively, within 24 h resupply, and by 86 and 72% after 96 h. In extracts of the white parts from Fe-deficient roots LDH and PDC activities decreased by 50-55% at 24 h of resupply. The new white root sections had at 96 h the lowest LDH activity measured, whereas that of PDC was similar to that of the white section of the Fe-deficient root tips before resupply (Table 4.5).

	0 h		24 h		96 h	
	0-5 mm yellow tip	5-10 mm white part	0-5 mm yellow tip	5-10 mm white part	0-5 mm new white tip	5-10 mm yellow part
MDH	112.1±24.6	25.3±2.0	162.2±27.6	50.6±9.3	46.2±3.4	59.7±2.3
CS	1.61±0.36	0.13±0.01	1.33±0.27	0.37±0.07	0.29±0.05	0.38±0.03
aconitase	1560±133	380±140	1684±368	940±237	578±81	560±74
ICDH	2.39±0.34	0.36±0.03	1.22±0.22	0.38±0.06	0.42±0.0	0.40±0.02
fumarase	3340±1100	594±112	3973±597	1300±260	1810±44	1689±165
PEPC	13.32±2.52	0.54±0.04	6.39±1.14	0.81±0.24	0.48±0.05	1.14±0.09
LDH	0.22±0.06	0.09±0.02	0.08±0.01	0.04±0.01	0.026±0.01	0.03±0.01
PDC	0.29±0.12	0.10±0.03	0.08±0.03	0.05±0.01	0.09±0.01	0.08±0.02

Table 4. 5. Enzymatic activities (nmol min^{-1} g^{-1} FW) in root tip extracts from Fe-deficient sugar beet plants at 0, 24 and 96 hours after Fe resupply. Data are means ± SE of 5 replications.

4. 4. 7. Changes in Root Nucleotide Concentrations with Fe Resupply

The total pyridine nucleotide pool increased with Fe resupply in both the yellow and white parts of the root tips from Fe-deficient plants (Table 4.6). Increases in NADPH, NADH, $NADP^+$ and NAD^+ were 1.5-, 2.1-, 2.3- and 2.8-fold, respectively, in the root yellow parts 24 h after Fe resupply. At 96 h after Fe resupply, concentrations of NADPH were 50% of the initial values, whereas NADH, $NADP^+$ and NAD^+ were 1.4-, 2.0- and 1.2-fold, respectively, of the initial values. As a result of these changes, in the yellow parts of Fe-deficient roots the NADPH/$NADP^+$ ratios were 0.59, 0.40 and 0.14 at 0, 24 and 96 h after Fe resupply, respectively (Table 4.6). The NADH/NAD^+ ratios in the yellow parts of Fe-deficient roots did not show significant changes in response to Fe resupply (Table 4.6).

	0 h		24 h		96 h	
	0-5 mm yellow tip	5-10 mm white part	0-5 mm yellow tip	5-10 mm white part	0-5 mm new white tip	5-10 mm yellow pa
NADPH	3.73±1.18	2.58±0.44	5.84±0.12	1.79±0.14	1.18±0.16	1.82±0.0
NADH	2.75±0.51	3.23±0.31	5.81±0.94	4.11±0.93	4.41±0.68	3.79±0.1
$NADP^+$	6.34±0.34	2.13±0.28	14.42±1.07	8.74±0.71	12.46±1.10	12.69±3.50
NAD^+	6.42±0.17	1.91±0.01	18.16±2.99	9.09±1.46	13.70±1.26	7.60±1.54
Total	19.24	9.85	44.23	23.73	31.75	25.90
ATP ($x10^{-3}$)	1.26±0.37	0.64±0.13	0.62±0.15	0.59±0.16	0.13±0.04	0.24±0.05
NADPH/$NADP^+$	0.59	1.21	0.40	0.20	0.09	0.14
NADH/NAD^+	0.43	1.69	0.32	0.45	0.32	0.50
%RED/TOTAL	33.7	59.0	26.3	24.9	17.6	21.7

Table 4. 6. Concentrations of pyridine nucleotides and ATP in root tips from Fe-deficient sugar beet plants at 0, 24 and 96 hours after Fe resupply. Data (in nmol g^{-1} FW) are means ± SE of 5 replications.

The white parts of Fe-deficient root tips had increased NADH, $NADP^+$ and NAD^+ concentrations with Fe resupply, whereas the concentration of NADPH concentration decreased (Table 4.6). This was associated to decreases in the

$NADPH/NADP^+$ and $NADH/NAD^+$ ratios within 24 h, from 1.2 to 0.2 and from 1.69 to 0.45, respectively. After 96 h of Fe resupply these ratios were 0.09 and 0.32, respectively, in the newly developed white tips.

The ATP concentrations in root yellow parts of Fe-deficient plants decreased by 51 and 81% within 24 and 96 h respectively of Fe resupply (Table 4.6). The white parts of Fe-deficient roots had ATP concentrations lower than those found in the yellow ones and did not change significantly (3%) within 24 h after Fe-resupply. The ATP concentration in the newly grown white tips was the lowest measured.

4. 4. 8. Redox Poise of the Q Pool

The total Q pool in the yellow parts of Fe-deficient roots did not show significant changes with Fe resupply (Table 4.7).

	0 h		24 h		96 h	
	0-5 mm yellow tip	**5-10 mm white part**	**0-5 mm yellow tip**	**5-10 mm white part**	**0-5 mm new white tip**	**5-10 mm yellow part**
Q_r (Q_{10})	6.48±2.80	2.79±0.48	4.67±0.19	2.49±0.61	1.19±0.55	1.73±0.33
Q_o (Q_{10})	6.01±3.07	7.54±2.80	6.53±0.11	5.41±0.16	5.58±0.99	10.33±0.54
Q_t (Q_{10})	12.49	10.33	11.20	7.90	6.77	12.06
Q_r/Q_t (Q_{10})	52%	27%	42%	31%	18%	14%

Table 4. 7. Concentrations of quinones (nmol g^{-1} FW) in root tips from Fe-deficient sugar beet plants at 0, 24 and 96 hours after Fe resupply. Data are means ± SE of 5 replications. Q_9 concentrations were negligible.

The reduced form of Q_{10} decreased with Fe resupply in the root yellow parts, whereas the oxidised Q_{10} increased (Table 4.7). As a result of these changes, the Q_{10} pools were 52, 42 and 14% reduced 0, 24 and 96 h after Fe resupply, respectively (Table 4.7). In the white parts of Fe deficient roots the average total Q pool decreased with Fe resupply (Table 4.7), due to decreases in both the reduced and oxidised forms of Q_{10} although the differences were not statistically significant (Table 4.7). The Q_{10} pools in the white root parts were

approximately 30% reduced before and after Fe resupply. The newly developed white tips had the Q_{10} pool 18% reduced (Table 4.7).

4. 5. Discussion

In the present work we have studied the kinetics of deactivation of the root responses to Fe deficiency in the model Strategy I plant species sugar beet. These responses included among others morphological changes and enhanced FC-R and proton extrusion activities. The results indicate that the kinetics of deactivation of the responses was relatively slow, occurring gradually in a time scale of several days.

Upon 24 h of Fe resupply the yellow root tips showed only a slight enlargement (approximately 1 mm) whereas root Fe concentrations had already increased several fold. By this time, the leaf Fe concentration had increased also significantly, with values half of those found in control leaves (López-Millán, chapter 7). At this resupply time, the FC-R and proton extrusion activities had decreased only by 20 and 30%, respectively. These data indicate that the deactivation of both responses 24 h after Fe resupply was significant but still not very marked. These changes were associated to 17 and 35% decreases in the root concentrations of the organic anions citrate and malate, respectively. One of the largest changes among all physiological parameters measured within 24 h of Fe resupply was a 50% decrease in PEPC activity.

After 96 h of Fe resupply to Fe-deficient plants roots had a new apical growth of approximately 5 mm. This newly developed root tip was white in colour, did not have root hairs and had organic acid contents and enzymatic activities similar to the values described for control, Fe-sufficient roots (López-Millán, chapter 3). The yellow root sections (those showing typical Strategy I responses to Fe deficiency before Fe resupply) still had a high flavin content, but the FC-R and proton extrusion activities had decreased by 80 and 70%, respectively. These values were still 2-fold higher than those of Fe-sufficient root tips (López-Millán, chapter 3). At this resupply time, citrate and malate concentrations were decreased in the yellow root sections by 70 and 50%, respectively. These values were still 4 to 6-fold higher than those of control root

tips (López-Millán, chapter 3). The decrease in PEPC activity was very marked (90%), associated with general decreases in MDH, CS, ICDH, fumarase and aconitase activities. From these data we can conclude that the deactivation kinetics of the sugar beet root responses is relatively slow.

It has been recently reported that Fe-deficient plants have an increased capacity to fix C in their roots through enhanced activities of PEPC, MDH and CS (López-Millán, chapter 3). The PEPC activity in yellow Fe-deficient root tips decreases markedly after Fe resupply, and this decrease was associated to significant decreases in the activities of MDH and CS. Concomitantly, there were general decreases in the concentrations of citrate and malate. These data suggests that the activity of PEPC is regulated by the concentrations of Fe in the growth medium, in agreement with the suggestion that the enhancement in the activity of this enzyme is a key step in the development of Fe deficiency responses in sugar beet.

Mitochondrial metabolic processes are known to be enhanced in Fe-deficient roots (Landsberg, 1994). The total mitochondrial quinone pool in yellow Fe-deficient root tips did not show significant changes when Fe was resupplied to Fe-deficient plants, whereas the total pyridine nucleotide pool increased within 24 h and decreased to the initial levels within 96 h of Fe resupply. These data may suggest that the relative amount of mitochondria in root tips did not change even after 96 h of Fe resupply. Both pools were initially more reduced than the controls and became gradually more oxidised after Fe resupply. Mitochondrial activity did not appear to change much after 24 h of resupply, but after 96 h it was markedly decreased, as indicated by the decreases in ATP, O_2 consumption and the activities of aconitase, fumarase and ICDH. Most of these parameters reached values similar to those of Fe-sufficient control plants within 96 h of Fe resupply.

The activities of PDC and LDH, two enzymes related to anaerobic metabolism, decreased by approximately 70% within 24 h of Fe resupply. The induction of enzymes associated to responses to hypoxia has been reported in roots of Fe-deficient barley (Suzuki et al., 1998), tomato (Herbik et al., 1996) and sugar beet (López-Millán, chapter 3). The rapid deactivation of the LDH and PDC enzymatic activities upon resupply may originate in increases in O_2 availability.

Root O_2 consumption decreased by 15% 24 h after resupply, and this decrease is likely to arise from the fact that both O_2 and Fe(III) could be acceptors for the FC-R (González-Vallejo et al., 1998, 1999). An alternative explanation for the deactivation of PDC and LDH could be a decrease in malic enzyme activity mediated by the decreases in malate occurring after Fe resupply. This would in turn cause a decrease in the capacity for regeneration of pyruvate, the substrate for PDC and LDH enzymatic activities.

In summary, Fe resupply to Fe-deficient sugar beet plants caused a progressive deactivation of the FC-R and proton extrusion activities, the two major root responses to Fe deficiency typical of Strategy I plant species. The kinetics of this deactivation was quite slow, being very moderate in 24 h and extensive in 96 h. Iron resupply produced rapid increases in root Fe and a progressive oxidation of the mitochondrial quinone and pyridine nucleotide pools. Changes occurring in 24 h included decreases in the activity of PEPC and the enzymes related to anaerobic metabolism PDC and LDH. Longer resupply times induced large decreases in PEPC and marked decreases in the activities of different enzymes involved in organic acid synthesis and in the size of the organic acid pools.

CAPÍTULO 5

EFECTOS DE LA DEFICIENCIA DE Fe SOBRE LAS COMPOSICIONES DEL FLUIDO APOPLÁSTICO DE HOJA Y DE LA SAVIA EN REMOLACHA. IMPLICACIONES EN EL TRANSPORTE DE Fe Y C

5. Effects of Fe Deficiency on the Composition of the Leaf Apoplastic Fluid and Xylem Sap in Sugar Beet. Implications for Fe and C transport

5. Effects of Iron Deficiency on the Composition of the Leaf Apoplastic Fuid and Xylem Sap in Sugar Beet

Implications for Iron and Carbon Transport

5. 1. Abstract

The effects of Fe deficiency on the chemical composition of two plant sites crucial for long-distance Fe transport, the xylem sap and the leaf apoplastic fluid, have been characterised in the model plant sugar beet. Apoplastic pH was estimated from direct pH measurements in the apoplastic fluid and xylem sap obtained by centrifugation and by fluorescence of leaves incubated with 5-carboxyfluorescein and fluorescein isothiocyanate-dextran. Iron deficiency caused a slight decrease in the pH of the leaf apoplast (from 6.3 to 5.9) and xylem sap (from 6.0 to 5.7) of sugar beet plants. The major organic acids found in leaf apoplastic fluid and xylem sap were malate and citrate. Total organic acid concentration in control plants was 4.3 mM in apoplastic fluid and 9.4 mM in xylem sap and increased to 12.2 and 50.4 mM, respectively, in Fe-deficient plants.

Total apoplastic inorganic cation concentrations (Ca, K and Mg) increased with Fe deficiency from 32 to 67 mM in apoplastic fluid and from 116 to 124 mM in xylem sap. Total apoplastic inorganic anion concentrations (chloride, nitrate, sulphate and phosphate) increased with Fe deficiency from 30 to 53 mM in apoplastic fluid and decreased in xylem sap from 78 to 38 mM. Iron concentrations decreased with Fe deficiency from 5.5 to 2.5 μM both in apoplastic fluid and xylem sap. The major Fe species predicted to exist by chemical speciation in the apoplastic fluid and xylem sap were $FeCitOH^-$ in the controls and $FeCit_2^{-3}$ in the Fe-deficient plants. Our data suggest the existence of an influx of organic acids from the roots to the leaves via xylem, probably associated to an anaplerotic carbon dioxide fixation by roots.

5. 1. 1. Abbreviations: FC-R, ferric-chelate reductase; FITC, fluorescein isothiocyanate; G6PDH, glucose-6-phosphate dehydrogenase; GABA, γ-amino-n-butyric acid; HPI, hexose phosphate isomerase; MDH, malate dehydrogenase; MS, mass spectroscopy; PAR, photosynthetic active radiation; PEPC, phosphoenol-pyruvate carboxylase; PM, plasma membrane; PPFD, Absorbed photosynthetic photon flux density; PVDF, polyvinyl fluoride; SPAD, portable Chl meter; 5-CF, 5 carboxyfluorescein.

5. 2. Introduction

When grown under limited Fe supply, many plant species develop Fe-acquisition mechanisms that are not expressed or under-expressed when Fe supply is sufficient. The most widespread Fe-acquisition mechanism in plants, Strategy I, has been found in dicotyledonous and non-graminaceous monocotyledonous species (Marschner et al., 1986; Römheld and Marschner, 1986; Bienfait, 1988; Brown and Jolley, 1988). This Strategy involves morphological changes, such as increased formation of lateral roots, root hairs and transfer cells, all of them increasing root surface for Fe uptake (Kramer et al., 1980; Landsberg, 1982; Schmidt, 1999). Strategy I also includes physiological changes, such as the development of an increased proton excretion which decreases rhizosphere pH (Brown, 1978), a release of reducing and/or chelating substances such as phenolics and flavins

(Welkie and Miller, 1960; Susín et al., 1994) and a two-step mechanism for Fe uptake, in which Fe(III) is first reduced by a plasma membrane-bound ferric reductase enzyme (Moog and Brüggemann, 1994; Susín et al., 1996; Robinson et al., 1999) and then absorbed as Fe(II) (Chaney et al., 1972). Once Fe enters the root cell it must be transported to the leaves. Iron is thought to be transported in the xylem as Fe(III), probably complexed by citrate (Tiffin, 1966; Brown and Chaney, 1971; White et al., 1981a; Cataldo et al., 1988). The mechanism of Fe uptake by leaf cells has been much less studied than the corresponding processes in the roots.

The apoplastic compartment occupies 5% or less of the plant tissue volume of aerial organs (Steudle et al., 1980; Parkhurst, 1982) and root cortexes (Vakhmistrov, 1967). Solute concentrations in the apoplast of aerial organs are determined by the balance of import via xylem, absorption by cells and export by phloem. Due to the small apoplastic volume, relatively small changes in these fluxes could result in large changes in the apoplastic composition. The apoplast contains enzymes (Li et al., 1989; Pinedo et al., 1993), high concentrations of metabolites such as ascorbic acid (Polle et al., 1990; Luwe et al., 1993) and sugars (Tetlow and Farrar, 1993), plays important roles in the transport and storage of mineral nutrients (Starrach and Mayer, 1989; Wolf et al., 1990; Zhang et al., 1991) and is involved in signal transmission (Hartung et al., 1992). Studies have been made on the composition of the apoplast under different conditions (Clarkson, 1984; Blatt, 1985; Bowling, 1987; Grignon and Sentenac, 1991; Speer and Kaiser, 1991; Tetlow and Farrar, 1993; Canny, 1995). Primary reactions that lead to symptoms of nutrient deficiency or toxicity take place in the apoplast (Mengel and Geurtzen, 1988; Speer and Kaiser, 1991).

Iron trafficking in the apoplast is mandatory for Fe uptake processes by root cells (Longnecker and Welch, 1990, Zhang et al., 1991). However, little is known so far about the changes induced in the leaf apoplast by Fe deficiency. Once in the leaf apoplast, Fe(III) has been shown to be reduced by a mesophyll plasma membrane bound ferric chelate reductase similar to that present in roots (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; González-Vallejo et al., 2000). It has been suggested that Fe reduction and transport across the plasma membrane of mesophyll cells is a crucial step that could be impaired by Fe deficiency through an increase of apoplastic pH (Mengel, 1995; Kösegarten et al.,

1999). Recently, it has been shown that mesophyll protoplasts have lower ferric chelate reductase activity on a protoplast surface basis when Fe-deficient (González-Vallejo et al., 2000).

The aim of this work was to investigate the effects of Fe deficiency on the composition of the apoplast and xylem sap of the model plant sugar beet, in order to understand the role of these compartments in the transport and acquisition of Fe by leaf cells.

5. 3. Materials and Methods

5. 3. 1. Plant Material

Sugar beet (*Beta vulgaris* L., Monohil hybrid from Hilleshög, Landskröna, Sweden) was grown in a growth chamber with a PPFD of 350 µmol m^{-2} s^{-1} PAR at a temperature of 25°C, 80% relative humidity and a photoperiod of 16 h light/ 8 h dark. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for two more weeks in half-strength Hoagland nutrient solution with 45 µM Fe and then transplanted to 20 L plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution (Terry, 1980) with either 0 or 45 µM Fe. The pH of the Fe-free nutrient solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$. This treatment simulates conditions usually found in the field leading to Fe deficiency (Susín et al., 1994). Young, fully-expanded leaves from plants grown for ten days in the presence or absence of Fe were used in all experiments.

5. 3. 2. Chlorophyll Determination

Chlorophyll (Chl) concentration was estimated non-destructively with a portable Chl meter (SPAD-502, Minolta, Osaka, Japan). For calibration, leaves with different degrees of Fe deficiency were first measured with the SPAD and then extracted with 100% acetone in the presence of Na ascorbate and Chl measured spectrophotometrically (Abadía and Abadía, 1993).

5. 3. 3. Apoplastic Fluid and Xylem Sap Isolation

Apoplastic fluid was obtained from whole sugar beet leaves by direct centrifugation as in Dannel et al. (1995) with some modifications. Leaves were excised at the base of the leaf lamina with a razor blade in the growth chamber and transported to the laboratory with the petiole immersed in de-ionized water. Once in the laboratory the petiole was excised under water. Each leaf was then rolled and placed into a plastic syringe barrel, with the petiole side at the narrow end of the syringe. Leaf-filled syringes were centrifuged at 4°C and a small volume of apoplastic fluid was obtained from the bottom of the centrifuge tube. Preliminary experiments were carried out to ascertain contamination by cytoplasmic components, by increasing centrifugal force in steps of 500*g* (15 min each) from 1500 to 6000*g*, the corresponding fluid being collected at each centrifugation step. In the final protocol, a first centrifugation was made at low speed (2500*g*, 15 min) to remove the xylem sap of the main vein and apoplastic fluid was obtained in a second centrifugation step (4000*g*, 15 min).

For xylem sap isolation, sugar beet petioles were excised under water with a razor blade at their base and near the leaf lamina. Three petioles were placed into a plastic syringe barrel upside down and xylem sap was collected by centrifugation for 15 min at 4000*g* and 4°C.

Cytosolic hexose phosphate isomerase (c-hpi; EC 5.3.1.9) and malate dehydrogenase (c-mdh; EC 1.1.1.37) were used as cytosolic contamination markers for both apoplastic fluid and xylem sap. The activity of c-hpi was determined using fructose-6-phosphate as substrate, which is converted by c-hpi into glucose-6-phosphate. This is then oxidised by exogenous G6P-DH and the simultaneous reduction of $NADP^+$ was measured from the increase in A_{340}. The final reaction mixture (pH 8.0) was 50 mM Tris, 5 mM $MgCl_2$, 1 mM NaCl, 0.40 mM $NADP^+$, 0.46 U/mL G6P-DH and 1.4 mM fructose-6-phosphate (Bergmeyer et al., 1974). The activity of c-mdh was determined using oxalacetate as substrate and measuring the decrease in A_{340} due to the enzymatic oxidation of NADH. The final reaction mixture (pH 9.5) was 46.5 mM Tris, 0.1 mM NADH and 0.4 mM oxalacetate (Dannel et al., 1995). The activity of these two markers in leaf apoplastic fluid and xylem sap was checked against the total activities in total homogenates of leaf tissue and petioles, respectively. To measure these enzymatic

activities one leaf or petiole was homogenised with 2 mL of a buffer (pH 8.0) containing 100 mM Hepes, 30 mM sorbitol, 2 mM DTT, 1 mM $CaCl_2$, 1% BSA and 1% PVP. The supernatant was collected and analysed immediately after 10 min centrifugation at 10000*g*.

5. 3. 4. pH Measurements

The pH of the apoplastic fluid and xylem sap was measured directly in apoplastic fluid and xylem sap obtained by centrifugation with a microelectrode (Physitemp, Clifton, New Jersey). Apoplastic pH was also measured *in vivo* by fluorescence according to Hoffman et al. (1992) with 5-carboxyfluorescein (5-CF) and FITC-dextran. The fluorescence emission at 540 nm of these dyes is pH-dependent when excited at 490 nm but almost pH-independent when excited at 460 nm. Therefore, the ratio of fluorescence intensities obtained with excitation at 490 and 460 nm is related to the pH of the compartment where the dye is located. Leaves were excised and the cut end of the petiole was exposed to incubation medium containing 5 µM 5-CF or 500 µM FITC-dextran (4000 D, 0.01 mol FITC per mol glucose, Sigma, St. Louis, Missouri), 1 mM KCl, 0.1 mM NaCl, 0.1 mM $CaCl_2$ at pH 5.5. The incubation was carried out for 5 h at 25°C at room ambient light (15-25 µmol photons m^{-2} s^{-1}). The level of auto-fluorescence was subtracted from total fluorescence. Two leaves per Chl level (each from different plant) were taken and four measurements were carried out in different areas of each leaf.

5. 3. 5. Organic Ion Analysis

Organic anions were quantified by HPLC with a 300 x 7.8 mm Aminex ion-exchange column (HPX-87H, Bio-Rad, Hercules, CA) in a HPLC Waters system, including a 600E multi-solvent delivery system, a 996 photodiode array detector and Millennium 2010 software. Apoplast and xylem samples were filtered with a 0.45 µm PVDF membrane (LIDA, Kenosha, WI). Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (8 mM sulphuric acid) was pumped with a 0.6 mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to cis-aconitate, citrate, 2-oxoglutarate, malate, succinate and

fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma. The identity of some peaks was further confirmed by UV-VIS and MS. Quantification was made with known amounts of each anion using peak areas.

5. 3. 6. Aminoacid Analysis

Aminoacids were quantified by HPLC (Stein et al., 1957). Chromatography was carried out in an Alpha plus aminoacid analyser (Pharmacia LKB Biotecnology, Upsala, Sweden) with a 200 x 4 mm column packed with a cation-exchanger resin (polystyrene divinil-sulphobencene). The mobile phase was citrate buffer with increasing pH. Aminoacids were detected at 570 nm after reaction with ninhydrin, and identified by comparison of their retention times with those of standards. Quantification was made from the peak areas.

5. 3. 7. Sugar Analysis

Sugars (glucose, fructose and sucrose) were analysed by HPLC with a 300 x 4 mm Spherisorb-NH_2 column (Waters) and a HPLC Waters system, including a 590 pump, a differential refractometer R401 detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (acetonitrile: water, 860:140) was pumped with a 3.5 mL min^{-1} flow rate. Peaks corresponding to glucose, fructose and sucrose were identified by comparison of their retention times with those of known standards from Sigma. Quantification was made from the peak areas.

5. 3. 8. Inorganic Ion Analysis

Ca and Mg were determined by atomic absorption spectrophotometry and K by emission spectrophotometry. Fe was determined in xylem sap and apoplastic fluid by graphite furnace atomic absorption spectrometry (Varian SpectrAA with Zeeman correction). Each sample was analysed in triplicate.

Inorganic anions (nitrate, sulphate, chloride and phosphate) were quantified by HPLC with a 4.6 x 75 mm IC-Pak A HR ion-exchange column (Waters, Milford, MA) in a HPLC Waters system, including a 600E pump, a 432 conductivity detector and Millennium 2010 software. Samples were injected with a Rheodyne injector (50 µL loop). Mobile phase (11 mM borate-gluconate) was pumped with a 1.0 mL min^{-1} flow rate. Quantification was made with known amounts of each anion using peak areas.

5. 3. 9. Chemical Speciation

Concentrations of the different Fe-chelate species were estimated with the software MinteqA2 (Allison et al., 1991) by using the ionic environment of the apoplastic fluid. Chelate formation constants used for citrate and malate were derived from those given by Holden et al. (1991) and Cline et al. (1982), respectively. At an ionic strength of 0 M, the $\log_{10}$ of the chelate formation constants used for the Fe-citrate species $[FeCit]^0$, $[FeCitH]^{+1}$, $[FeCitOH]^{-1}$, $[FeCit_2]^{-3}$ and $[Fe_2Cit_2(OH)_2]^{-2}$ were 13.13, 14.43, 10.11, 20.13 and 24.51, respectively. The $\log_{10}$ of the chelate formation constants for the Fe-malate species $[FeMal]^{+1}$, $[Fe_2(MalOH)_2]^0$, $[Fe_2Mal(MalOH)_2]^{-2}$ and $[Fe_3Mal_5OH_4]^{-3}$ were 8.39, 15.32, 20.33 and 27.75, respectively.

5. 4. Results

5. 4. 1. Apoplastic Fluid Isolation

The volume of apoplastic fluid obtained by centrifugation increased gradually when the centrifugal forces increased, in both Fe-deficient and control, Fe-sufficient sugar beet leaves (Fig. 5.1). The activities of the cytosolic marker enzymes in the apoplastic fluid were low at centrifugal forces lower than 4000*g* and increased markedly thereafter. When using seven successive steps of centrifugation, the activities of c-mdh were less than 7 and 4% of the total leaf homogenate activities at 4000*g* in control and Fe-deficient sugar beet leaves,

respectively (Fig. 5.1). At higher centrifugation forces the activities of c-mdh reached values of 70 and 43% of the total leaf homogenate activities, indicating loosening or rupture of cell membranes. Similar results were obtained for c-hpi activity at centrifugal forces of 4000*g* or lower, with activities equivalent to less than 7 and 10% of the total leaf homogenate activities in control and Fe-deficient sugar beet, respectively.

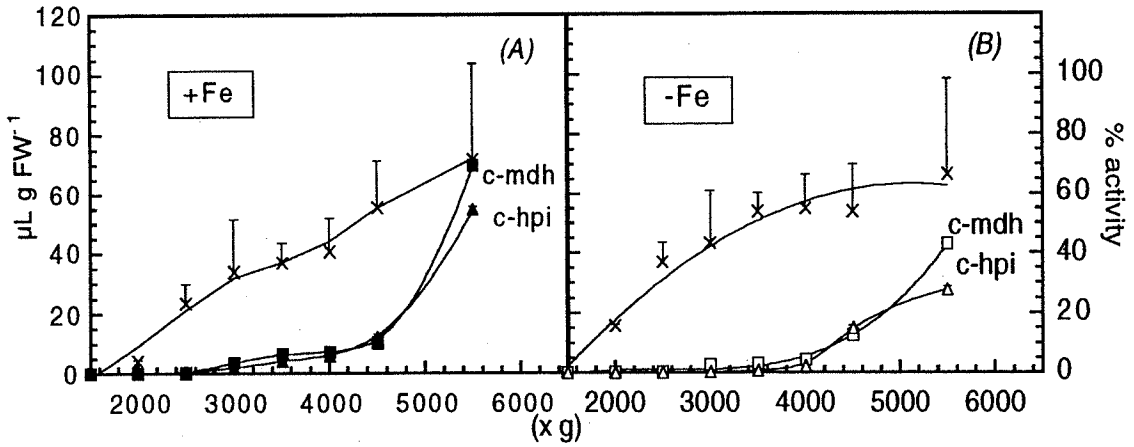

Figure 5. 1. Effects of the centrifugal force on the total volume of apoplastic fluid (x) and on the activity of the cytosolic enzymes malate dehydrogenase (squares) and hexose phosphate isomerase (triangles) in apoplastic fluid obtained by centrifugation from Fe-sufficient (A) and Fe-deficient (B) sugar beet leaves. Data are means ± SE of 10 replications.

When using only two centrifugation steps at 2500 and 4000*g* the c-mdh and c-hpi activities in apoplastic fluid were 2-3% of those found in total leaf homogenates (Table 5.1). In routine experiments apoplastic fluid was collected at 4000g, after carrying out a preliminary centrifugation of the leaves at 2500g to discard fluid containing the xylem sap of the main vein. In the case of xylem sap obtained from the centrifugation of petioles the activities of the cytosolic marker enzymes were always 1% or less of those found in total petiole homogenates (Table 5.1). Contamination by cytosolic enzymes was always assessed in each sampling.

	Fe-sufficient c- mdh	c-hpi	Fe-deficient c-mdh	c-hpi
Leaf	0.472±0.200	84±10	0.626±0.321	57±3
Petiole	0.071±0.009	10.8±5.7	0.174±0.030	24.6±9.0
Apoplast	0.013±0.004 (3%)	2.5±1.8 (3%)	0.014±0.010 (2%)	1.7±0.5 (3%)
Xylem sap	ND (0%)	0.07±0.01 (0.6%)	0.001±0.0007 (0.7%)	0.24±0.01 (1%)

Table 5. 1. Activities of c-mdh and c-hpi in whole extracts of leaves and petioles (in nmol g^{-1} FW s^{-1}), apoplastic fluid and xylem sap (in nmol mL^{-1} s^{-1}) of Fe-sufficient (300 µmol Chl m^{-2}) and Fe-deficient -Fe (50 µmol Chl m^{-2}) sugar beet. Values in brackets represent cytosolic contamination of apoplastic fluid and xylem sap. Data are means ± SE of 5 replicates.

5. 4. 2. Apoplastic Fluid and Xylem Sap pH

The apoplastic pH of sugar beet leaves was measured using two different methods, pH determination in the apoplastic fluid obtained by centrifugation and *in vivo* estimation by means of fluorescent dyes (Hoffman et al., 1992) (Fig. 5.2). When determined with a microelectrode the pH of the apoplastic fluid was slightly decreased by Fe deficiency from approximately 6.3 in control leaves to 5.9 in markedly Fe-deficient leaves (Fig. 5.2A). The pH of sugar beet xylem sap obtained by centrifugation decreased with Fe deficiency from 6.0 to 5.7 (Fig. 5.2B).

In vivo pH measurements were carried out with the fluorescent dyes 5-CF and FITC-dextran (Fig. 5.2A). The pH values estimated *in vivo* using 5-CF were very similar to those found by direct pH measurement in the apoplastic fluid, with values of approximately 6.5 in control leaves and 6.0 in markedly Fe-deficient leaves. With FITC-dextran, pH was in the range 5.4-5.6 in all leaves. The different pH values obtained using 5-CF and FITC-dextran are possibly related to their different size and permeability through biological membranes, since the larger size of FITC-dextran may difficult its access to the whole of the apoplastic space.

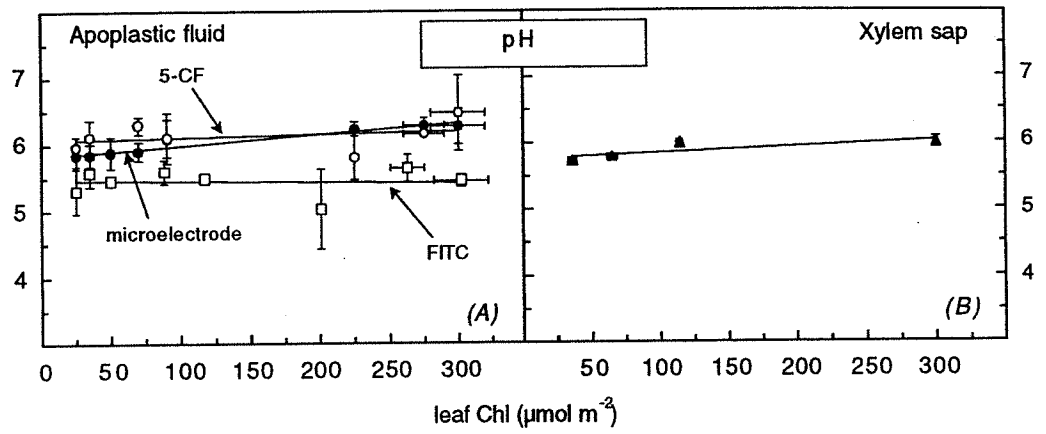

Figure 5. 2. Effects of Fe deficiency on the xylem sap and apoplastic pH in sugar beet leaves. Measurements were made with a microelectrode in apoplastic fluid (solid circles in A) and xylem sap (solid triangles in B), obtained by centrifugation and *in vivo* by fluorescence with the dyes 5-CF (open circles in A) and FITC-dextran (open squares in A). Data are means ± SE of 3 replications.

5. 4. 3. Organic Anion Composition

Organic anions were quantified by HPLC. Peaks corresponding to cis-aconitate, citrate, 2-oxoglutarate, malate, succinate and fumarate were identified in both apoplastic fluid and xylem sap (Fig. 5. 3). Succinate co-eluted with another unidentified compound with absorption maxima at 261 and 210 nm.

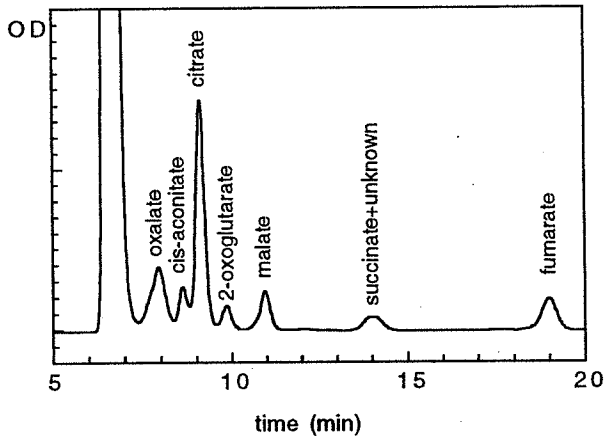

Figure 5. 3. Separation of organic acids by ion exchange high pressure liquid chromatography. Organic acids were detected at 210 nm.

Apoplastic fluid from control and Fe-deficient sugar beet contained concentrations of citrate, malate and succinate in the mM range, and of cis-aconitate, 2-oxoglutarate, and fumarate in the µM range (Fig. 5.4). Iron deficiency caused a general increase in organic anion concentrations in the apoplastic fluid, reaching a maximum in chlorotic leaves with approximately 35 µmol Chl m^{-2}. Maximum increases in the concentration of the three major anions in apoplastic fluid were 6-fold for citrate (0.7 to 4.4 mM), 3-fold for malate (from 0.7 to 2.2 mM) and 2-fold for succinate (from 1.4 to 2.6 mM) (Fig. 5.4A). For the minor organic anions the maximum increases in concentration were 4-fold for cis-aconitate (from 72 to 300 µM), 5-fold for 2-oxoglutarate (from 32 to 145 µM) and 11-fold for fumarate (from 0.8 to 9 µM) (Fig. 5.4B).

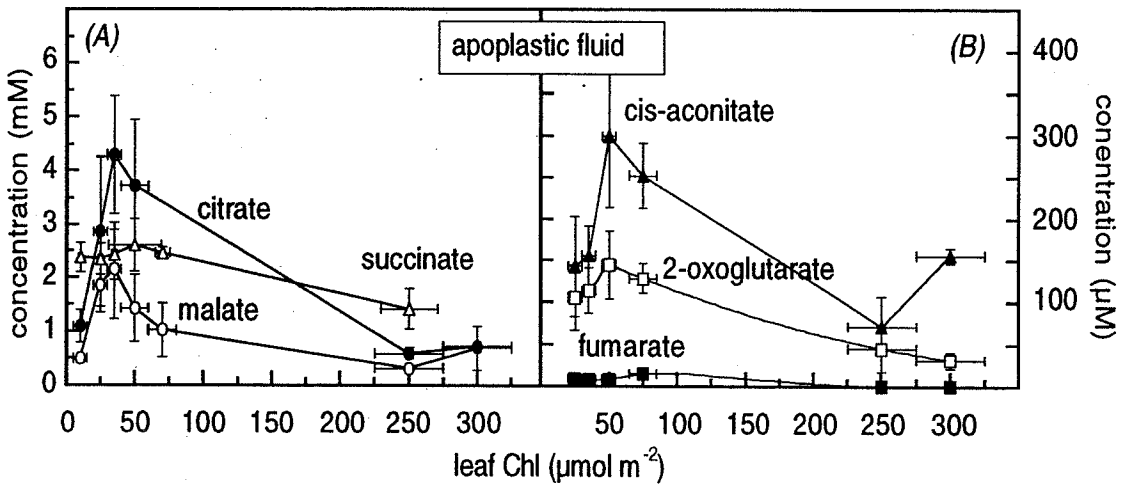

Figure 5. 4. - Effects of Fe deficiency on the organic anion concentrations in apoplastic fluid of sugar beet leaves. A. Major organic anions (in mM): citrate (solid circles), malate (open circles) and succinate (open triangles). B. Minor organic anions (in µM): 2-oxoglutarate (open squares), cis-aconitate (solid triangles) and fumarate (solid squares). Data are means ± SE of 10 replications.

The major organic anions in xylem sap were also citrate, malate and succinate (Fig. 5.5). The concentrations of citrate, malate and succinate in xylem sap increased with Fe deficiency 24-fold (from 0.2 to 4.7 mM), 14-fold (from 2.1 to 30.2 mM) and 2-fold (from 3.5 to 7.0 mM), respectively (Fig. 5.5A), when compared to the controls. The highest organic anion concentrations were found in leaves with approximately 35 µmol Chl m-2. Cis-aconitate increased with Fe deficiency 47-fold

(from 4 to 190 µM), 2-oxoglutarate 14-fold (from 43 to 630 µM) and fumarate 23-fold (from 27 to 645 µM) (Fig. 5.5B).

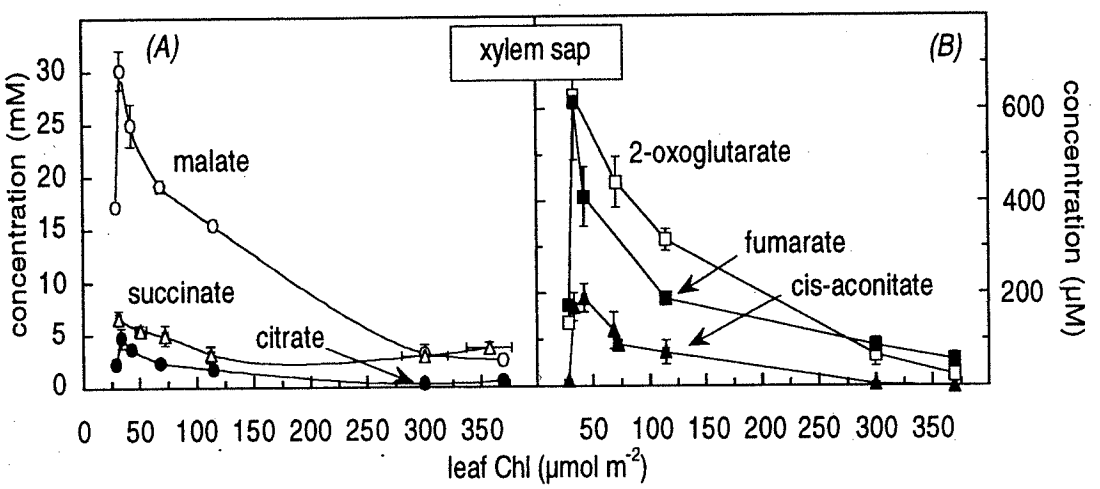

Figure 5. 5 Effects of Fe deficiency on the organic anion concentrations in xylem sap from sugar beet leaves. A. Major organic anions (in mM): citrate (solid circles), malate (open circles) and succinate (open triangles). B. Minor organic anions (in µM): 2-oxoglutarate (open squares), cis-aconitate (solid triangles) and fumarate (solid squares). Data are means ± SE of 10 replications.

5. 4. 4. Aminoacid Composition

Aminoacid concentrations in apoplastic fluid were always in the µM range. Iron deficiency caused increases in the apoplastic concentration of total aminoacids of approximately 40%. Major aminoacids in apoplastic fluid were Asp, Ser, Glu, Gln, Pro, Ala, Val and GABA (Table 5.2). The concentrations of Asp, Gln and Ala in apoplastic fluid decreased with Fe deficiency, whereas those of Ser, Glu, Val, Pro and GABA increased. Among the minor aminoacids in apoplastic fluid, the concentration of Lys, Thr, Asn, Gly and Ile decreased with Fe deficiency, whereas those of Leu and His increased (Table 5.2).

	Fe-sufficient (+Fe)	Fe-deficient (-Fe)	-Fe/+Fe
Asp	262 ± 20	177 ± 28	0.7
Ser	121 ± 42	182 ± 56	1.5
Glu	320 ± 3	538 ± 83	1.7
Gln	450 ± 180	396 ± 90	0.9
Pro	ND	77 ± 43	–
Ala	303 ± 130	243 ± 52	0.8
Val	60 ± 55	510 ± 50	8.5
GABA	56 ± 11	166 ± 12	3.0
Thr	44 ± 15	39 ± 8	0.9
Asn	59 ± 10	33 ±3	0.5
Gly	56 ± 8	48 ± 12	0.8
Ile	16 ± 16	9 ± 4	0.6
Leu	ND	14 ± 7	–
Lys	31± 0.5	12 ± 0.1	0.4
His	ND	4 ± 2	–
Total aminoacid	1,778	2,448	1.4

Table 5. 2. Concentrations of aminoacids (in µM) in sugar beet apoplastic fluid from Fe-deficient (-Fe, 50 µmol Chl m^{-2}) and Fe-sufficient (+Fe, 300 µmol Chl m^{-2}) leaves. Data means ± SE of five replications.

5. 4. 5. Sugars

Iron deficiency caused changes in the sugar concentrations of the apoplastic fluid (Fig. 5.6A). Moderate Fe deficiency caused a 4.5-fold decrease in Suc concentration (from 3 7 to 0.8 mM), although severely deficient leaves had Suc concentrations similar to the controls (2.6 mM). Glc concentrations decreased with Fe deficiency (from 16.4 to 9.0 mM), whereas Fru concentrations increased 2-fold with Fe deficiency (from 4.5 to 9.1 mM).

The sugar concentrations were higher in xylem sap than in apoplastic fluid. In xylem sap moderate Fe deficiency caused a 2-fold increase in Fru and a marked decrease in Glc (Fig. 5.6B). In severely deficient leaves, however, the concentrations of Fru and Glc were, respectively, 60% and 2-fold of those found in the control leaves. The concentration of Suc did not show major changes with Fe deficiency (Fig 5.6B).

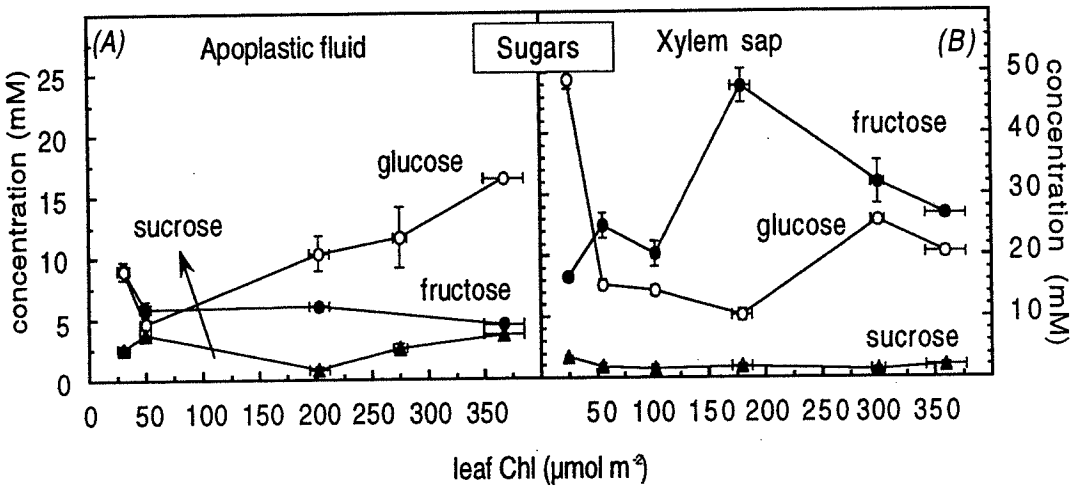

Figure 5. 6. Effects of Fe deficiency on the concentration of sugars in leaf apoplastic fluid (A) and xylem sap (B) of sugar beet plants. Glucose (open circles), fructose (solid circles) and sucrose (solid triangles) were in mM. Data are means ± SE of 5 replications.

5. 4. 6. Inorganic Ion Composition

The concentrations of inorganic cations (Ca, K and Mg) in apoplastic fluid of sugar beet leaves increased with Fe deficiency with a similar trend to that found for the organic anions. The maximum K, Ca and Mg concentrations were found in apoplastic fluid of Fe-deficient leaves with approximately 50 µmol Chl m^{-2} (Fig. 5.7A). K, Ca and Mg in apoplastic fluid were 30, 0.5 and 2 mM in control plants and reached maximal concentrations of 50, 10 and 7 mM in Fe-deficient plants. Therefore, the largest increase in apoplastic fluid concentrations with Fe deficiency was 20-fold for Ca, followed by 4-fold for Mg and 2-fold for K.

Nitrate, Cl^- and SO_4^{2-} concentrations in the apoplastic fluid increased with Fe deficiency (Fig. 5.7B). Increases were maximal in Fe-deficient leaves with approximately 100 µmol Chl m^{-2}. The increases were 1.6-fold for Cl^- (from 9 to 14.4 mM), 1.5-fold for NO_3^- (from 15 to 23 mM) and 11.5-fold for SO_4^{2-} (from 1.4 to 16 mM). Phosphate decreased 1.7-fold with Fe deficiency, from 4.5 to 2.7 mM.

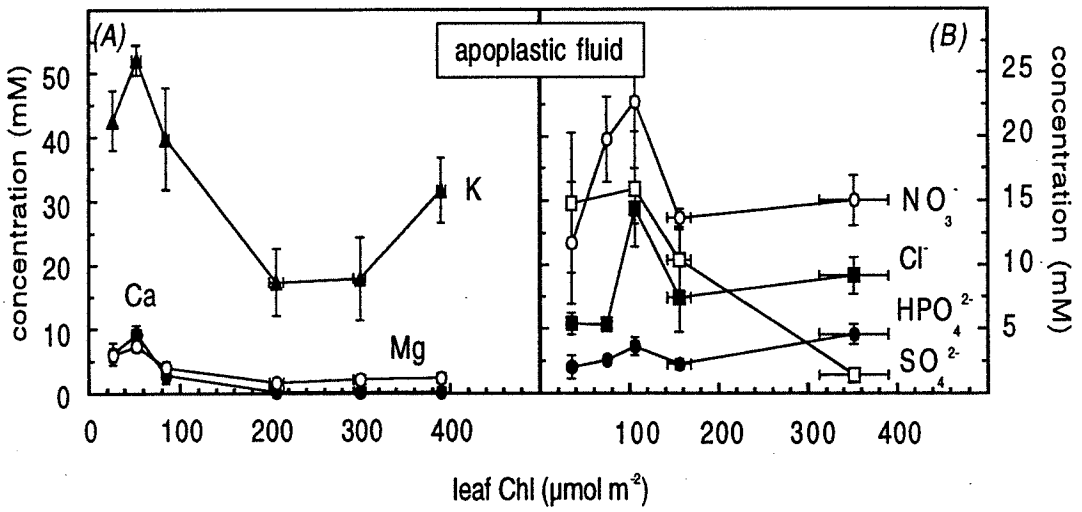

Figure 5. 7. Effects of Fe deficiency on the concentration of cations and anions in apoplastic fluid from sugar beet leaves. A. K (solid triangles), Ca (solid circles) and Mg (open circles). B. Chloride (solid squares), phosphate (solid circles), nitrate (open circles) and sulphate (open squares). All data are in mM. Data are means ± SE of 10 replications.

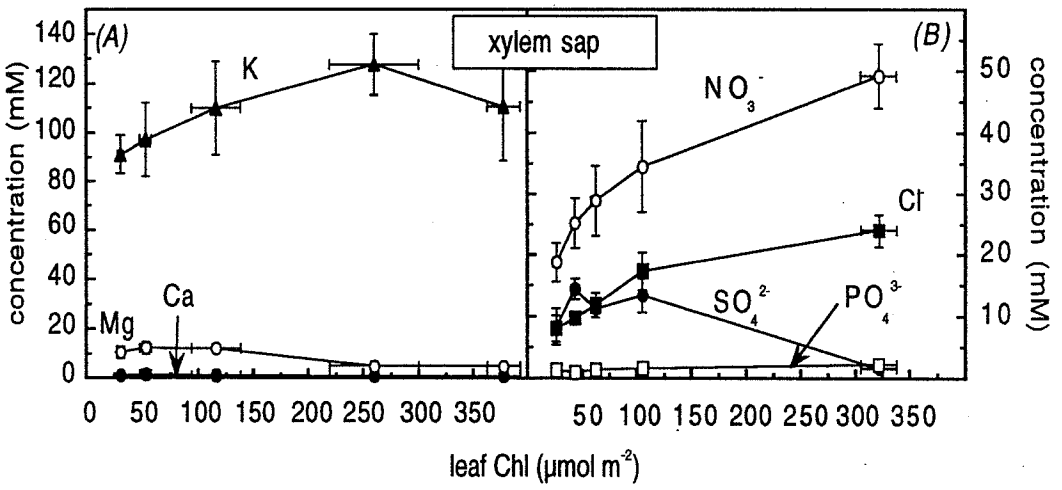

Figure 5. 8. Effects of Fe deficiency on the concentration of cations and anions in xylem sap from sugar beet leaves. A. K (solid triangles), Ca (solid circles) and Mg (open circles). B. Chloride (solid squares), phosphate (open squares), nitrate (open circles) and sulphate (solid circles). All data are in mM. Data are means ± SE of 10 replications.

The concentrations of Mg and K were higher in xylem sap than in the apoplastic fluid, whereas Ca had the opposite behaviour (Fig. 5.8A). Xylem sap concentrations of Ca and Mg increased with Fe deficiency, whereas those of K were

quite constant (70-130 mM). The increases were 2.4-fold for Mg (from 4.9 to 11.9 mM) and 2.4-fold for Ca (from 0.8 to 1.9 mM).

Nitrate, Cl^- and HPO_4^{2-} concentrations in the xylem sap decreased with Fe deficiency (Fig. 5.8B). The decreases were 2.5-fold for NO_3^- (from 49 to 19 mM), 3-fold for Cl^- (from 24 to 8.1 mM) and 1.7-fold for HPO_4^{2-} (from 2.3 to 1.3 mM). Sulfate increased 5-fold with Fe deficiency from 1.7 to 8.4 mM.

When expressed in meq, total cation/anion concentrations in apoplastic fluid were 33/34 in Fe-sufficient plants and 67/74 in Fe-deficient plants. Total cation/anion concentrations (in meq) in xylem sap were 120/80 in Fe-sufficient plants and 120/115 in Fe-deficient plants.

5. 4. 7. Iron Concentrations

The concentration of Fe was in the µM range in leaf apoplastic fluid and xylem sap of sugar beet (Fig. 5. 9).

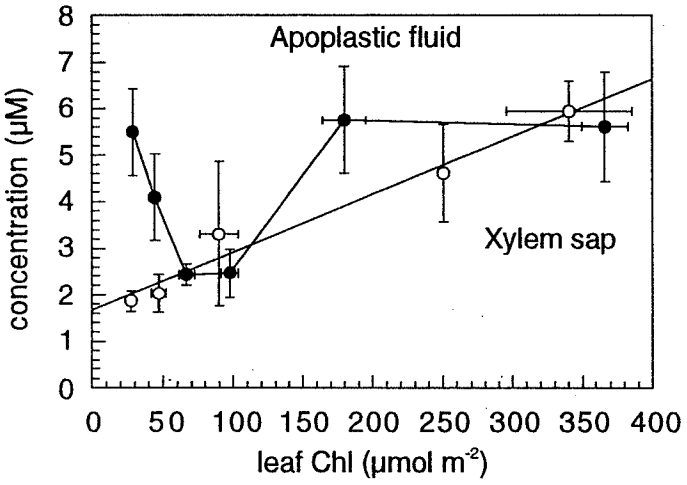

Figure 5. 9. Effects of Fe deficiency on the concentration of iron in xylem sap (open circles) and leaf apoplastic fluid (solid circles) of sugar beet plants. Data are means ± SE of 5 replications.

Apoplastic Fe first decreased with Fe deficiency (from 5.9 µM to 2.5 µM), but then increased in extremely chlorotic leaves (30 µmol Chl m^{-2}) to values similar to those found in control leaves. Iron concentrations in xylem sap decreased

linearly with Fe deficiency from 5.6 µM in Fe-sufficient leaves to approximately 1.9 µM in extremely deficient sugar beet plants.

5. 4. 8. Chemical Speciation

Iron was predicted by chemical speciation to be complexed mainly by citrate in both apoplastic fluid and xylem sap. In Fe-sufficient and deficient leaf apoplastic fluid Fe was distributed evenly between $FeCitOH^-$ and $FeCit_2^{3-}$ (Table 5.3). Iron deficiency caused changes in the predicted distribution of total Fe between both complexes. With Fe deficiency the amount of Fe predicted to exist as $FeCitOH^-$ decreased from 50 to 24%, whereas the $FeCit_2^{3-}$ form would increase from 46 to 76%. The predicted amount of Fe-malate complexes in the apoplastic fluid was always very low, with the most abundant species ($[FeMal]^{+1}$) being in the 10^{-13} M range.

The major predicted complexes of Fe in xylem sap were $FeCitOH^-$ and $FeCit_2^{3-}$, in both Fe-sufficient and deficient samples (Table 5.3). Iron predicted to be present as $FeCitOH^-$ decreased from 66.2 to 4.2% with Fe deficiency, whereas the $FeCit_2^{3-}$ species increased from 25.9 to 95.8%. The predicted amount of Fe-malate complexes in xylem sap was always very low, with the most abundant species ($[Fe_2Mal_3OH_2]^{-2}$) being in the 10^{-10} M range.

	Apoplastic fluid		Xylem sap	
	+Fe	-Fe	+Fe	-Fe
$FeCitOH^-$	2.69 (49.9%)	0.57 (23.8%)	3.57 (66.2%)	0.10 (4.2%)
$FeCit_2^{3-}$	2.71 (46.3%)	1.81 (75.7%)	1.40 (25.9%)	2.30 (95.8%)
$Fe2(CitOH)_2^{2-}$	0.20 (3.7%)	0.01 (0.4%)	0.42 (7.8%)	0.01

Table 5. 3. Predicted chemical distribution of Fe in the different Fe-chelate species found in Fe-deficient and sufficient apoplastic fluid and xylem sap. Data are in µM. Numbers in brackets are percentages of total Fe.

5. 5. Discussion

We have characterised the effects of Fe deficiency on the chemical composition of two plant sites crucial for long-distance Fe transport, the xylem sap and the leaf apoplastic fluid, in the model plant sugar beet. The major change caused by Fe deficiency at both sites is an increase in the concentrations of organic anions, especially malate and citrate. This increase was accompanied by other changes in inorganic anions and was balanced by an increase in cations, especially K. Changes found are consistent with previously reported effects of Fe deficiency on bulk concentrations of organic acids and cations in plant shoots (see Welkie and Miller, 1993, for a review). Sugar and aminoacid concentrations are also affected by Fe deficiency.

Iron-citrate complexes were the major predicted Fe chemical species in both xylem sap and apoplastic fluid of sugar beet. This agrees with previous data obtained from tomato and soybean stem exudates (White et al., 1981a, b) and supports that citrate plays a major role in long distance Fe transport, as first proposed by Tiffin (1966). Our data, however, indicate that Fe deficiency causes significant changes in the chemical speciation of Fe-citrate in sugar beet. Under Fe sufficiency conditions the major predicted Fe species was $Fe(CitOH)^{-}$ both in xylem sap (66% of total Fe) and in apoplastic fluid (50% of total Fe). Conversely, under Fe deficiency the major species was $FeCit_2^{3-}$ both in xylem sap (96% of total Fe) and apoplastic fluid (76% of total Fe). From our data it seems that the citrate:Fe molar ratio could be the major factor controlling Fe speciation. Other factors that change significantly with Fe deficiency, such as malate concentrations, pH, and the concentrations of other cations and anions have only minor effects on Fe speciation. The formation of citrate-Fe polymers (Spiro, 1961a) is frequently assumed to occur in plant shoots (Bienfait and Scheffers, 1991; Moog and Brüggemann, 1994; Schmidt, 1999), although no experimental evidence is available in support of this theory. The formation of citrate-Fe polymers in the xylem and leaf apoplast of sugar beet is unlikely, because of the high citrate:Fe ratios found in these compartments. Excess citrate competes effectively with the formation of the citrate-Fe polymers, therefore inhibiting polymerisation (Spiro et al., 1961b).

The concentration of Fe in apoplastic fluid was approximately 5.6-5.9 μM in severely deficient (30 μmol Chl m^{-2}) and control sugar beet leaves, whereas in leaves with 100 μmol Chl m^{-2} it was approximately 2.2 μM. The relatively high Fe concentrations in the apoplast of severely Fe-deficient leaves suggest that Fe deficiency is associated to a progressive impairment of the Fe acquisition mechanisms in mesophyll cells. This agrees with the low FC-R activity of Fe-deficient sugar beet protoplasts reported recently (González-Vallejo et al., 2000).

The large citrate:Fe molar ratios found in the leaf apoplastic fluid of Fe-deficient plants may impair significantly Fe uptake by mesophyll cells. The citrate:Fe molar ratios increased with Fe deficiency from 120 to 1750 in apoplastic fluid and from 35 to 2000 in xylem sap of sugar beet. The activity of the FC-R leaf PM enzyme has been recently shown to decrease markedly when the citrate:Fe ratio increases, activities decreasing 5-fold when the citrate:Fe molar ratio increased from 100 to 500 (González-Vallejo et al., 1999). It should be also mentioned that increases in the malate:Fe molar ratio above 10 do not affect the activity of the leaf PM FC-R (González-Vallejo et al., 1999). The marked decrease in FC-R activities at high citrate:Fe ratios could be related to the fact that the major chemical species under these conditions is the strongly charged $FeCit_2^{3-}$ species, which may experience a strong electrostatic repulsion with the negatively charged PM.

Our sugar beet data do not provide support for the hypothesis (Mengel, 1995; Kössegarten et al., 1999) that apoplast pH changes induced by Fe deficiency could modulate the activity of the FC-R enzyme of the mesophyll cell plasma membrane. Iron deficiency did not increase the bulk apoplastic pH in sugar beet. Conversely, Fe deficiency caused small decreases in the pH of the apoplast, as judged from direct pH measurements in apoplastic fluid obtained by centrifugation and *in vivo* measurements with fluorescent dyes. These pH decreases could possibly originate from a Fe deficiency-induced enhancement of the activity of leaf plasma membrane ATPases. The apoplastic pH decreases caused by Fe deficiency would tend to increase FC-R activities associated to the PM, which were shown to be maximum at a pH of approximately 5.5 in isolated, Fe-deficient sugar beet leaf protoplasts (González-Vallejo et al., 2000).

Malate was a major organic anion in both Fe-sufficient and deficient xylem sap. In the apoplastic fluid, however, malate was present in much lower concentrations than in xylem sap, whereas citrate concentrations were very similar in both compartments. This suggests that the concentration of malate in the apoplast could be depleted by a malate transporter located in the mesophyll cell plasma membrane. Several mechanisms have been reported for malate transport in different cell organelles such as mitochondria and chloroplasts, including the malate-oxalacetate shuttle and various antiport systems with phosphate, tricarboxilates and 2-oxoglutarate (see Martinoia and Rentsch, 1994, for a review). However, there are little references of malate transport mechanisms across the leaf plasma membrane. The mechanisms described so far for the leaf plasma membrane include an anion channel (Martinoia and Rentsch, 1994), that could be part of a plant CO_2 sensor (Hedrich and Marten, 1993).

The high concentrations of organic anions in the xylem sap indicate that anaplerotic, non-autotrophic C export from roots could be significant in Fe-deficient plants. Concentrations of 30 mM malate, 7 mM succinate and 5 mM citrate in the xylem sap of Fe-deficient plants would be equivalent to approximately 4.8 μmol C m^{-2} s^{-1}, from the measured water transpiration rate of 2 mmol m^{-2} h^{-1} in the same leaves. This rate of C export could be several fold higher than the rate of photosynthetic CO_2 fixation in the deficient leaves, that could reach values of approximately 3 μmol C m^{-2} s^{-1} at light saturation and less than 1 μmol C m^{-2} s^{-1} at the PPFD occurring in the growth chamber (Terry, 1983). Conversely, in the controls the rates of C export from roots would be lower than 1.0 μmol C m^{-2} s^{-1}, less than 1% of the maximum leaf photosynthesis in the same leaves, in line with the current view that C fixation by roots is negligible under normal conditions (Farmer and Adams, 1991). The occurrence of a significant anaplerotic C fixation in the roots of Fe-deficient plants could provide an explanation for the relatively small effect of Fe deficiency on sugar beet leaf growth under controlled conditions (Terry, 1979), in spite of the markedly reduced photosynthetic rates of the same leaves (Terry, 1980). This non-autotrophic, anaplerotic C fixation is associated to an increased PEP carboxylase activity in root tips (Rabotti et al., 1995; López-Millán, chapter 3), that uses bicarbonate, readily available in natural environments leading to Fe deficiency such as calcareous soils, as substrate (Alhendawi et al., 1997).

In Fe-deficient leaves the apoplastic concentrations of cations increased respect to controls, thus tending to balance the organic acid increases. Jacobson reported as early as 1955 that an increase in cation uptake by Fe-deficient roots accounted for the increase in malate concentrations. Concentrations of inorganic cations have been generally reported to increase in Fe-deficient leaves (Nagarajah and Ulrich, 1965; Welkie and Miller, 1993). Also, the increase observed in total aminoacid concentration (1.4-fold) in the apoplast of Fe-deficient leaves suggests that part of the CO_2 fixed by PEPC could be incorporated into aminoacids. Transamination of oxalacetate may result in increases in Glu (Cramer et al., 1993), such as that observed in the xylem sap of Fe-deficient plants. Val, the aminoacid having the largest increase with Fe deficiency, is synthesised via pyruvate (Goodwin and Mercer, 1983).

In summary, Fe deficiency decreases by approximately 0.3-0.4 units the pH of the xylem sap and apoplastic fluid of sugar beet leaves. The major increases in organic anion concentrations induced by Fe deficiency in both apoplastic fluid and xylem sap suggest the existence of an influx of organic anions from the roots to the shoot via xylem, that could be important for the maintenance of basic processes in leaves with low photosynthetic rates. The major predicted Fe chemical species in both xylem sap and apoplastic fluid of sugar beet plants were iron-citrate complexes, with the citrate:Fe ratio being the major factor controlling Fe speciation. On the other hand, the large citrate:Fe molar ratios found in the leaf apoplastic fluid of Fe-deficient plants may impair significantly Fe uptake by mesophyll cells. These data indicate the importance of citrate in the long distance Fe transport and subsequent uptake by the mesophyll cell.

CAPÍTULO 6

CAMBIOS INDUCIDOS POR LA DEFICIENCIA DE Fe EN LA COMPOSICIÓN DEL FLUIDO APOPLÁSTICO DE HOJAS DE PERAL (Pyrus communis L.) CULTIVADO EN CAMPO

6. Changes Induced by Iron Deficiency in the Composition of the Leaf Apoplastic Fluid from Field-Grown Pear (*Pyrus communis* L.) Trees

6. Changes Induced by Iron Deficiency in the Composition of the Leaf Apoplastic Fluid from Field-Grown Pear (*Pyrus communis* L.) Trees.

6. 1. Abstract

Experiments have been carried out with field-grown, pear leaves to investigate the effect of iron chlorosis on leaf apoplastic composition. Iron deficiency caused an increase in the apoplastic pH of pear leaves from the control values of 5.5-5.9 to 6.5-6.6, as judged from direct pH measurements in apoplastic fluid obtained by centrifugation and by fluorescence of leaves incubated with 5-carboxy fluorescein. The major organic acids found in leaf apoplastic fluid of iron-deficient and iron-sufficient pear leaves were malate, citrate and ascorbate. The total concentration of organic acids was 2.9 mM and increased to 5.5 mM in Fe-deficient leaves. The total apoplastic concentration of inorganic cations (Ca, K and Mg) increased from 15 to 20 mM. The total apoplastic concentration of inorganic anions (Cl^-, NO_3^-, SO_4^{2-} and HPO_4^{2-}) did not change with Fe deficiency. Iron concentrations in the apoplastic fluid decreased from 4 to 1.6 µM with Fe deficiency.

The major Fe species predicted by the chemical speciation software MinteqA2 for the apoplastic ionic environment was $FeCitOH^-$ in both Fe-sufficient and deficient apoplastic samples, indicating that citrate plays a major role in the long distance Fe transport. Organic acids in whole leaf homogenates increased from 20 to 40 nmol m^{-2} with Fe deficiency. The activities of several enzymes involved in organic acid metabolism (fumarase, isocitrate-dehydrogenase, aconitase and citrate synthase) increased in total leaf extracts with Fe deficiency, whereas the activity of phosphoenol pyruvate carboxylase did not change.

6. 1. 1. Abbreviations: CS, citrate synthase; DTNB, 5-5´dithio-bis-2-nitrobenzoic acid; FC-R, ferric-chelate reductase; FDH, formate dehydrogenase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MS, mass spectroscopy; PDC, pyruvate decarboxylase; PEPC, phosphoenol pyruvate carboxylase; PM, plasma membrane; PVDF, polyvinyl fluoride; SPAD, portable Chl meter; 5-CF, 5-carboxy fluorescein.

6. 2. Introduction

Iron deficiency is one of the major abiotic stresses affecting fruit tree crops in the Mediterranean area. One of the most obvious characteristics of the plants affected by Fe deficiency is that their leaves become chlorotic (Terry and Abadía, 1986; Abadía and Abadía, 1993). In a geographical region of great agricultural importance in northeastern Spain, such as the Ebro river basin, many fruit tree crops are affected by Fe chlorosis. Crops affected include peach, pear, apple, cherry, citrus, grape and plum (Sanz et al., 1992). Pear is one of the fruit tree species most affected by Fe chlorosis, that causes decreases in yield and shortens the productive lifetime of the orchards by 5-6 years (Sanz et al., 1992). In the Ebro river basin, approximately 67% of pear orchards (over 13200 ha) are currently being treated with Fe compounds to correct Fe chlorosis.

Iron chlorosis is caused by low Fe availability in calcareous, high pH soils (Lindsay and Schwab, 1982). Iron deficiency produces physiological and biochemical responses at the plant root level in dicotyledoneous plants. The

physiological responses include higher proton extrusion which decreases the pH of the rhizosphere (Brown, 1978), a release of reducing and/or chelating substances such phenolics and flavins (Welkie and Miller, 1960; Susín et al., 1996) and a two-step mechanism for Fe uptake in which Fe is first reduced by a plasma membrane (PM)-bound ferric-chelate reductase (FC-R) (Moog and Brüggemann, 1994; Susín et al., 1996; Robinson et al., 1999) and subsequently absorbed as Fe(II) (Chaney et al., 1972; Eide et al., 1996). Among the biochemical responses, dicotyledoneous plants accumulate organic acids, mainly citrate and malate, both in leaves (Iljin, 1951; Palmer et al., 1963; Landsberg, 1981) and roots (de Kock and Morrison, 1958; Brown, 1966; de Vos et al., 1986).

In the xylem, Fe is transported to the leaves as Fe(III), probably chelated by citrate (Tiffin, 1966; Brown and Chaney, 1971; White et al., 1981, Schmidt, 1999). The way Fe enters the leaf cell has been much less studied than the corresponding processes in the roots. Once in the leaf apoplast, Fe(III) is reduced before uptake by the leaf cell. Reduction of Fe-chelates are mediated by a PM-bound FC-R enzyme (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; González-Vallejo et al., 2000). Therefore, the composition of the apoplast is of crucial importance to understand the processes involved in the mechanism of Fe uptake by leaves. For instance, apoplastic pH could be important for the mobility of Fe and the activity of the leaf PM FC-R (Mengel, 1995; Schmidt, 1999; Kösegarten et al., 1999). No report has been made so far, to our knowledge, on the effects of Fe deficiency on the composition of the leaf apoplast. Fe trafficking in the apoplast is also mandatory for Fe uptake by the root cells (Longnecker and Welch, 1990; Zhang et al., 1991).

The leaf apoplast is involved in transmission of signals (Hartung et al., 1992), transport and storage of mineral nutrients (Starrach and Mayer, 1989; Wolf et al., 1990; Zhang et al., 1991), trace gas exchange with the atmosphere (Kesselmeier, 1998; Gabriel et al., 1999) and different responses to plant environmental stresses (Polle et al., 1990; Pfanz et al., 1991; Dietz et al, 1997). Studies have been made on the composition of the apoplast under different environmental conditions (Clarkson, 1984; Blatt, 1985; Bowling, 1987; Speer and Kaiser, 1991; Grignon and Sentenac, 1991; Tetlow and Farrar, 1993; Canny, 1995). The apoplast contains significant concentrations of inorganic and organic

anions (Petersen and Böttger,1991; Hedrich et al., 1994; Schurr and Schulze, 1995; Dietz et al., 1997).

The aim of this work was to investigate the effects of Fe deficiency on the composition of the leaf apoplast in pear trees grown in the field, in order to understand the role of this compartment in the transport and acquisition of Fe by leaf cells. We have investigated the changes induced by Fe deficiency on the concentrations of different inorganic and organic ions in pear leaf apoplast. Chemical speciation has been carried out with the MinteqA2 software. The activities of different enzymes, including PEPC and several TCA-cycle enzymes, and the concentrations of pyridine nucleotides and organic anions have been measured in leaf extracts of Fe-deficient and control leaves.

6. 3. Materials and Methods

6. 3. 1. Plant Material

Leaves were sampled from pear trees (*Pyrus communis* L.) growing in calcareous soils and affected by iron chlorosis. The orchard was located in El Temple (Huesca) in the Ebro basin in north-eastern Spain. The soil has a clay-loamy texture, with 32% total calcium carbonate, 12.6% active lime, 1.89% organic matter and pH in water 8.4. The pear cultivar was 'Blanquilla' grafted on quince 'BA29'. The trees were 17 years old, trained as palmette, with a frame of 3x4 m. The orchard had not been treated with Fe-chelates in the last two or three years. Under these conditions chlorosis became more marked in the orchards studied every year. In 1997 growing season there were trees with marked chlorosis symptoms (average leaf chlorophyll 200 μmol m^{-2}) and other trees that still remained green (average leaf chlorophyll 600 μmol m^{-2}). Samplings were conducted during the summer of 1997. Young, fully-expanded leaves, showing no interveinal chlorosis and almost homogeneous colour throughout the leaf, were chosen for all measurements.

6. 3. 2. Chlorophyll Determination

Chlorophyll concentration was estimated non-destructively with a SPAD-502 device (Minolta, Osaka, Japan). For calibration, leaf disks with different degrees of Fe deficiency were first measured with the SPAD, then extracted with 100% acetone in presence of Na ascorbate and chlorophyll measured spectrophotometrically (Abadía and Abadía, 1993).

6. 3. 3. Apoplastic Fluid Isolation

Apoplastic fluid was obtained from whole pear leaves by direct centrifugation as in Dannel et al. (1995) with some modifications. Leaves were excised at the base of the leaf lamina with a razor blade in the field and transported to the laboratory with the petiole immersed in de-ionized water. Once in the laboratory the petiole was excised under water. Five leaves were then rolled and placed into a plastic syringe barrel, with the petiole side at the narrow end of the syringe. Leaf-filled syringes were centrifuged at 4°C. A small volume of apoplastic fluid was obtained from the bottom of the centrifuge tube. A first centrifugation was made at low speed (2500*g*, 15 min) to remove the xylem sap of the main vein. Apoplastic fluid was obtained after a second centrifugation for 15 min at 4000*g* and 4°C.

Malate dehydrogenase (c-mdh; EC 1.1.1.37) was used as a cytosolic contamination marker for apoplastic fluid. The activity of c-mdh was determined using oxalacetate as substrate and measuring the decrease in A_{340} due to the enzymatic oxidation of NADH. The final reaction mixture (pH 9.5) was 46.5 mM Tris, 0.1 mM NADH and 0.4 mM oxalacetate (Dannel et al., 1995). The activity of the marker in leaf apoplast was related to the total activity in whole leaf homogenates. To measure enzymatic activities in total leaf homogenates 3 leaf disks of 0.95 cm^{-2} were homogenised with 2 mL of buffer (pH 8.0) containing 100 mM Hepes, 30 mM sorbitol, 2 mM DTT, 1 mM $CaCl_2$, 1% BSA and 1% PVP. The supernatant was collected and analysed immediately after 10 min centrifugation at 10000*g*.

6. 3. 4. pH Measurements

The pH of the apoplastic fluid was measured directly in the fluid obtained by centrifugation with a microelectrode (Physitemp, USA). Apoplastic pH was also measured *in vivo* by fluorescence according to Hoffman et al. (1992) with 5-carboxyfluorescein (5-CF). Fluorescence emitted by 5-CF at 540 nm is pH-dependent when excited at 490 nm but nearly pH-independent when excited at 450 nm. Therefore, the ratio of fluorescence intensities obtained with excitation at 490 and 450 nm is an estimate of the pH of the compartment where the dye is located. Leaves were excised and the cut end of the petiole was exposed to incubation medium containing 5 µM 5-CF, 1 mM KCl, 0.1 mM NaCl and 0.1 mM $CaCl_2$ at pH 5.5. The incubation was carried out for 5 h at room ambient light and temperature (15-25 µmol photons m^{-2} s^{-1}, 20-25°C). The level of auto-fluorescence was subtracted from total fluorescence. Three leaves per Chl level were taken and four measurements were carried out in different areas of each leaf.

6. 3. 5. Inorganic Ion Determination

Ca and Mg were determined by atomic absorption spectrophotometry and K by emission spectrophotometry. Fe was determined in the apoplastic fluid by atomic absorption spectrometry with a graphite furnace (Varian SpectrAA with Zeeman correction). Each sample was analysed in triplicate.

Inorganic anions (NO_3^-, SO_4^{2-}, Cl^- and HPO_4^{2-}) were quantified by HPLC with a 4.6 x 75 mm IC-Pak A HR ion-exchange column (Waters, Milford, MA, USA) in a HPLC Waters system, including a 600E pump, a 432 conductivity detector and Millennium 2010 software. Samples were injected with a Rheodyne injector (50 µL loop). Mobile phase (11 mM borate-gluconate) was pumped with a 1.0 mL min^{-1} flow rate. Quantification was made with known amounts of each anion using peak areas.

6. 3. 6. Organic Ion Analysis

Leaf samples (3 leaf disks of 0.95 cm^2 taken with a calibrated cork borer) were frozen in liquid N_2 and ground in a mortar with 8 mM sulphuric acid. Homogenates were boiled for 30 min, centrifuged 10 min at 10000*g*, filtered with a 0.2 µm PVDF filter (LIDA, Kenosha, WI, USA), taken to a final volume of 2 mL with 8 mM sulphuric acid and kept at -80°C until analysis. Apoplast samples were filtered (0.45 µm PVDF, LIDA) before HPLC analysis.

Organic anions were analysed by HPLC with a 300 x 7.8 mm Aminex ion-exchange column (HPX-87H from Bio-Rad, Hercules, CA, USA) with a HPLC system (Waters, Milford, MA, USA), including a 600E pump, a 996 photodiode array detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (8 mM sulphuric acid) was pumped with a 0.6 mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, citrate, 2-oxoglutarate, ascorbate, malate, shikimate and fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma (Fig. 6.1). The peak with retention time of 12 min was composed of succinate and an unidentified compound with maxima at 210 and 262 nm. Because of this reason, succinate was not quantified. Another peak with a retention time of approximately 14 min, with absorption maxima at 219 and 281 nm, was tentatively identified as p-coumaric acid. The identity of some peaks was further confirmed by UV-VIS and MS. Quantification was made with known amounts of each anion using peak areas.

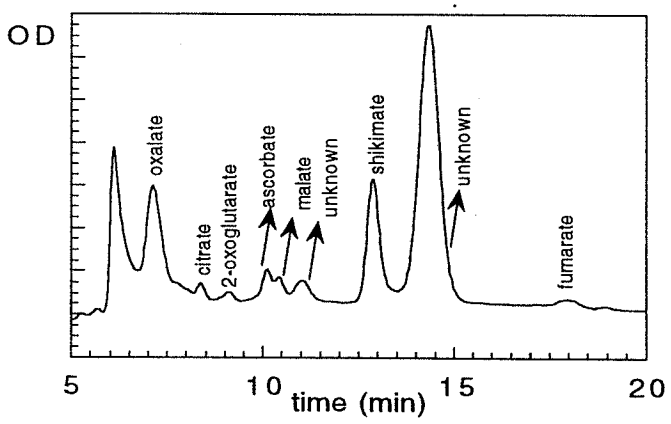

Figure 6. 1. Separation of organic anions by ion exchange high pressure liquid chromatography. Organic anions were detected at 210 nm.

6. 3. 7. Sugar Analysis

Sugars (glucose, fructose and sucrose) were analysed by HPLC with a 300 x 4 mm Spherisorb-NH_2 column (Waters) and a HPLC system (Waters), including a 590 pump, a differential refractometer R401 detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (acetonitrile: water 860:140) was pumped with a 3.5 mL min^{-1} flow rate. Peaks corresponding to glucose, fructose and sucrose were identified by comparison of their retention times with those of known standards from Sigma. Quantification was related to peak areas of known amounts of the standards.

6. 3. 8. Enzyme Assays

Extracts for measuring enzyme activities were made by grinding 3 leaf disks of 0.95 cm^2 each in a mortar with 2 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10000*g* and 4°C, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 1 mL final volume, of the media indicated below.

Malate dehydrogenase (MDH; EC 1.1.1.37) was determined as described above. Citrate synthase (CS; EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1967) by monitoring the reduction of acetyl CoA to CoA with 5-5'-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. The reaction was carried out with 50 µL of extract in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalacetate and 100 mM Tris-HCl, pH 8.1. Aconitase (EC 4.2.1.3) was measured from the formation of cis-aconitate, monitored at 240 nm (Bacon et al., 1961) with 60 µL of extract in 500 mM sucrose, 50 mM isocitrate and 100 mM Tris-HCl (pH 8.5). Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was determined with 50 µL of extract by monitoring the reduction of $NADP^+$ at 340 nm in a reaction mixture containing 3.5 mM $MgCl_2$, 0.41 mM $NADP^+$, 0.55 mM isocitrate and 88 mM imidazole at pH 8.0 (Bergmeyer et al., 1974). Fumarase (EC 4.2.1.2) was assayed with 50 µL of extract following the increase in optical density at 240 nm due to the formation of fumarate (Bergmeyer et al., 1974). The reaction buffer was 50 mM malate and 100 mM phosphate, pH 7.4.

Phosphoenol pyruvate carboxilase (PEPC; EC 4.1.1.31) was measured in a coupled enzymatic assay with MDH according to Vance et al. (1983) with 75 µL of extract in 2 mM phosphoenol pyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH, and 100 mM bicine-HCl, pH 8.5. For the determination of lactate dehydrogenase (LDH; EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1) the oxidation of NADH was monitored at 340 nm with 50 µL of extract. LDH was assayed in a reaction buffer containing 94.5 mM phosphate buffer (pH 9.5), 0.77 mM pyruvate and 0.2 mM NADH. PDC was determined in 190 mM citrate-KOH buffer (pH 6.0), 30 mM pyruvate, 0.32 mM NADH and 33 µg mL^{-1} alcohol dehydrogenase.

6. 3. 9. Nucleotide Analysis

Pyridine nucleotides were extracted from liquid N_2-frozen leaf disks (0.95 cm^2) in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA (for $NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12000 g for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 100 mM bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method of Matsumura and Miyachi (1980).

6. 3. 10. Chemical Speciation

Concentrations of the different Fe-chelate species were estimated with the software MinteqA2 (Allison et al., 1991) by using the ionic environment of the apoplastic fluid. Chelate formation constants used for citrate and malate were derived from those given by Holden et al. (1991) and Cline et al. (1982), respectively. At an ionic strength of 0 M, the $\log_{10}$ of the chelate formation constants used for the Fe-citrate species $[FeCit]^0$, $[FeCitH]^{+1}$, $[FeCitOH]^{-1}$, $[FeCit_2]^{-3}$ and $[Fe_2Cit_2(OH)_2]^{-2}$ were 13.13, 14.43, 10.11, 20.13 and 24.51, respectively. The $\log_{10}$ of the chelate formation constants for the Fe-malate species $[FeMal]^{+1}$, $[Fe_2(MalOH)_2]^0$, $[Fe_2Mal(MalOH)_2]^{-2}$ and $[Fe_3Mal_5OH_4]^{-5}$ were 8.39, 15.32, 20.33 and 27.75, respectively.

6. 4. Results

6. 4. 1. Isolation of Apoplastic Fluid

The volume of apoplastic fluid obtained by centrifugation increased gradually when the centrifugal forces increased (Fig. 6.2A).

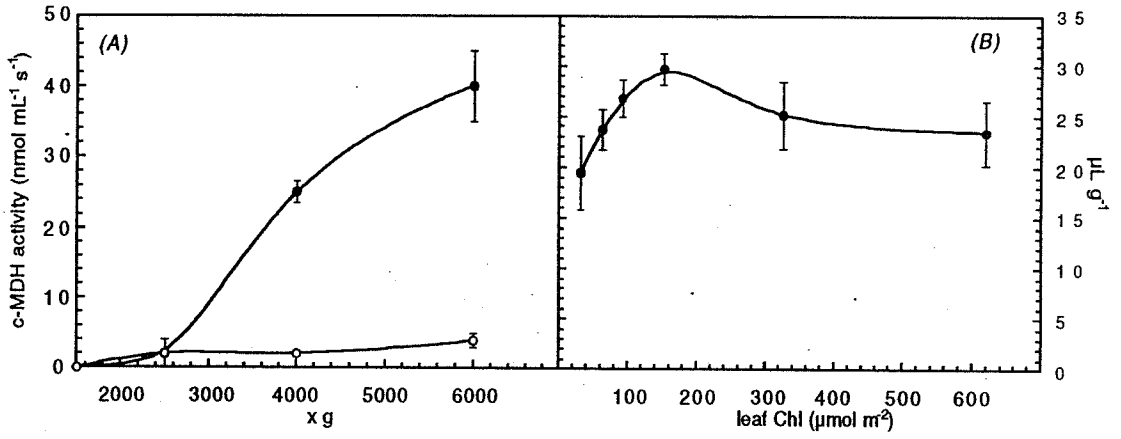

Figure 6. 2. (A) Total apoplastic fluid volume (solid circles) and activity of the cytosolic marker malate dehydrogenase (open circles) in apoplastic fluid obtained at different centrifugal forces from pear leaves. (B) Changes with Fe deficiency in total leaf apoplastic fluid volume (µL g^{-1}). Data are means ± SE of 10 replicates.

In routine experiments apoplastic fluid was collected at 4000*g*, after carrying out a preliminary centrifugation of the leaves at 2500*g* to discard fluid containing the xylem sap of the main vein. The activity of the cytosolic marker c-mdh was approximately 2 nmol NADH s^{-1} mL^{-1} both at 2500 and 4000*g* (Fig. 6.2A). This value was less than 2% of the total activity found in leaf homogenates (approximately 100-120 nmol NADH s^{-1} mL^{-1}), indicating a low degree of cytosolic contamination (Dannel et al., 1995). Compared to the controls moderate and severe Fe deficiency caused 25% increases and 15% decreases, respectively, in the volume of apoplastic fluid recovered per g of leaf fresh weight (Fig. 6.2B).

6. 4. 2. Apoplastic Fluid pH

The pH of the apoplastic compartment of pear leaves was measured by two different methods, direct pH determination in the apoplastic fluid obtained by centrifugation and *in vivo* estimation by means of the fluorescent dye 5-CF (Hoffman et al., 1992). When determined with a microelectrode the pH of the apoplastic fluid increased with Fe deficiency by one unit, from approximately 5.5 in control to 6.6 in Fe-deficient leaves (Fig. 6.3).

The changes in pH values estimated *in vivo* using 5-CF follow a similar trend than that found by direct pH measurement in the apoplastic fluid, with values of approximately 5.9 in control and 6.5 in Fe-deficient leaves (Fig. 6.3). In this case, however, pH changes were not statistically significant at the $p<0.01$ level.

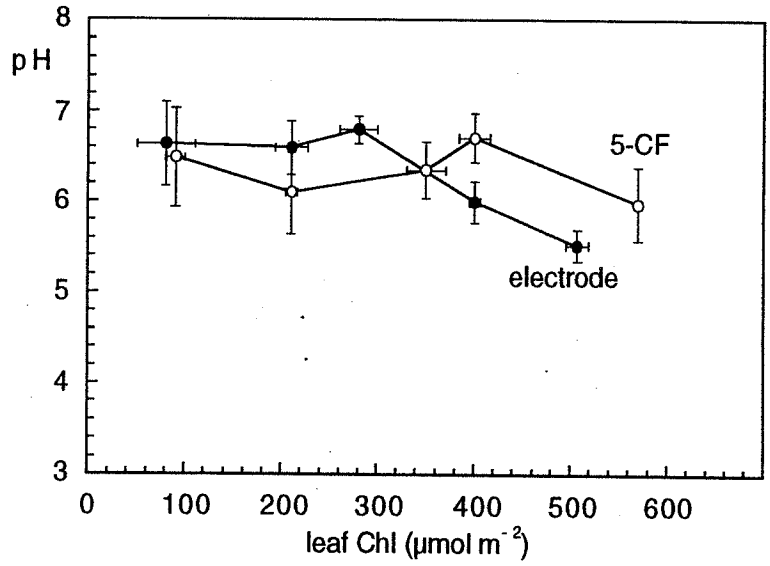

Figure 6. 3. Effects of Fe deficiency on the apoplastic pH of pear leaves. Measurements were made with a microelectrode in apoplastic fluid obtained by centrifugation (solid circles) and *in vivo* by fluorescence with the dye 5-CF (open circles). Data are means ± SE of 3 and 5 replicates in the fluorescence and electrode measurements, respectively.

6. 4. 3. Ionic Composition of the Apoplastic Fluid

Calcium and Mg concentrations in apoplastic fluid increased in response to Fe deficiency (Fig 6.4A). Increases were 10% fold for Ca (from 5.3 to 6.0 mM) and 4.3-fold for Mg (from 0.6 to 2.6 mM). The K concentration increased from 9 mM in the controls to 11 mM in moderately deficient leaves and decreased to 5.2 mM in severely deficient leaves.

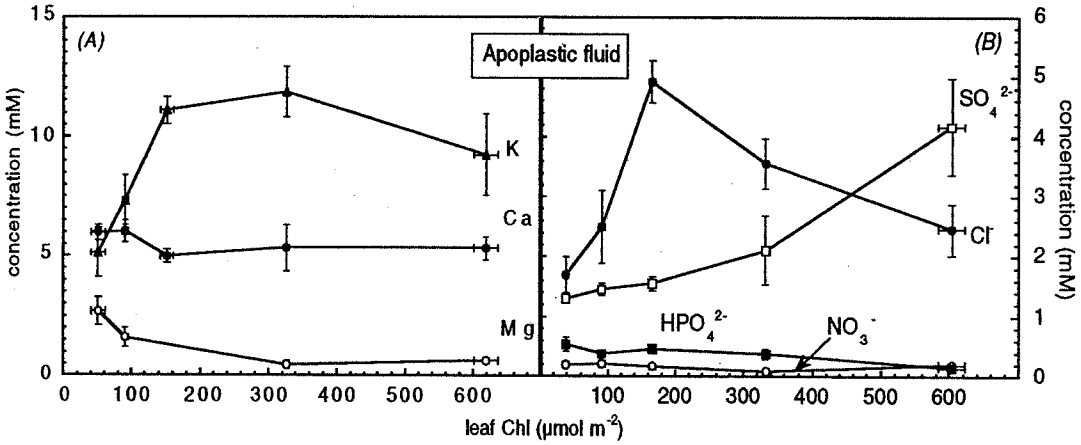

Figure 6. 4. Effects of Fe deficiency on the concentration of cations and anions in apoplastic fluid from pear leaves. (A) Cations: K (solid triangles), Ca (solid circles) and Mg (open circles). (B) Anions: chloride (solid circles), phosphate (solid squares), nitrate (open circles) and sulphate (open squares). All data are in mM. Data are means ± SE of 5 replicates.

Iron deficiency caused a 3-fold decrease in SO_4^{2-} concentration (from 4.2 to 1.4 mM) in apoplastic fluid of pear leaves (Fig. 6.4B). Phosphate concentration increased 3.6-fold with Fe deficiency (from 0.15 to 0.54 mM) (Fig. 6.4B). Concentrations of Cl^- increased 2-fold in moderately deficient leaves (from 2.4 to 4.9 mM) and decreased again to values slightly lower than the controls in severely deficient leaves (Fig. 6.4B). Iron deficiency did not cause significant changes in the NO_3^- concentrations in leaf apoplastic fluid (Fig. 6.4B).

The concentration of Fe in the apoplastic fluid of pear leaves was 4.0-4.4 µM in leaves with 700 and 300 µmol Chl m^{-2} (Fig. 6.5). Below this Chl concentration there was a linear decrease in Fe in apoplastic fluid with Fe deficiency, down to 1.6 µM in leaves with 30 µmol Chl m^{-2} (Fig. 6.5).

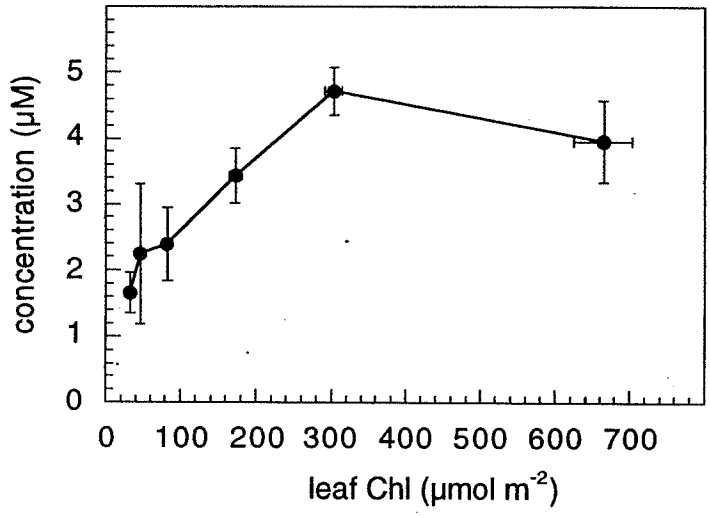

Figure 6. 5. Effects of Fe deficiency on the Fe concentration in leaf apoplastic fluid of pear trees. Data are means ± SE of 5 replicates.

Iron deficiency caused a general increase in organic anion concentrations in the apoplastic fluid, reaching maximal values in chlorotic leaves with a Chl concentration between 100-150 µmol m^{-2} (Fig. 6.6A). The major organic anions found in pear leaf apoplast were citrate, ascorbate and malate, the latter being the major anion. Maximum increases in the concentrations of the three major anions in the apoplastic fluid were 2.2-fold for malate (from 1.1 to 2.5 mM), 2-fold for citrate (from 0.7 to 1.4 mM) and 1.4-fold for ascorbate (from 1.1 to 1.6 mM). Minor anions in the leaf apoplastic fluid were shikimate, 2-oxoglutarate and fumarate (Fig. 6.6B). Iron deficiency caused a 2-fold increase in fumarate concentration (from 28 to 56 µM) and a marked decrease in 2-oxoglutarate concentration (from 24 to 6 µM) (Fig. 6.6B). Shikimate concentrations in apoplastic fluid ranged between 250 and 150 µM (Fig. 6.6B).

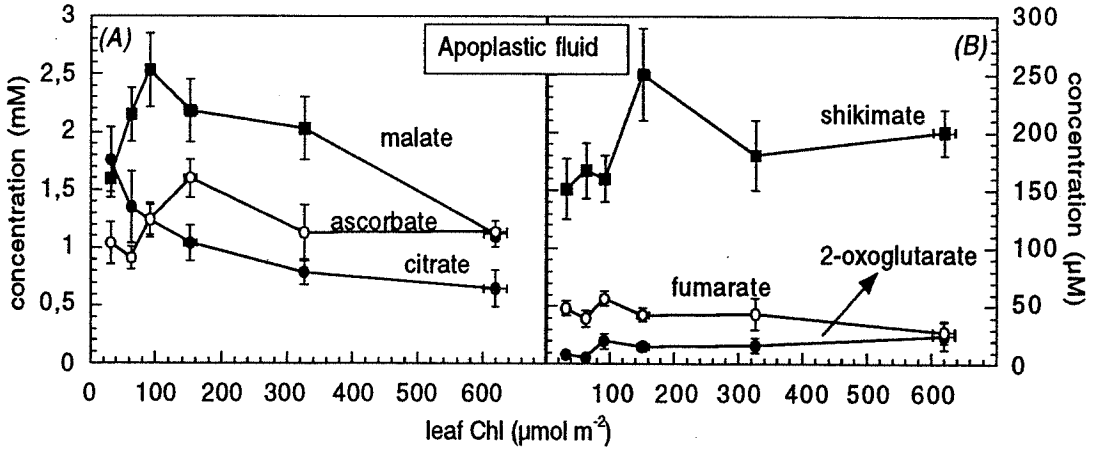

Figure 6. 6 Effects of Fe deficiency on the organic anion concentrations in pear leaf apoplastic fluid. (A) Major organic anions (in mM): citrate (solid circles), malate (solid squares) and ascorbate (open circles). (B) Minor organic anions (in µM): 2-oxoglutarate (solid circles), shikimate (solid squares) and fumarate (open squares). Data are means ± SE of 10 replicates.

6. 4. 4. Sugar Composition of the Apoplastic Fluid

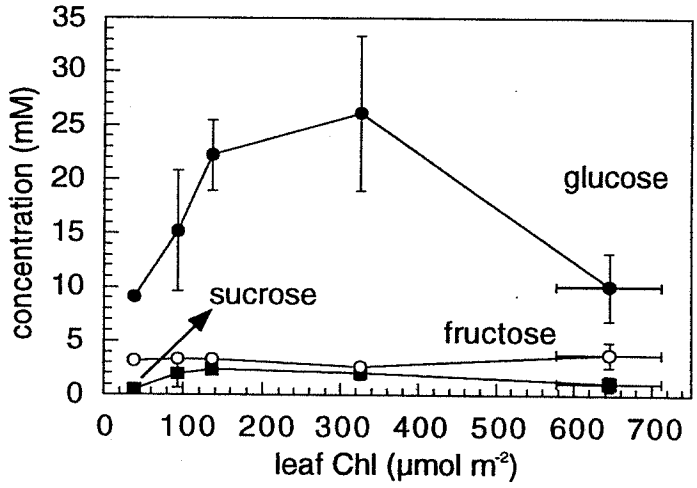

Figure 6. 7. Effects of Fe deficiency on the concentration of sugars in leaf pear apoplastic fluid. Glucose (solid circles), fructose (open circles) and sucrose (solid squares). All data are in mM. Data are means ± SE of 5 replicates.

Iron deficiency caused changes in the sugar concentrations of the apoplastic fluid (Fig. 6.7). Moderate Fe deficiency caused 2.6- and 2-fold increases in concentrations of glucose (from 10 to 26 mM) and sucrose (from 1.1 to 2.0 mM) (Fig. 6.7). Severely deficient samples had glucose concentrations similar to the controls (9 mM) and sucrose concentrations 2-fold lower than the control values (0.5 mM) (Fig. 6.7). Fructose concentrations decreased only slightly with Fe deficiency (Fig. 6.7).

6. 4. 5. Iron Chemical Speciation in the Apoplastic Fluid

The major chemical species of Fe predicted to exist in equilibrium in the apoplastic fluid (by the MinteqA2 software) were mainly complexes with citrate (Table 6.1).

	Fe-sufficient (+Fe)	**Fe-deficient (-Fe)**	**-Fe/+Fe**
FeCitOH	2.49 (62.2%)	1.85 (84.3%)	0.7
$FeCit_2^{3-}$	1.18 (29.6%)	0.16 (7.5%)	0.1
$Fe_2(CitOH)_2^{2-}$	0.16 (8.0%)	0.09 (8.1%)	0.6
Σ Cations	17.4	18.4	
Σ Anions	11.3	11.7	

Table 6. 1. Concentrations (in μM) of the major chemical Fe species in apoplastic fluid of Fe-deficient and Fe-sufficient pear leaves, predicted by the MinteqA2 speciation software, and total concentrations (in mM) of cations and anions. Values in brackets represent percentages with respect to the total Fe concentration in apoplastic fluid.

The major Fe:citrate complex predicted in both Fe-sufficient and deficient leaf apoplastic fluid was $FeCitOH^-$. Iron deficiency caused changes in the predicted distribution of total Fe. With Fe deficiency the amount of Fe predicted to exist as $FeCitOH^-$ increased from 62.2 to 84.3%. Iron predicted to be present as $FeCit_2^{3-}$ decreased with Fe deficiency from 29.6 to 7.5%. The amount of the $Fe_2(CitOH)_2^{2-}$ species accounted for 8% of the total Fe in both Fe-sufficient and deficient apoplastic fluid (Table 6.1).

6. 4. 6. Organic Anion Concentration in Leaves

The total organic anion concentrations in pear leaves increased 1.6-fold with Fe deficiency. Major organic anions found in whole leaf homogenates were citrate, oxalate, malate and ascorbate. Leaves with 50 µmol Chl m^{-2} had 15-, 1.7- and 1.3-fold increases in citrate, ascorbate and malate concentrations, respectively, when compared to control leaves (Fig. 6.8A). Oxalate decreased markedly (from 5 to 1 mmol m^{-2}) with Fe deficiency (Fig. 6.8A).

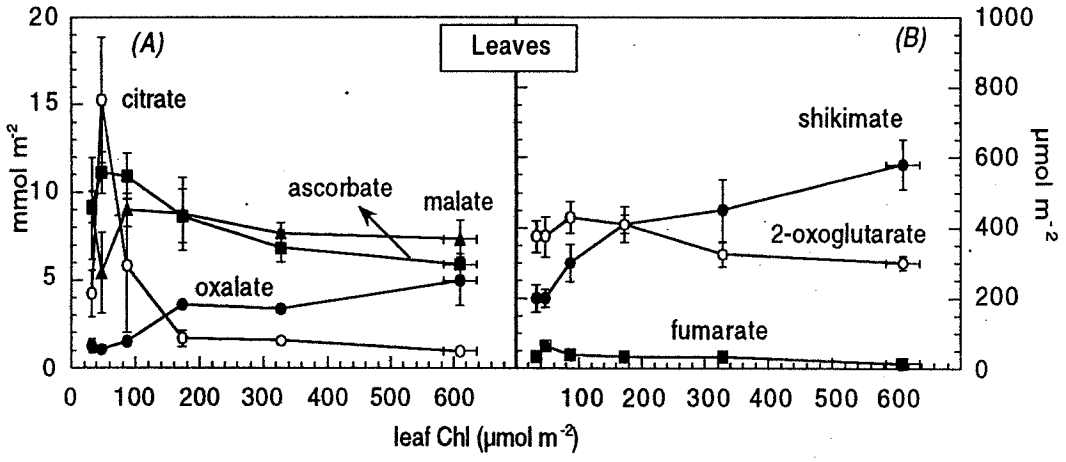

Figure 6. 8. Effects of Fe deficiency on the organic anion concentrations in pear leaves. (A) Major organic anions (in mmol m^{-2}): citrate (open circles), malate (solid triangles), oxalate (solid circles) and ascorbate (solid squares). (B) Minor organic anions (in µmol m^{-2}): 2-oxoglutarate (open circles), shikimate (solid circles) and fumarate (solid squares). Data are means ± SE of 5 replicates.

Minor organic acids in the apoplastic fluid were shikimate, 2-oxoglutarate and fumarate (Fig. 6.8B). Iron deficiency caused a 3-fold decrease in shikimate concentration (from 580 to 200 µmol m^{-2}), whereas 2-oxoglutarate and fumarate concentrations increased 1.3- (from 300 to 380 µmol m^{-2}) and 4.3- fold (from 15 to 65 µmol m^{-2}), respectively. The peak at approximately 14 min, tentatively identified as p-coumaric acid, decreases 3-fold with Fe deficiency.

6. 4. 7. Enzyme Activities in Leaf Extracts

We measured the activities in leaf homogenates of five enzymes involved in the TCA cycle. Enzymatic activities were increased by moderate Fe deficiency (Fig. 6.9A, B), and maximal activities were found with leaves of approximately 100 µmol Chl m^{-2}. Increases were 2.4-fold for fumarase (from 1.6 to 3.8 nmol s^{-1} m^{-2}), 2.3-fold for ICDH (from 0.6 to 1.3 nmol s^{-1} m^{-2}), 1.4-fold for aconitase (from 3.6 to 5.1 nmol s^{-1} m^{-2}) and 3.4-fold for CS (from 70 to 246 nmol s^{-1} m^{-2}). Malate dehydrogenase activity did not show significant changes with Fe deficiency.

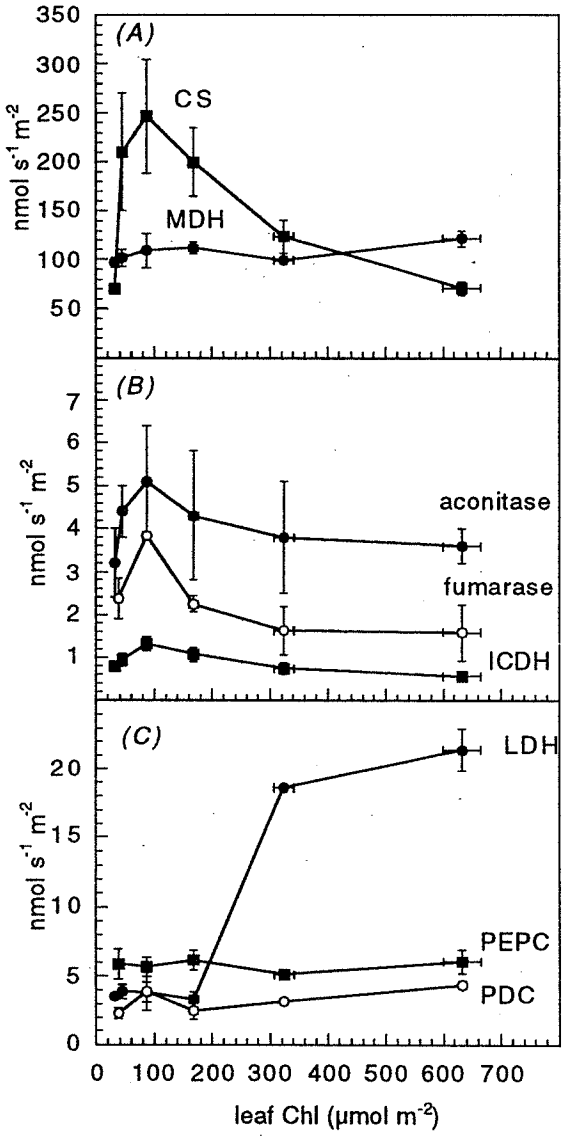

Figure 6. 9. Effects of Fe deficiency on different enzymatic activities in pear leaf extracts. (A) CS and MDH. (B) aconitase, fumarase and ICDH. (C) LDH, PDC, PEPC. All data are in nmol of substrate s^{-1} m^{-2}. Data are means ± SE of 5 replicates.

The activities of PEPC, an enzyme related to C-fixation, and of LDH and PDC, two enzymes involved in anaerobic metabolism, were also measured in leaf homogenates. The activity of PEPC was approximately 5.5 nmol s^{-1} m^{-2} and did not show significant changes with Fe deficiency (Fig. 6.9C). The activity of LDH decreased with Fe deficiency 5.6-fold (from 21.4 to 3.5 nmol s^{-1} m^{-2}) whereas the PDC activity decreased only slightly (from 4.3 to 3.8 nmol s^{-1} m^{-2}) (Fig. 6.9C).

6. 4. 8. Leaf Nucleotide Concentrations

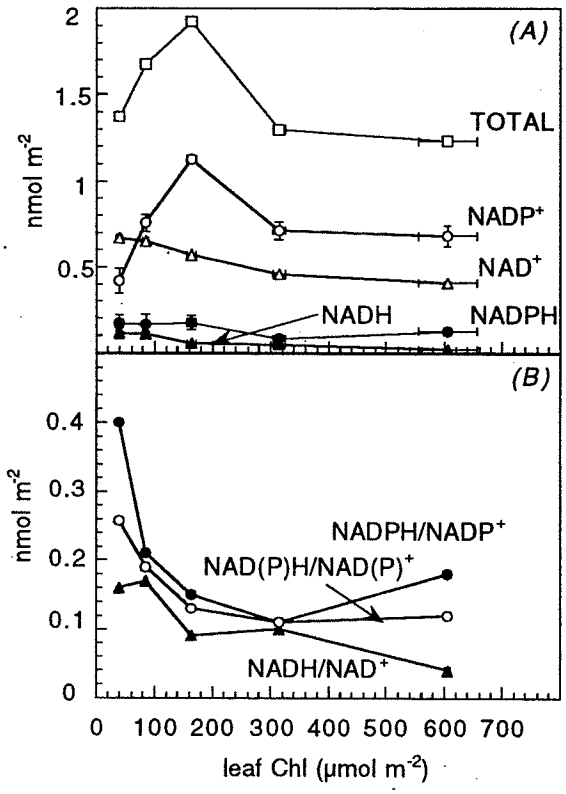

Figure 6. 10. Effects of Fe deficiency on (A) pyridine nucleotide concentrations in pear leaves (in nmol m^{-2}) and (B) NADH/NAD^+, NADPH/$NADP^+$ and NAD(P)H/$NAD(P)^+$ ratios. Data are means ± SE of 5 replicates.

The pool of pyridine nucleotides increased 1.6-fold in the leaves of Fe-deficient plants (100-150 µmol Chl m^{-2}) when compared to the controls. Iron deficiency increased the concentrations of both reduced and oxidised nucleotide forms. The largest relative increase was 5.5-fold for NADH (from 0.02 to 0.11 nmol m^{-2}),

followed by 1.7-fold for $NADP^+$ (from 0.68 to 1.13 nmol m^{-2}), 1.6-fold for NAD^+ (from 0.41 to 0.67 nmol m^{-2}) and 1.4-fold for NADPH (from 0.12 to 0.17 nmol m^{-2}) (Fig. 6.10A). As a result of these changes, the $NADH/NAD^+$ and $NADPH/NADP^+$ ratios increased 4.2- and 2.2-fold in response to Fe deficiency (Fig. 6.10B).

6. 5. Discussion

We have characterised for the first time the effects of Fe deficiency on the chemical composition of leaf apoplastic fluid in field-grown pear trees. Several reports have investigated the ionic composition of the apoplast in leaves (see Grignon and Sentenac, 1991; Canny, 1995 and Sattelmacher et al., 1998 for reviews), but little is known about the composition of this leaf compartment in field-grown trees. No report so far has been published on the changes induced by Fe deficiency in this compartment.

Iron deficiency caused a marked increase in the apoplastic pH of pear leaves as judged from direct pH measurements in apoplastic fluid obtained by centrifugation and by fluorescence of leaves incubated with 5-CF. The increase in pH was almost one unit, from 5.5-5.9 to 6.5-6.6. These data support Mengel's view (Mengel, 1995) that Fe deficiency could indeed be associated to pH increases of the leaf apoplastic space in field-grown plant species. This pH increase, however, is not likely to cause major problems in Fe acquisition by mesophyll cells (see discussion below). The pH increase found in pear contrasts with the significant decrease (0.4 pH units) found recently with Fe deficiency in the apoplastic fluid of sugar beet (López-Millán, chapter 5).

Our data confirm that leaves from Fe-deficient trees are under a short supply of apoplastic Fe. Iron deficiency caused a marked (2-fold) decrease in the Fe concentration of the apoplastic fluid, similar to the decrease in total leaf Fe concentration (data expressed on a leaf volume basis) in the pear leaves in the same orchard (Morales et al., 1998). The major Fe-chelate species predicted to exist in the apoplastic fluid of Fe-deficient and Fe-sufficient leaves were $FeCitOH^-$ and $FeCit_2^{3-}$. Iron deficiency causes significant changes in the chemical speciation of Fe-citrate in pear apoplast, with the monomeric $FeCitOH^-$ form increasing from 60 to 85% of the total Fe with Fe deficiency. This chemical species may favour Fe

acquisition by the mesophyll plasma membrane FC-R, since it would experience less electrostatic repulsion with the negatively charged PM than $FeCit_2^{3-}$. The formation of citrate-Fe polymers (Spiro et al., 1961a) is frequently assumed to occur in plant shoots (Bienfait and Scheffers, 1991; Moog and Brüggemann, 1994; Schmidt, 1999). However, no experimental evidence is currently available in support of this theory. In fact, excess citrate would compete effectively with the formation of the citrate-Fe polymers, therefore inhibiting polymerisation (Spiro et al., 1961b). Given the high citrate:Fe ratios, the formation of citrate-Fe polymers in the pear apoplast is unlikely.

The marked increases in the organic anion:Fe ratios with Fe deficiency may constitute a major factor decreasing Fe availability by mesophyll cells. The malate:citrate:Fe molar ratios increased with Fe deficiency from 300:150:1 to 1750:900:1 in the apoplastic fluid. The large citrate:Fe molar ratios found in the leaf apoplastic fluid of Fe-deficient plants may impair significantly Fe uptake by mesophyll cells, since the activity of the FC-R leaf PM enzyme has been recently shown to decrease markedly when the citrate:Fe ratio increases. Plasma membrane FC-R activities decreased 5-fold when the citrate:Fe molar ratio increased from 100 to 500 (González-Vallejo et al., 1999). On the other hand, high malate:Fe ratios in apoplastic fluid may favour Fe acquisition by mesophyll cells, because the activity of the FC-R enzyme increases markedly with increases in the malate:Fe ratio (González-Vallejo et al., 1999). Chemical speciation, however, indicates that Fe-malate complexes are unlikely to be formed in significant amounts in the apoplast to be substrates for the FC-R. It should be also taken into account that Fe deficiency may decrease largely by itself leaf FC-R activities, both in protoplasts and in mesophyll tissue (Gonzalez-Vallejo et al., 2000; Larbi et al., 1999).

Several other factors that change with Fe deficiency may modulate Fe availability from the apoplastic space to a lower extent than the citrate:Fe ratios. It has been hypothesised that an increase in apoplastic pH could affect the FC-R of the plasma membrane of mesophyll cells, leading to Fe immobilisation in the apoplastic space (Mengel, 1995). The one-unit pH change found in pear apoplast with Fe-deficiency would lead to 30-50% decreases in FC-R enzyme activity of sugar beet leaf protoplasts (González-Vallejo et al., 2000) and to approximately 20% increases in the FC-R enzyme activity of sugar beet leaf disks (Larbi, 1999).

A second factor to be considered is the Fe speciation changes associated to the changes in chemical composition of the pear apoplast. The fact that Fe deficiency led to a predicted increase in pear apoplast of the Fe:citrate species less charged, $FeCitOH^-$, would tend to diminish electrostatic repulsion with the negatively charged PM, thus increasing Fe availability in spite of the increase in apoplastic pH. A third factor modulating FC-R activities could be changes in pyridine nucleotide redox state. The increases found in mesophyll $NADPH/NADP^+$ and $NADH/NAD^+$ ratios (2.2 and 4.2-fold, respectively) would also tend to increase PM FC-R activities.

The organic acid increases found in the apoplastic fluid and whole leaf homogenates are unlikely to be caused by an increase of the rate of C fixation in leaves. The leaf photosynthetic rate of fruit tree leaves (Hurley et al., 1986) and other plant species (Terry, 1980) is markedly decreased by Fe deficiency. Also, the activity of leaf PEPC, that might fix bicarbonate, does not increase with Fe deficiency. Instead, our data suggest that the increase in organic anion concentrations in the leaves could be due to C import via xylem. Iron-deficient roots have increased activities of PEPC and other enzymes, accompanied by metabolite changes suggesting the existence of a non-autotrophic, anaplerotic C fixation in roots (López-Millán, chapter 3). This C can be exported via xylem to the leaves (Bialzyk and Lechowski, 1992; López-Millán, chapter 5) thus contributing to the increases in organic anions found in apoplastic fluid and whole leaves.

The increase in leaf organic anion concentrations with Fe deficiency may be the cause underlying other leaf biochemical changes. This includes the slight increases in leaf TCA cycle enzymatic activities, the 60% increase in the leaf pyridine nucleotide pool and the increases in the leaf $NADPH/NADP^+$ and $NADH/NAD^+$ ratios. In general, these changes seem to improve the conditions for an enhancement of leaf PM FC-R activity. Iron deficiency, however, was associated only to a 50% decrease in the concentration of Fe in pear apoplast, suggesting that mesophyll FC-R activities were not markedly enhanced by Fe deficiency. The absence of a leaf FC-R activity enhancement with Fe deficiency in protoplasts (González-Vallejo et al., 2000) and mesophyll tissue (Bruggemann et al., 1993; de la Guardia and Alcántara, 1996; Larbi, 1999) would suggest that other limiting steps for Fe uptake are present in Fe-deficient mesophyll.

Ionic concentrations of organic and inorganic ions in the apoplast of control pear leaves are in the same range than those reported in the literature for leaves of *Quercus*, sunflower, pea, spinach and bean (Bowling, 1987; Speer and Kaiser, 1991; Dannel et al., 1995; Gabriel et al., 1999). Sulphate concentrations were, however, much higher, possibly due to agrochemical treatments made usually in the area. The total anion: cation ratios for Fe-sufficient and deficient apoplast fluid were 0.64 and 0.63. Such a large excess of cations compared to anions in the apoplast is a common observation, mediated by the strong negative charge of the cell wall and PM (Dietz et al, 1997).

In summary, iron deficiency in pear was associated to organic anion accumulation in the apoplast and also in whole leaf tissue. This organic anion accumulation, however, does not appear to improve the conditions for Fe availability from the apoplast, since the predicted major chemical species was the same in Fe-sufficient and Fe-deficient leaves, in spite of the one-unit increase in apoplast pH with Fe deficiency. Instead, citrate accumulation could potentially be deleterious for Fe availability through a decrease of the PM FC-R activity caused by the high citrate:Fe ratios. We hypothesise that the accumulation of organic anions in the Fe-deficient plants is a consequence of C transport via xylem and that this response does not provide any evident advantage for Fe long distance transport. The production and accumulation of organic acids could have instead two significant advantages. First, it would improve organic anion release to the rhizosphere, thus improving Fe-chelation and microbial growth. Second, it would provide substrates for respiratory and other maintenance processes to leaves with very low photosynthetic activity.

CAPÍTULO 7

CAMBIOS INDUCIDOS POR LA DEFICIENCIA DE Fe Y APORTE POSTERIOR DE Fe EN EL METABOLISMO DE LOS ÁCIDOS ORGÁNICOS EN HOJAS DE REMOLACHA (Beta vulgaris L.)

7. Changes Induced by Fe Deficiency and Fe Resupply in the Organic Acid Metabolism of Sugar Beet Leaves (*Beta vulgaris* L.)

7. Changes Induced by Fe Deficiency and Fe Resupply in the Organic Acid Metabolism of Sugar Beet Leaves (*Beta vulgaris* L.).

7. 1 Abstract

The effects of iron deficiency and iron resupply on the metabolism of leaf organic acids have been investigated in hydroponically grown sugar beet. Measurements included organic acid concentrations and activities in leaf extracts of several enzymes related to organic acid metabolism. These included phosphoenol-pyruvate carboxylase (EC 4.1.1.31), different Krebs cycle enzymes (malate dehydrogenase (EC 1.1.1.37), aconitase (EC 4.2.1.3), fumarase (EC 4.2.1.2), citrate synthase (EC 4.1.3.7) and isocitrate dehydrogenase (EC 1.1.1.42), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and two enzymes related to anaerobic metabolism (lactate dehydrogenase, EC 1.1.1.27 and pyruvate decarboxylase, EC 4.1.1.1). Iron concentration in leaves was severely decreased by iron deficiency. Iron resupply caused an increase in iron concentrations, reaching levels similar to the controls in 96 h. Iron deficiency induced a 2.3-fold

(from 16 to 37 mmol m^{-2}) increase in leaf total organic acid concentration. Organic anion concentrations were still 4-fold higher than the controls 24 h after resupply and decreased to values similar to those found in the controls after 96 h. All measured enzymes had increased activities in extracts of iron-deficient leaves when compared to the controls and generally decreased to control values 24 h after iron addition. These data provide evidence that organic acid accumulation in the leaf is likely not due to an enhancement in leaf carbon fixation. Instead, this accumulation seems to be due to organic acid export from the roots to the leaves via xylem.

7. 1. 1. Abbreviations: CS, citrate synthase; DTNB, 5-5´dithio-bis-2-nitobenzoic acid; FC-R, ferric-chelate reductase; FDH, formate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MS, mass spectroscopy; PDC, pyruvate decarboxylase; PEPC, phosphoenol pyruvate carboxylase; PM, plasma membrane; PVDF, polyvinyl fluoride; SPAD, portable Chl meter; 5-CF, 5-carboxy fluorescein.

7. 2. Introduction

When plants are grown under Fe-deficient conditions their youngest leaves develop a characteristic greenish-yellow colour, often referred to as iron chlorosis. Leaves have decreases in leaf chlorophyll (Chl) concentration and drastically reduced photosynthetic rates (Terry and Abadía, 1986; Abadía and Abadía, 1993). Without sufficient Chl, leaves are unable to produce enough photosynthates for growth and development (Miller et al., 1984). The characteristic yellow colour of chlorotic leaves is a consequence of a relative enrichment in carotenoids (Bolle-Jones and Notton, 1953; Terry, 1980; Morales et al., 1990). Other changes caused by Fe deficiency on leaf physiology were described in several reviews (Terry and Abadía, 1986; Abadía, 1992; Terry and Zayed, 1995).

Most of the information available on the effects of Fe deficiency in the leaf physiology is relevant to chloroplast structure and function. Less information is available so far on other biochemical responses of leaf cells, such as the accumulation of organic acids and the changes in the activities of the enzymes involved in their metabolism. When grown under Fe deficiency, plants accumulate organic acids, mainly citrate and malate, in leaves and xylem sap (Iljin, 1951; Palmer et al., 1963; Bindra, 1980; Landsberg, 1981; Welkie and Miller, 1993). It is commonly assumed that citrate could play an important role in the translocation of Fe in the roots (Tiffin, 1966a) and in its long-distance transport through the xylem to the leaves (Tiffin, 1966b; Brown, 1971; White et al., 1981; Cataldo et al., 1988; Schmidt, 1999). However, both the role(s) and the source of organic acids in Fe-deficient leaves have not been well established so far (Schmidt, 1999).

Iron deficiency causes nutrient imbalances in different parts of the plant. For instance, leaf mineral composition and nutrient ratios are generally affected (Alloush et al., 1990; Köseoglu, 1995). The concentration of bipyridyl-extractable Fe, total K and the ratios K/Ca and P/Fe of peach leaves are significantly affected by Fe chlorosis (Abadía et al., 1985). When plants grow under Fe deficiency in controlled environments the leaf Fe concentration (on dry matter or area basis) is generally low when compared to Fe-sufficient plants (Terry, 1980; Terry and Low, 1982; Kolesch et al., 1984). However, in leaves of field-grown Fe-deficient plants the concentration of Fe is relatively high in many cases (Abadía et al., 1989; Morales et al., 1998). The fact that Fe-chlorotic leaves often have a high Fe concentration has been recently termed the (Fe) chlorosis paradox (Römheld, 1997). This may suggest that, when Fe-deficient, part of the Fe acquired from the soil by the FC-R could be immobilised and accumulated as inactive forms somewhere in the leaf (Morales et al., 1998).

Iron resupply to Fe-deficient plants restores plant physiology. For instance, it increases Chl concentrations and restores photosynthetic activity in sugar beet

(Nishio and Terry, 1983; Nishio et al., 1985) and increases Fe concentrations in roots and leaves of sugar beet (Young and Terry, 1982) and maize (Lobréaux et al., 1992, 1993, Thoiron et al., 1997). Also, Fe resupply to Fe-deficient sunflower plants was shown to cause decreases in the citrate concentrations in the xylem sap (Brown and Tiffin, 1965; Tiffin, 1966a,1966b).

The aim of this work was to investigate the effects of Fe resupply on the organic acid metabolism of Fe-deficient sugar beet leaves to further understand the biochemical responses of plants to Fe deficiency. We measured the concentrations of mineral nutrients and organic acids in leaves of sugar beet affected by Fe deficiency and their changes with Fe resupply. Changes with Fe resupply on the activity of the enzyme glucose-6-phosphate dehydrogenase and on the activities of several enzymes related to organic acid metabolism (PEPC and different TCA cycle related enzymes) were measured. The activities of LDH and PDC, two enzymes related to anaerobic metabolism, were also determined in Fe-deficient sugar beet leaves before and after Fe resupply.

7. 3. Materials and Methods

7. 3. 1. Plant Material

Sugar beet (*Beta vulgaris* L. Monohil hybrid from Hilleshög, Landskröna, Sweden) was grown in a growth chamber with a PPFD of 350 µmol m^{-2} s^{-1} PAR at a temperature of 25°C, 80% relative humidity and a photoperiod of 16 h light/ 8 h dark. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for two more weeks in half-strength Hoagland nutrient solution with 45 µM Fe and then transplanted to 20 L plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution (Terry, 1980) with either 0 or 45 µM Fe. The pH of the Fe-free nutrient solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$. This treatment simulates conditions usually found in the field leading to Fe deficiency (Susín et al., 1994). In Fe resupply experiments, plants grown for 10 d in absence of Fe were transferred to 20 L plastic buckets containing half-strength Hoagland nutrient solution, pH

5.5, with 45 µM Fe and no $CaCO_3$. Leaf samples were taken at 0 (before adding Fe), 24 and 96 h after the addition of Fe.

7. 3. 2. Chlorophyll Determination

Leaf chlorophyll concentration was estimated non-destructively with an SPAD-502 device (Minolta, Osaka, Japan). For calibration, leaf disks with different degrees of Fe deficiency were first measured with the SPAD, then extracted with 100% acetone in presence of Na ascorbate and chlorophyll measured spectrophotometrically (Abadía and Abadía, 1993).

7. 3. 3. Mineral Content Analysis

The leaf mineral composition was determined as described previously (Abadía et al., 1985). Potassium was measured by emission spectrophotometry and Ca, Mg, Fe, Mn, Cu and Zn by atomic absorption spectrophotometry.

7. 3. 4. Organic Anion Analysis

Leaf samples (1 leaf disk of 9.64 cm^2, taken with a calibrated cork borer) were frozen in liquid N_2 and ground in a mortar with 8 mM sulphuric acid. Homogenates were boiled for 30 min, filtered with a 0.2 µm PVDF filter (LIDA, Kenosha, WI, USA), taken to a final volume of 2 mL with 8 mM sulphuric acid and kept at -80°C until analysis.

Organic anions were analysed by HPLC with a 300 x 7.8 mm Aminex ion-exchange column (HPX-87H from Bio-Rad, Hercules, CA, USA) with a HPLC Waters system, including a 600E pump, a 996 photodiode array detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (8 mM sulphuric acid) was pumped with a 0.6 mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, cis-aconitate, citrate, 2-oxoglutarate, malate, succinate and fumarate were identified by comparison of their retention times with those of known standards from Bio-

Rad and Sigma. Succinate coeluted with an unidentified compound. The identity of some peaks was further confirmed by UV-VIS and MS (Waters). Quantification was made with known amounts of each organic anion using peak areas.

7. 3. 5. Enzyme Assays

Extracts for measuring enzyme activities were made by grinding 3 leaf disks of 1 cm^2 each, in a mortar with 1 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10000*g* and 4°C, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 1 mL (final volume) of the media indicated below.

Malate dehydrogenase (MDH; EC 1.1.1.37) was determined with oxalacetate as substrate (Dannel et al., 1995) by measuring the decrease in A_{340} nm due to the enzymatic oxidation of NADH. The reaction was carried out with 5 µL of extract in 0.1 mM NADH, 0.4 mM oxalacetate and 46.5 mM Tris-HCl, pH 9.5. Citrate synthase (CS; EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1967) by monitoring the reduction of acetyl CoA to CoA with 5-5'-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. The reaction was carried out with 50 µL of extract in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalacetate and 100 mM Tris-HCl, pH 8.1. Aconitase (EC 4.2.1.3) was measured from the formation of cis-aconitate, monitored at 240 nm (Bacon et al., 1961) with 60 µL of extract in 500 mM sucrose, 50 mM isocitrate and 100 mM Tris-HCl (pH 8.5). Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was determined with 50 µL of extract by monitoring the reduction of $NADP^+$ at 340 nm in a reaction mixture containing 3.5 mM $MgCl_2$, 0.41 mM $NADP^+$, 0.55 mM isocitrate and 88 mM imidazole, pH 8.0 (Bergmeyer et al., 1974). Fumarase (EC 4.2.1.2) was assayed with 50 µL of extract following the increase in A_{240} due to the formation of fumarate (Bergmeyer et al., 1974) in 50 mM malate and 100 mM phosphate, pH 7.4.

Phosphoenol pyruvate carboxilase (PEPC; EC 4.1.1.31) was measured in a coupled enzymatic assay with MDH according to Vance et al. (1983) with 75 µL of extract in 2 mM phosphoenol-pyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16

mM NADH and 100 mM bicine-HCl, pH 8.5. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was determined with D glucose-6-phosphate as substrate (Bergmeyer et al., 1974) by measuring the increase in A_{340} due to the enzymatic reduction of $NADP^+$. The reaction was carried out with 50 µL of extract in 138 mM $MgCl_2$, 20 mM glucose-6-phosphate, 7.8 mM $NADP^+$ and 100 mM HEPES, pH 7.6. For the determination of lactate dehydrogenase (LDH; EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1) the oxidation of NADH was monitored at 340 nm with 50 µL of extract. LDH was assayed in a reaction buffer containing 94.5 mM phosphate buffer (pH 9.5), 0.77 mM pyruvate and 0.2 mM NADH. PDC was determined in 190 mM citrate-KOH buffer (pH 6.0), 30 mM pyruvate, 0.32 mM NADH and 33 µg mL^{-1} alcohol dehydrogenase.

7. 4. Results

7. 4. 1. Changes in Leaf Mineral Nutrient Composition Induced by Fe Deficiency and Fe Resupply

Fe deficiency induced changes in the mineral nutrient composition of sugar beet leaves. Iron-deficient leaves had 50 and 37% decreases in total P and K concentrations, respectively, and 140 and 50% increases in Ca and Mg concentrations, respectively, when compared to the controls (Table 7.1). Among the microelements, Fe decreased by 70% in Fe-deficient leaves, whereas the concentrations of Mn, Cu and Zn were not significantly different from those of Fe-sufficient leaves (Table 7.1). The high nutrient concentrations found agree with data reported before for a different sugar beet genotype grown in nutrient solution (Nagarajah and Ulrich, 1965).

Iron resupply to Fe-deficient plants induced slight increases in leaf K and Mg concentrations, whereas leaf P and Ca did not change significantly (Table 7.1). After 24 of Fe resupply there were no significant changes in K, whereas Mg increased by 33%. After 96 of Fe resupply K increased by 14%, whereas Mg returned to values similar to those found in Fe-deficient leaves. Iron concentrations increased 1.9- and 3.5-fold at 24 and 96 h after Fe resupply,

respectively, reaching values similar to control leaves (Table 7.1). The concentrations of Mn and Zn decreased after 24 h of Fe resupply. After 96 h of Fe resupply Zn returned to the values found before resupply, whereas Mn reached values higher than those found in Fe-deficient leaves. Cu concentrations decreased slightly with Fe resupply.

	Fe-sufficient	Fe-deficient	Fe resupplied 24 h	Fe resupplied 96 h
P	1.0±0.1	0.5±0.1	0.5±0.1	0.6±0.1
K	8.1±04	5.1±0.3	5.2±0.1	5.8±0.3
Ca	1.8±0.2	4.4±0.3	4.9±0.2	4.5±0.3
Mg	1.2±0.0	1.8±0.1	2.4±0.1	2.0±0.1
Fe	153±33	46±8	88±16	160±20
Mn	248±14	254±3	234±5	272±6
Cu	22±3	25±2	24±1	19±2
Zn	83±4	87±3	60±1	78±7

Table 7. 1. Mineral composition of Fe-sufficient (300 µmol Chl m^{-2}), Fe-deficient (50-70 µmol Chl m^{-2}) and Fe-deficient Fe resupplied leaves after 24 and 96 h. P, K, Ca and Mg are in g/100 g DW and Fe, Mn, Cu and Zn are in µg g^{-1} DW. Data are means ± SE of 5 replicates.

7. 4. 2. Changes in Leaf Chlorophyll with Fe Resupply

Iron deficiency was associated to low leaf Chl concentrations in sugar beet. The Chl concentrations in the youngest leaves from plants grown for 10 d in absence of Fe ranged from 50 to 200 µmol m^{-2}, whereas control values were approximately 300 µmol m^{-2}. Resupply of Fe to these plants caused a general leaf regreening (Fig. 7.1). Chl increases after 24 h were 26, 8 and 2% of the initial values in leaves with 50, 100 and 200 µmol m^{-2}, respectively (Fig. 7.1). After 96 h of Fe resupply Chl increased further to values between 153 and 233 µmol m^{-2} (Fig. 7.1). Plants after Fe resupply are shown in Fig 7.2.

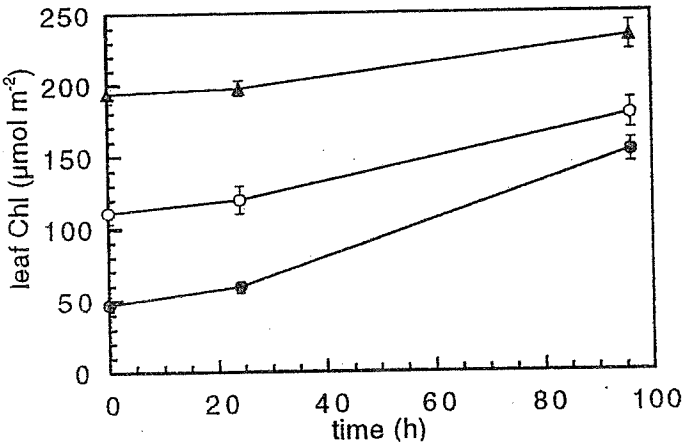

Figure 7. 1 Changes in chlorophyll concentrations with Fe resupply in leaves affected by different degrees of Fe chlorosis. Data are means ± SE of 10 replicates.

Figure 7. 2. Sugar beet plants after 96 h of Fe resupply

7. 4. 3. Changes in Leaf Organic Anion Concentrations with Fe Deficiency and Fe Resupply

	Fe-sufficient	Fe-deficient	Fe resupplied 24h	Fe resupplied 96h
Oxalate	14.0±3.1 (300)	11.6±0.7 (50)	18.1±0.3	14.8±0.6
		14.5±2.6 (100)	35.5±1.4	18.1±1.4
		21.3±0.5(200)	35.7±1.6	22.9±1.3
Citrate	0.8±0.6 (300)	5.8±0.4 (50)	1.5±0.6	0.2±0.0
		6.6±0.9 (100)	4.0±1.0	0.3±0.1
		9.9±1.9 (200)	4.4±2.0	1.5±0.3
Malate	0.9±0.1 (300)	3.7±0.1 (50)	1.8±0.3	1.0± 0.2
		5.4±0.1 (100)	2.9±0.8	0.9±0.2
		6.1±0.8 (200)	2.8±1.2	1.3±0.1
Succinate	0.41±0.10 (300)	1.21±0.19 (50)	1.75±0.76	4.49±0.69
		1.06±0.14 (100)	0.59±0.27	2.67±0.55
		0.33±0.11 (200)	1.25±0.31	2.93±0.30
2-oxoglut	9.6±2.3 (300)	29.1±4.6 (50)	45.0±15.0	17.0±8.0
		61.0±8.0 (100)	27.0±10.4	25.2±3.9
		53.5±18.2 (200)	26.9±6.0	25.1±7.0
cis-aconitate	<0.1(300)	37.0±12.8 (50)	<0.1	<0.1
		120.7±52.4 (100)	<0.1	<0.1
		31.3±16.1 (200)	<0.1	<0.1
Fumarate	14.7±3.3 (300)	109.5±6.4 (50)	15.5±0.1	6.8±0.9
		151.6±21.2 (100)	51.0±12.0	5.3±0.7
		292.0±7.2 (200)	38.8±22.0	7.8±0.9

Table 7. 2. Concentrations of oxalate, citrate, malate and succinate (in mmol m^{-2}) and 2-oxoglutarate, cis-aconitate and fumarate concentrations (in µmol m^{-2}) in Fe-sufficient (300 µmol Chl m^{-2}) and Fe-deficient (50, 100 and 200 µmol Chl m^{-2}) leaves of sugar beet at 0, 24 and 96 h after Fe resupply. Values in parenthesis are leaf Chl concentrations. Data are means ± SE of 5 replicates.

In both Fe-sufficient and deficient leaves the four major organic acid species were oxalate, citrate, malate and succinate, accounting for 98% of the total organic anion pool. Organic anions generally increased with Fe deficiency, major increases being found for oxalate, citrate and malate in moderately Fe-deficient leaves with a Chl content of 200 µmol m^{-2} (Table 7.2). Concentration increases with Fe deficiency were 12.4-fold for citrate (from 0.8 to 9.9 mmol m^{-2}), 6.8-fold for

malate (from 0.9 to 6.1 mmol m^{-2}) and 1.5-fold for oxalate (from 14 to 21 mmol m^{-2}) (Table 7.2). Severely Fe-deficient leaves had lower concentrations of citrate and malate than those found in moderately Fe-deficient ones, although these values were still higher than those of Fe-sufficient leaves (Table 7.2). Succinate was maximal in severely Fe-deficient leaves.

Maximum concentrations of the minor organic anions cis-aconitate and 2-oxoglutarate (12- and 6-fold increases over the control values) were found in Fe-deficient leaves with a Chl concentration of 100 μmol Chl m^{-2} (Table 7.2). Fumarate concentrations also increased 20-fold in moderately deficient leaves (200 μmol Chl m^{-2}) and decreased in severely deficient leaves (50 μmol Chl m^{-2}) which still showed fumarate concentrations 8-fold higher than those found in Fe-sufficient leaves (Table 7.2).

Iron resupply to Fe-deficient plants decreased progressively leaf organic anion concentrations, except for oxalate and succinate. After 24 h of Fe resupply citrate concentrations decreased by 74, 39 and 56% in Fe-deficient leaves with initial Chl concentrations of 50, 100 and 200 μmol m^{-2}, respectively. After 96 h of Fe resupply citrate concentrations had decreased to values similar to or even lower than those found in the controls (Table 7.2). Malate decreased in all leaves approximately to 46-54% and 17-21% of the initial values, 24 and 96 h after adding Fe, respectively (Table 7.2). Oxalate had major increases 24 h after Fe resupply and then decreased at 96 h, whereas succinate generally increased at both resupply times (Table 7.2). The concentrations of the minor organic acids cis-aconitate, fumarate and 2-oxoglutarate decreased with Fe resupply. Cis-aconitate could not be detected after Fe resupply, similarly to what happens in the controls. Fumarate concentrations decreased to 13-34% and 3-6% of the initial values, 24 and 96 h after adding Fe, respectively (Table 7.2). At 24 h after Fe resupply the concentration of 2-oxoglutarate increased 1.5-fold in leaves with 50 μmol Chl m^{-2} and decreased to 44-50% in leaves with a lower degree of Fe deficiency. At 96 h 2-oxoglutarate was 41-58% of the initial values.

7. 4. 4. Changes in Leaf Enzymatic Activities with Fe Deficiency and Fe Resupply

Five enzymatic activities involved in organic acid metabolism (MDH, ICDH, CS, aconitase and fumarase) were measured in extracts of Fe-sufficient and Fe-deficient leaves of sugar beet.

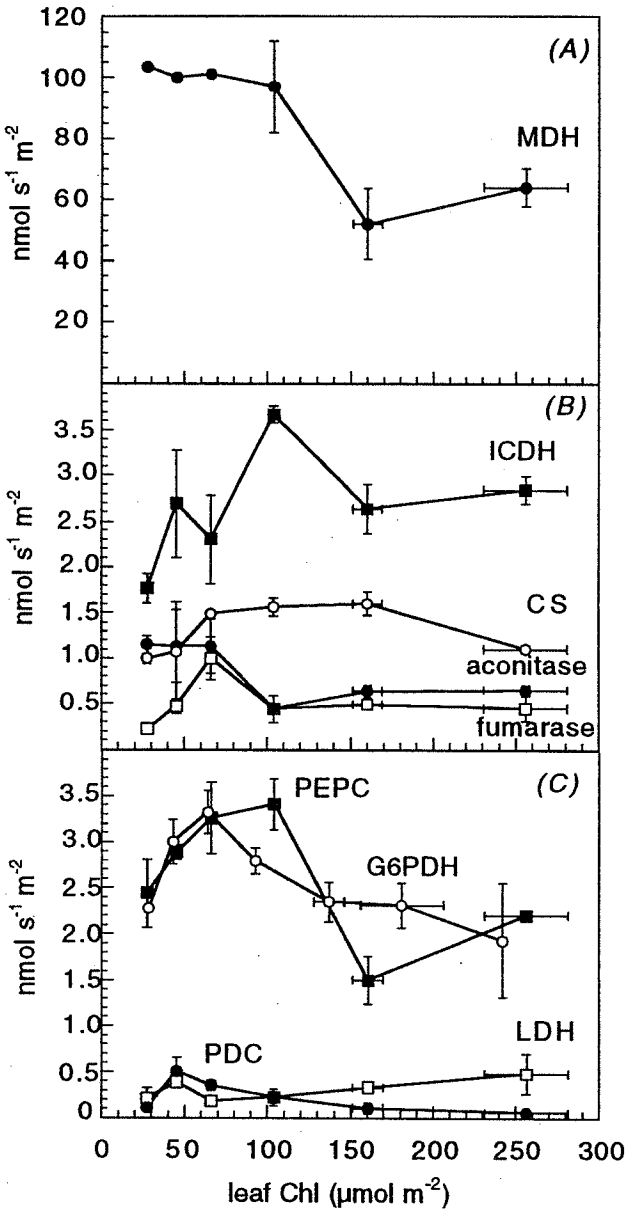

Figure 7. 3. Changes in enzymatic activities of leaf extracts with Fe deficiency (in nmol s^{-2} m^{-2}). MDH (A), ICDH (solid squares), CS (open circles), aconitase (solid circles), fumarase (open squares) (B), PEPC (solid squares), G6PDH (open circles), PDC (solid circles) and LDH (open squares) (C). Data are means ± SE of 5 replicates.

Enzymatic activities increased moderately with Fe deficiency, with the highest activities being found in leaves with 40-100 μmol Chl m^{-2} (Fig. 7.3). Increases over the control values were 2.0-fold for fumarase (from 0.5 to 1.0 nmol s^{-1} m^{-2}), 1.7-fold for aconitase (from 0.6 to 1.1 nmol s^{-1} m^{-2}) and 1.6-fold for MDH (from 64 to 100 nmol s^{-1} m^{-2}). Fumarase activity decreased in leaf extracts with lower Chl concentrations, whereas MDH and aconitase activities remained fairly constant. The activity of CS showed a moderate increase with Fe deficiency, whereas ICDH increased 1.3-fold (from 2.8 to 3.7 nmol s^{-1} m^{-2}) in leaves with 100 μmol Chl m^{-2} and decreased in leaves with lower Chl concentrations.

Activities of PEPC and G6PDH in leaf extracts also increased moderately with Fe deficiency. Major increases in PEPC and G6PDH activities were found in leaf extracts with 100 and 70 μmol Chl m^{-2}, respectively (Fig. 7.3C). Enzymatic activity increases with Fe deficiency were 1.5-fold for PEPC (from 2.2 to 3.3 nmol s^{-1} m^{-2}) and 1.7-fold for G6PDH (from 1.9 to 3.3 nmol s^{-1} m^{-2}) (Fig. 7.3C).

We have also measured the activities of LDH and PDC, two enzymes related to the anaerobic metabolism. LDH activity decreased with Fe deficiency from 0.5 to 0.2 nmol s^{-1} m^{-2}, whereas PDC activity increased 10-fold in leaves with 50 μmol Chl m^{-2} when compared to the controls and decreased in leaves with lower Chl concentrations (Fig. 7.3C).

The effects of Fe resupply were only studied in those enzymes which increased in Fe-deficient leaf extracts more than 50% when compared to the controls (MDH, fumarase, aconitase, PEPC, G6PDH and PDC; Table 7.3). Except for fumarase all these enzymatic activities decreased 24 h after Fe resupply, reaching values even lower than those found in control leaves (Table 7.3). At 96 h after Fe resupply enzyme activities measured did not change significantly with respect to those activities found before resupply. Fumarase activity increased 2.2- and 1.9-fold, 24 and 96 h after adding Fe. These values were 4 to 5-fold higher than the activities found in the Fe-sufficient controls.

	Fe-sufficient (+Fe)	Fe-deficient (-Fe)	ratio -Fe/+Fe	Fe resupplied 24 h	96 h
MDH	64.0±6.22	100±0.3	1.6	33.1±1.8.	37.6±1.3
CS	1.10±0.01	1.49±0.05	1.3	nm	nm
aconitase	0.65±0.06	1.13±0.40	1.7	0.19±0.01	0.18±0.02
ICDH	2.84±0.15	2.69±0.58	0.9	nm	nm
fumarase	0.45±0.14	1.00±0.23	2.2	2.20±0.29	1.90±0.14
PEPC	2.20±0.05	3.26±0.39	1.5	0.33±0.03	0.37±0.02
G6PDH	1.93±0.62	3.30±0.23	1.7	0.10±0.01	0.11±0.01
LDH	0.48±0.06	0.38±0.3	0.8	nm	nm
PDC	0.05±0.01	0.50±0.15	10	0.04±0.00	0.04±0.00

Table 7. 3. Enzymatic activities (in nmol m^{-2} s^{-1}) in leaf extracts from Fe-sufficient (250 µmol Chl m^{-2}) and Fe-deficient (50-70 µmol Chl m^{-2}) sugar beet plants at 0, 24 and 96 h after Fe resupply. Data are means ± SE of 5 replicates.

nm. not measured

7. 5. Discussion

Concentrations of Fe in leaves were severely decreased by Fe deficiency, down to 46 µg g^{-1} dry mass. This confirms that Fe chlorosis in sugar beet is mediated by a true Fe shortage, and that Fe-immobilisation processes are not involved, conversely to what happens in some instances under field conditions (Morales et al., 1998). Iron resupply caused a rapid increase in leaf Fe concentrations, reaching values half of that found in control leaves 24 h after Fe resupply and values similar to the controls in 96 h. Similar rapid Fe concentration increases upon Fe resupply have been reported in sugar beet (Young and Terry, 1982) and maize leaves (Thoiron et al., 1997).

The total pools of leaf organic anions were much larger in Fe-deficient leaves than in the controls. Increases were particularly large for citrate (12-fold) and malate (7-fold). This agrees with data obtained with other plant species (Iljin, 1951; de Kock and Morrison, 1958; Palmer et al., 1963; Landsberg, 1981). Maximal organic anion concentrations occurred in moderately Fe-deficient leaves having 200 µmol Chl m^{-2}. Contrary to reports for other plant species and in agreement with previous studies in sugar beet (Nagarajah and Ulrich, 1965), Fe deficiency

caused an increase in leaf Ca and Mg concentrations but did not increase leaf K or HPO_4^{2-} concentrations. Increases in divalent cation concentrations could counteract the increases in organic anions. Upon Fe-resupply, the pools of leaf organic anions decreased gradually. By 24 h of Fe resupply the pools of these anions decreased approximately by half, and by 96 h of resupply concentrations reached values similar to those found in the controls.

The activities of different enzymes in extracts of Fe-deficient leaves were higher than in those of Fe-sufficient leaves. The largest increase was found for PDC (10-fold), whereas MDH, aconitase, fumarase and PEPC increased approximately 1.5 to 2-fold. Maximal enzymatic activities occurred in Fe-deficient leaves with 50-100 μmol Chl m^{-2}. Generally, Fe resupply caused a rapid decrease in the activities of these enzymes, and by 24 h activities were similar or even lower than those found in the Fe-sufficient controls.

The increased organic acid pools in Fe-deficient leaves are unlikely to be due to an increased leaf C fixation. First, Fe-deficient leaves have markedly decreased leaf photosynthetic rates (Terry, 1980; Abadía, 1992), caused by concomitant decreases in all the components of the photosynthetic machinery (Winder and Nishio, 1995). An alternative possibility to increase leaf C fixation would be a coordinated increase in the activities of PEPC, MDH and CS, that would cause carboxylation of PEP to oxalacetate and subsequently conversion into malate and citrate. The relationship between enzymatic activities and organic anion concentrations in Fe-deficient leaves, however, does not appear to be direct. In moderately Fe-deficient leaves PEPC and MDH activities did not increase whereas malate and citrate increases were approximately 7- and 12-fold, respectively. In extracts of severely Fe-deficient leaves PEPC and MDH activities increase 2-fold whereas malate and citrate increases were approximately 4- and 7-fold. Also, the possibility that PEPC may fix C in Fe-deficient leaves would depend on the existence of a source of PEP in Fe-deficient leaves, that have otherwise markedly reduced leaf photosynthetic rates (Terry, 1980) and low sugar contents (Arunalantham et al., 1990).

Instead, malate and citrate could be provided anaplerotically via xylem from roots. In Fe-deficient sugar beet roots the increased PEPC activity triggers other enzyme and metabolite changes that lead to enhancement of non-autotrophic,

anaplerotic C fixation in the form of organic anions (López-Millán, chapter 3), similar to that reported previously in P-stressed *Lupinus* (Vance et al., 1994). Furthermore, the xylem and apoplast of Fe-deficient sugar beet are highly enriched in organic anions, supporting the significance of this process (López-Millán, chapter 5). This pathway implies several ecological advantages for the Fe-deficient leaves. The anaplerotically-fixed organic anion pool could provide the energy to maintain basic metabolic processes in Fe-deficient leaves that have drastically reduced leaf photosynthetic rates (Terry, 1980). On the other hand, organic anions could play an important role in the translocation and transport of Fe from roots to leaves (Tiffin, 1966 a, b; Brown et al., 1971; White et al., 1981; Cataldo et al., 1988).

The increased activities of MDH and aconitase in Fe-deficient sugar beet leaves could be rather the consequence and not the cause of the increased leaf organic pools. The increases in malate and citrate, substrates for MDH and aconitase respectively, could be responsible for the increases in enzyme activities. Aconitase activity was 1.7-fold higher in Fe-deficient sugar beet leaves when compared to the controls, indicating that citrate accumulation was not due to a decrease in the activity of this enzyme, conversely to what has been proposed previously (de Kock and Morrison, 1958; Bacon et al., 1961). The absence of decreases in aconitase activity with Fe deficiency has been also observed in roots of bean (Landsberg, 1981), red pepper (de Vos et al., 1986), tomato (Pich and Scholz, 1993) and sugar beet (López-Millán, chapter 3).

Within the first 24 h of Fe resupply to Fe-deficient leaves there were increases in leaf Fe and decreases in PEPC and MDH activities to the values found in controls. However, malate and citrate concentrations still remained 2- to 5-fold higher than in control leaves and did not decrease to control values until 96 h after Fe resupply. These results indicate that the enzymatic activities are tightly regulated by Fe concentrations. The relatively slow decrease in organic acid concentrations with Fe resupply may result either from the still high xylem organic anion concentrations at this resupply time or be mediated by limitations in the utilization of the large amount of leaf stored C in organic acid forms.

From these data one may hypothesise that organic acid synthesis in roots could be down regulated by the Fe concentration in the leaves. This would provide a

significant advantage for the Fe-deficient plants, since organic acids would contribute to the transport of Fe in the xylem to the leaves and would provide carbon molecules for energy production in the leaf, which is highly affected by Fe deficiency. This regulation could be mediated, as suggested by Landsberg (1982), by auxin which has been shown to stimulate malate synthesis in oat coleoptile segments (Haschke and Lüttge, 1975) as well as CS in bean root tips (Sarkissian, 1972).

In Fe-deficient leaves PDC activity increased 10-fold whereas LDH decreased slightly with Fe deficiency. Several enzymes involved in the anaerobic metabolism, such as PDC, LDH and FDH, have been shown to increase their activities in Fe-deficient roots (Suzuki et al., 1998; López-Millán, chapter 3) This increase is possibly a consequence of the markedly increased O_2-consumption rates found in these roots, which may lead to a certain degree of root hypoxia. The increase in PDC activity observed in Fe-deficient leaves could also be associated to O_2 limitation, which has been suggested to occur in Fe-deficient leaves (Belkhodja et al., 1998). Another possible explanation for this increase in Fe-deficient leaves is that changes observed in TCA cycle enzymatic activities as well as in organic acid concentrations could lead to increases in pyruvate and subsequently to an increased PDC activity. Conversely, LDH, that uses also pyruvate as substrate, had decreased activities with Fe deficiency. Cytoplasmic acidosis, associated to increases in organic acid concentration, may lead to LDH inhibition (Drew, 1997).

In summary, Fe-deficient sugar beet leaves had large increases in the concentrations of organic acids and moderate increases in the activities of PEPC and several TCA cycle enzymes when compared to the controls. Several lines of evidence indicate that these organic anion increases arise from an external supply of C via xylem: a) the increase in organic acids occurred in leaves with a markedly decreased Chl concentration with very low photosynthetic rates and sugar contents; b) the increases observed in the activities of the enzymes involved in organic anion synthesis were lower than the increases observed in citrate and malate, especially in moderately Fe-deficient leaves; c) maximal increases in organic anions and enzymatic activities occur in leaves with different Chl concentrations; and d) Fe resupply caused a decrease in the organic anion pool down to control values in 96 h, whereas the enzymatic activities decreased to control values in 24 h. These data are in good agreement with the existence of an

export of organic acids from the roots to the Fe-deficient leaves via xylem, possibly associated to the enhanced anaplerotic fixation of CO_2 reported to occur in the roots of Fe-deficient plants (López-Millán, chapter 5).

CAPÍTULO 8

CAMBIOS INDUCIDOS POR LA DEFICIENCIA DE Fe EN EL METABOLISMO DE LOS ÁCIDOS ORGÁNICOS EN TOMATE (Lycopersicon esculentum L.)

8. 1. Changes Induced by Iron Deficiency in the Organic Acid Metabolism of Tomato (*Lycopersicon esculentum* L.)

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8. 1. Abstract

The effects of Fe deficiency on the organic acid metabolism have been characterised in roots, xylem sap and leaves of tomato. Iron deficiency increased the pH of the tomato leaf apoplast estimated with 5-carboxyfluorescein fluorescence from 5.4 to 6.4. The major organic acids found in roots, xylem sap and leaves were malate and citrate. Total organic acid concentrations in control plants were 14 and 31 nmol g^{-1} FW in roots and leaves, respectively and 0.7 mM in xylem sap. In Fe-deficient plants total organic acid concentrations were 13 and 65 nmol g^{-1} FW in roots and leaves, respectively and 4.8 mM in xylem sap. The largest increases were found for citrate in roots, xylem sap and leaves and for malate in xylem sap and leaves. Phosphoenol pyruvate carboxylase activity increased by 10 and 1.2-fold in root and leaf extracts, respectively, with Fe deficiency.

Ferric chelate reductase activity increased with Fe deficiency 2.4-fold in root tips and 7-fold in intact whole root systems. The activities of malate dehydrogenase, citrate synthase, isocitrate dehydrogenase, fumarase, aconitase, lactate dehydrogenase and pyruvate carboxylase, increased with Fe deficiency both in root and leaf extracts, with increases being largest in roots. Total pyridine nucleotide pools did not change significantly with Fe deficiency in roots and leaves. Also, O_2 consumption rates were similar in Fe-deficient and sufficient roots. Our data suggest the existence of an influx of organic acids from the roots to the leaves via xylem, probably associated to an anaplerotic carbon dioxide fixation by roots.

8. 1. 1. Abreviations: CS, citrate synthase; DTNB, 5-5´dithio-bis-2-nitrobenzoic acid; FC-R, ferric-chelate reductase; FDH, formate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MS, mass spectroscopy; PAR, photosynthetic active radiation; PDC, pyruvate decarboxylase; PEPC, phosphoenol pyruvate carboxylase; PM, plasma membrane; PPFD, Absorbed photosynthetic photon flux density; PVDF, polyvinyl fluoride; SPAD, portable Chl meter; 5-CF, 5-carboxy fluorescein.

8. 2. Introduction

Iron is an essential microelement for plant growth and development. Soils normally contain high amounts of Fe but in well aerated and alkaline soils the availability of Fe for plant uptake is very limited (Lindsay and Schwab, 1982; Mengel and Geurtzen, 1988). Plants may develop two different Strategies in response to Fe shortage (Marschner et al., 1986; Römheld and Marschner; 1986). Strategy II plants, which include *Graminaceae* species, increase the synthesis and secretion of phytosiderophores to the rhizosphere, inducing Fe uptake as a Fe (III)-phytosiderophore complex (Marschner and Römheld, 1994). The most widespread Fe-acquisition mechanism in plants, Strategy I, occurs in dicotyledonous and non-graminaceous monocotyledonous species (Marschner et

al., 1986; Römheld and Marschner, 1986; Bienfait, 1988; Brown and Jolley, 1988). In Strategy I plants, adaptive mechanisms include both morphological and physiological responses. Morphological changes associated with Fe deficiency include an increased formation of lateral roots, root hairs and transfer cells (Kramer et al., 1980; Landsberg, 1982; Schmidt, 1999). Strategy I also includes physiological changes, such as the development of an increased proton extrusion from roots resulting in acidification of the medium (Brown, 1978), a two step mechanism for Fe uptake, in which Fe (III) is first reduced by a plasma membrane-bound ferric chelate reductase enzyme (Moog and Brüggemann, 1994; Susín et al., 1996; Robinson et al., 1999) and then absorbed as Fe(II) (Chaney et al., 1972; Eide et al., 1996), a release of reducing and/or chelating substances such as phenolics and flavins (Welkie and Miller, 1960; Susín et al., 1994) and an accumulation of organic acids, mainly citrate and malate, both in leaves (Iljin, 1951; de Kock and Morrison, 1958; Palmer et al., 1963; Landsberg, 1981) and roots (Brown, 1966; de Vos et al., 1986; Venkat Raju et al., 1972; van Egmond and Atkas, 1977).

Iron is transported in the xylem as Fe(III), probably chelated by citrate (Tiffin, 1966; Brown, 1971; White et al., 1981a,b; Cataldo et al., 1988; Schmidt, 1999). Several authors have reported an increase in xylem sap organic acid concentrations with Fe deficiency (White et al., 1981a; Bialzyk and Lechowsky, 1995), although the precise role of these organic acids in Fe transport and uptake by mesophyll cells is still unclear. Once in the leaf apoplast, Fe(III) has been shown to be reduced before uptake by the leaf cell. Reduction of Fe-chelates may be mediated by a plasma membrane-bound ferric chelate reductase similar to that present in roots (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; González-Vallejo et al., 2000). It has been suggested that Fe reduction by mesophyll cells could be impaired by Fe deficiency through an increase of apoplastic pH (Mengel 1995; Kösegarten et al., 1999). In many cases leaves from Fe-deficient plants have total Fe concentrations similar to those of Fe-sufficient leaves (Abadía, 1992; Römheld, 1997; Morales et al., 1998), indicating that Fe could be immobilised in inactive forms. The lack of Fe causes marked changes in the structure and function of leaves (Terry and Abadía, 1986; Abadía, 1992; Abadía and Abadía, 1993). Most of the information available on the effects of Fe deficiency in the leaf physiology is relevant to chloroplast structure and function,

whereas little information is available on other biochemical responses of leaf cells, such as organic acid metabolism changes.

The aim of this work was to investigate changes in organic acid metabolism with Fe deficiency in tomato plants. We measured organic acid concentrations in root tips, xylem sap and leaves of tomato affected or not by Fe deficiency. Several enzymes related to organic acid metabolism such as PEPC, different TCA cycle related enzymes and LDH and PDC, two enzymes related to anaerobic metabolism, and the size and redox poise of the pyridine nucleotide pools were determined in root tips and leaves. The effects of Fe deficiency on the pH of tomato leaf apoplast were also measured.

8. 3. Materials and Methods

8. 3. 1. Plant Material

Tomato (*Lycopersicon esculentum* L. cv. Tres cantos from Spain) was grown in a growth chamber with a PPFD of 350 µmol m^{-2} s^{-1} PAR at a temperature of 25°C, 80% relative humidity and a photoperiod of 16 h light/ 8 h dark. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for two more weeks in half-strength Hoagland nutrient solution with 45 µM Fe and then transplanted to 20 L plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution (Terry, 1980) with either 0 or 45 µM Fe. The pH of the Fe-free nutrient solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$. This treatment simulates conditions usually found in the field leading to Fe deficiency (Susín et al., 1994). Young leaves and root tips (approximately 0-5 mm from the root apex) from plants grown for 15 d in the presence or absence of Fe were used in all experiments.

8. 3. 2. Chlorophyll Determination

Leaf chlorophyll concentration was estimated non-destructively with a SPAD-502 device (Minolta Corp., Osaka, Japan). For calibration, leaf disks with different degrees of Fe deficiency were first measured with the SPAD, then extracted with 100% acetone in presence of Na ascorbate and Chl measured spectrophotometrically (Abadía and Abadía, 1993).

8. 3. 3. pH Measurements

Apoplastic pH was measured *in vivo* by fluorescence according to Hoffman et al. (1992) with 5-carboxyfluorescein (5-CF). Fluorescence emitted at 540 nm is pH dependent when excited at 490 nm but nearly pH independent when excited at 450 nm. Therefore, the ratio of fluorescence intensities obtained with excitation at 490 and 450 nm is related to the pH of the compartment where the dye is located. Leaves were excised and the cut end of the petiole was exposed to incubation medium containing 5 µM 5-CF, 1 mM KCl, 0.1 mM NaCl, 0.1 mM $CaCl_2$ at pH of 5.5. The incubation was carried out for 5 h at 25°C at room ambient light (15-25 µmol photons $m^{-2} s^{-1}$). The level of auto-fluorescence was subtracted from total fluorescence. Three leaves per Chl level (each from different plant) were taken and four measurements were carried out in different areas of each leaf.

8. 3. 4. Xylem Sap Collection

Xylem sap was collected by cutting the stem with a razor blade at approximately 7 cm above the roots. The decapitated stumps were wiped with a paper towel and fitted at approximately 1 min after decapitation with a plastic tubing. The stumps were allowed to bleed into the plastic tube and samples were collected 15 min after decapitation. Each sample was filtered immediately (0.45 µm, LIDA, Kenosha, WI, USA) and frozen until analysis.

8. 3. 5. Organic Anion Analysis

Root material (100 mg FW) or leaf samples (3 leaf disks of 0.96 cm^2 each, taken with a calibrated cork borer) were frozen in liquid N_2 and ground in a mortar with 8 mM sulphuric acid. Homogenates were boiled for 30 min, filtered with a 0.2 µM PVDF filter (LIDA, Kenosha, WI, USA), taken to a final volume of 2 mL with 8 mM sulphuric acid and kept at -80°C until analysis. Xylem sap samples were filtered with a 0.2 µM filter (Millipore, Bedford, MA, USA) before organic anion analysis.

Organic anions were analysed by HPLC with a 300 x 7.8 mm Aminex ion-exchange column (HPX-87H from Bio-Rad, Hercules, CA, USA) with a HPLC Waters system, including a 600E pump, a 996 photodiode array detector and Millenium 2010 software. Samples were injected with a Rheodyne injector (20 µL loop). Mobile phase (8 mM sulphuric acid) was pumped with a 0.6 mL min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, cis-aconitate, citrate, 2-oxoglutarate, malate and fumarate were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma. Succinate co-eluted with an unidentified compound. Identification was confirmed by their peaks in the UV spectra and/or HPLC-MS (Waters) (in this latter case formic acid was used as mobile phase instead of sulphuric acid). Quantification was made with known amounts of each organic anion using peak areas.

8. 3. 6. Enzyme Assays

Extracts for measuring enzyme activities were made by grinding 100 mg FW of root tip material (or 3 leaf disks of 0.96 cm^2) in a mortar with 1 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10000g and 4°C, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 1 mL (final volume) of the media indicated below.

Malate dehydrogenase (MDH; EC 1.1.1.37) was determined with oxalacetate as substrate (Dannel et al., 1995) by measuring the decrease in A_{340}

due to the enzymatic oxidation of NADH. The reaction was carried out with 5 µL of extract in 0.1 mM NADH, 0.4 mM oxalacetate and 46.5 mM Tris-HCl, pH 9.5. Citrate synthase (CS; EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1967) by monitoring the reduction of acetyl CoA to CoA with 5-5'-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. The reaction was carried out with 50 µL of extract in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalacetate and 100 mM Tris-HCl, pH 8.1. Aconitase (EC 4.2.1.3) was measured from the formation of cis-aconitate, monitored at 240 nm (Bacon et al., 1961) with 60 µL of extract in 500 mM sucrose, 50 mM isocitrate and 100 mM Tris-HCl (pH 8.5). Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was determined with 50 µL of extract by monitoring the reduction of $NADP^+$ at 340 nm in a reaction mixture containing 3.5 mM $MgCl_2$, 0.41 mM $NADP^+$, 0.55 mM isocitrate and 88 mM imidazole, pH 8.0 (Bergmeyer et al., 1974). Fumarase (EC 4.2.1.2) was assayed with 50 µL of extract following the increase in A_{240} due to the formation of fumarate (Bergmeyer et al., 1974). The reaction buffer was 50 mM malate and 100 mM phosphate, pH 7.4.

Phosphoenol pyruvate carboxilase (PEPC; EC 4.1.1.31) was measured in a coupled enzymatic assay with MDH according to Vance et al. (1983) with 75 µL of extract in 2 mM phosphoenol-pyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH, and 100 mM bicine-HCl, pH 8.5. For the determination of lactate dehydrogenase (LDH; EC 1.1.1.27) and pyruvate decarboxylase (PDC; EC 4.1.1.1) the oxidation of NADH was monitored at 340 nm with 50 µL of extract. LDH was assayed in a reaction buffer containing 94.5 mM phosphate buffer (pH 9.5), 0.77 mM pyruvate and 0.2 mM NADH. PDC was determined in 190 mM citrate-KOH buffer (pH 6.0), 30 mM pyruvate, 0.32 mM NADH and 33 µg mL^{-1} alcohol dehydrogenase.

8. 3. 7. Nucleotide Analysis

Pyridine nucleotides were extracted from liquid N_2-frozen root material (approximately 30 mg FW) and from leaf disks (0.96 cm^2) in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA (for $NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12000g for 6 min. Samples were adjusted to

pH 8.0 with HCl or NaOH and 100 mM bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method of Matsumura and Miyachi (1980).

8. 3. 8. Root O_2 Consumption

Roots were excised under water at room temperature from plants illuminated for several h. Root O_2 consumption rates were measured from the decrease in O_2 concentration in an aqueous phase with a Clark-type O_2 electrode (Hansatech, Kings Lynn, UK). Calibration was made from the difference in signal between air and N_2-saturated water (Walker, 1987). The effects of the respiration inhibitors KCN and hydroxi-salycilic acid (SHAM) were studied at different concentrations. Sequential additions of KCN were made directly to roots in the measurement cuvette. Roots were pre-incubated with different concentrations of SHAM for 30 min prior to measurement. A new batch of root material was used for each SHAM concentration.

8. 3. 9. Ferric Chelate Reductase Activity

The FC-R activity of root tips and whole root systems was monitored by measuring the formation of the Fe(II)-BPDS complex from Fe(III)-EDTA at 535 nm according to Zouari et al. (2000).

8. 4. Results

8. 4. 1. Changes in Apoplastic pH with Fe Deficiency

The apoplastic pH of tomato leaves was measured *in vivo* by means of the fluorescent dye 5-CF (Hoffman et al., 1992). Iron deficiency caused an increase in the pH of the apoplastic fluid, from approximately 5.4 in control leaves to 6.4 in markedly Fe-deficient leaves (Fig. 8. 1).

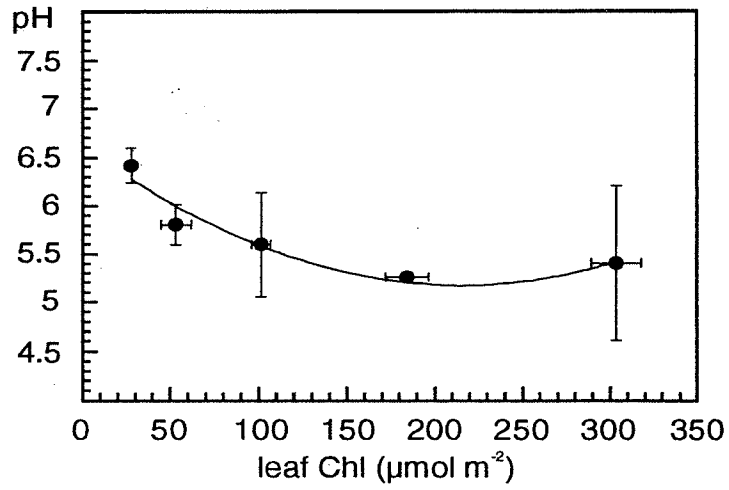

Figure 8. 1. Effects of Fe deficiency on the apoplastic pH of tomato leaves. Data are means ± SE of 3 replicates.

8. 4. 2. Changes in Organic Anion Concentrations with Iron Deficiency

Iron deficiency caused increases in the root concentrations of citrate, fumarate and cis-aconitate and decreases in oxalate, 2-oxoglutarate and malate. The increases were 19.6-fold for citrate, 3-fold for fumarate and 1.9-fold for cis-aconitate (Table 8. 1). Decreases were 70% for malate, 90% for oxalate and 96% for 2-oxoglutarate (Table 8.1). As a result of these changes the total concentration of organic acids did not change with Fe deficiency in roots of tomato plants (Table 8.1).

Iron deficiency caused a 2-fold increase in the total organic acid pool in tomato leaves. Major organic acids in both Fe-sufficient and deficient leaves were citrate and malate. Concentrations were increased by approximately 2-fold with Fe deficiency when compared to the controls (Table 8.1). Fumarate and 2-oxoglutarate concentrations in Fe-deficient leaves increased by 30 and 70%, respectively, when compared to the controls (Table 8.1). Oxalate concentration in Fe-deficient leaves decreased by 50%, whereas that of cis-aconitate did not show any change with Fe deficiency (Table 8.1).

Iron deficiency caused a 6.9-fold increase in the concentration of the total organic acid pool in the xylem sap of tomato (Table 8.1). Major acids in xylem sap

were citrate and malate in both Fe-sufficient and deficient tomato plants. Their concentrations were increased by 20.1-fold for citrate and 6.4-fold for malate with Fe deficiency (Table 8.1). Oxalate, 2-oxoglutarate and fumarate concentrations in xylem sap increased 3.3-, 1.9- and 4-fold, respectively, with Fe deficiency (Table 8.1). Cis-aconitate was not detected in control xylem sap but reached concentrations of approximately 26 µM in Fe-deficient xylem sap samples.

	Root tips		Leaves		Xylem sap	
	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe
oxalate	2.51±0.10	0.25±0.12	0.93 ± 0.19	0.51 ± 0.04	19.3 ± 1.1	64.5 ± 7.2
cis-aco	0.37±0.03	0.69±0.18	1.01 ± 0.25	1.04 ± 0.08	ND	25.7 ± 6.1
citrate	0.52±0.08	10.18±2.14	15.90 ± 0.03	35.30 ± 0.85	36 ± 10	725 ± 123
2-oxoglut	5.57±0.92	0.21±0.03	0.15 ± 0.03	0.26 ± 0.05	21.6 ± 13.0	41.5 ± 7.3
malate	4.48±0.02	1.49±0.39	13.11 ± 2.65	27.73 ± 3.43	611 ± 300	3930 ± 723
fumarate	0.03±0.01	0.09±0.00	0.16 ± 0.02	0.21 ± 0.03	5.5 ± 3.0	21.7 ± 2.3
total	13.48	12.91	31.26	65.05	693	4808

Table 8.1. Organic anion concentrations in root tips, leaves (nmol gr^{-1} FW) and xylem sap (µM) of Fe-sufficient (+Fe, 300 µmol Chl m^{-2}) and Fe deficient (-Fe, 12 µmol m^{-2}) tomato plants. Data are means ± SE of 5 replicates.

8. 4. 3. Changes in Enzymatic Activities with Iron Deficiency

We measured five enzymatic activities involved in the TCA cycle (MDH, CS, ICDH, aconitase and fumarase), PEPC activity and two enzymes (LDH and PDC) related to the anaerobic metabolism (Table 8.2).

The activities of the TCA related enzymes increased in the extracts of Fe-deficient roots when compared to the values obtained in Fe-sufficient roots (Table 8.2). Increases over the control values in Fe-deficient root extracts were 5.0-fold for MDH, 3.5-fold for fumarase, 10.5-fold for ICDH, 7.2-fold for aconitase and 2.5-fold for CS (Table 8.2).

Iron deficiency caused a 10.2-fold increase in PEPC activity in root extracts (Table 8.2). The enzymatic activities of LDH and PDC were increased by 6- and 2.9-fold with Fe deficiency (Table 8.2).

Iron deficiency caused increases in the leaf enzymatic activities of MDH, CS, ICDH and aconitase. Increases in Fe-deficient leaf extracts over the control values were 1.4-fold for MDH, 2.3-fold for ICDH, 1.4-fold for aconitase and 3.4-fold for CS (Table 8.2). Fumarase activity decreased by 50% with Fe deficiency (Table 8.2). Leaf PEPC, PDC and LDH activities were not significantly different in extracts of Fe-deficient and Fe-sufficient leaves (Table 8.2).

	Root tips			Leaves		
	+Fe	-Fe	-Fe/+Fe	+Fe	-Fe	-Fe/+Fe
MDH	16.4±1.0	82.8±8.4	(5.0)	11.5±1.6	16.6±1.3	(1.4)
CS	0.46±0.07	1.15±0.09	(2.5)	5.15±0.77	17.40±2.22	(3.4)
ICDH	0.13±0.05	1.36±0.21	(10.5)	0.07±0.01	0.16±0.03	(2.3)
aconitase	256±15	1836±32	(7.2)	186±28	254±58	(1.4)
fumarase	336±150	1183±305	(3.5)	584±92	289±52	(0.5)
PEPC	0.24±0.06	2.44±0.25	(10.2)	0.17±0.02	0.21±0.04	(1.2)
LDH	0.038±0.008	0.230±0.041	(6.0)	0.045±0.007	0.042±0.006	(0.9)
PDC	0.069±0.006	0.199±0.046	(2.9)	0.088±0.003	0.089±0.025	(1.0)

Table 8. 2. Enzymatic activities in root tips and leaf extracts (μmol min^{-1} g^{-1} FW) of Fe-sufficient (+Fe, 300 μmol Chl m^{-2}) and Fe deficient (-Fe, 12 μmol m^{-2}) tomato plants. Data are means ± SE of 5 replicates.

8. 4. 4. Changes in Nucleotide Concentrations with Iron Deficiency

The size of the total pyridine nucleotide pool in roots did not change significantly with Fe deficiency (Table 8.3). Fe deficiency increased the concentrations of $NAD(P)^+$ and decreased the concentrations of NAD(P)H in Fe-deficient root tips. Increases were 3.8-fold for $NADP^+$ and 1.6-fold for NAD^+ and decreases were 0.58-fold for NADH and 0.51-fold for NADPH (Table 8.3). As a

result of these changes, the NADH/NAD$^+$ and NADPH/NADP$^+$ ratios decreased by 62 and 86% , respectively, with Fe deficiency.

The size of the total pyridine nucleotide pool in leaves did not change significantly with Fe deficiency. Iron deficiency increased by 60% the leaf concentration of NADP$^+$ and decreased by 12% the leaf concentration of NADPH. As a result of these changes the NADPH/NADP$^+$ ratio decreased by 45% (Table 8.3). The leaf concentration of NADH did not change with Fe deficiency whereas the NAD$^+$ concentration decreased by 42%. These changes lead to an 83% increase in the NADH/NAD$^+$ ratio in Fe-deficient leaves when compared to the controls.

	Root tips		Leaves	
	+Fe	-Fe	+Fe	-Fe
NADH	1.51±0.08	0.88±0.07	1.11±0.08	1.19±0.03
NADPH	13.49±1.32	6.97±0.58	8.38±0.33	7.41±0.08
NAD$^+$	0.87±0.06	1.35±0.19	2.06±0.39	1.20±0.17
NADP$^+$	1.70±0.14	6.40±1.03	5.95±0.40	9.51±0.79
TOTAL	17.57	15.60	17.50	19.31
NADPH/NADP$^+$	7.93	1.09	1.41	0.78
NADH/NAD$^+$	1.73	0.65	0.54	0.99

Table 8. 3. Pyridine nucleotide concentrations in root tips and leaves (nmol g^{-1} FW) of Fe-sufficient (+Fe, 300 µmol Chl m^{-2}) and Fe deficient (-Fe, 12 µmol Chl m^{-2}) tomato plants. Data are means ± SE of 5 replicates.

8. 4. 5. Changes in Root tip O_2 Consumption Rates Induced by Fe Deficiency

Roots from Fe-deficient and sufficient tomato plants had similar O_2 consumption rates. Iron-deficient roots consumed 240±63 nmol O_2 min^{-1} g^{-1} FW and control roots consumed 260±49 nmol O_2 min^{-1} g^{-1} FW.

Cyanide resistant O_2 consumption was approximately 30% of the total consumption in Fe-deficient roots and 70% in Fe-sufficient roots (Fig. 8.2). In Fe-

deficient roots SHAM at concentrations of 4 and 20 mM decreased root O_2 consumption by 50 and 80%, respectively (Fig. 8.2A). In the controls 4 mM SHAM decreased O_2 consumption by 60%, whereas 20 mM SHAM inhibited completely O_2 consumption (Fig. 8.2B). Residual O_2 consumption, the fraction of oxygen uptake that is resistant to the combination of KCN and SHAM (Ribas-Carbó et al., 1997), was practically zero in both Fe-deficient and sufficient roots.

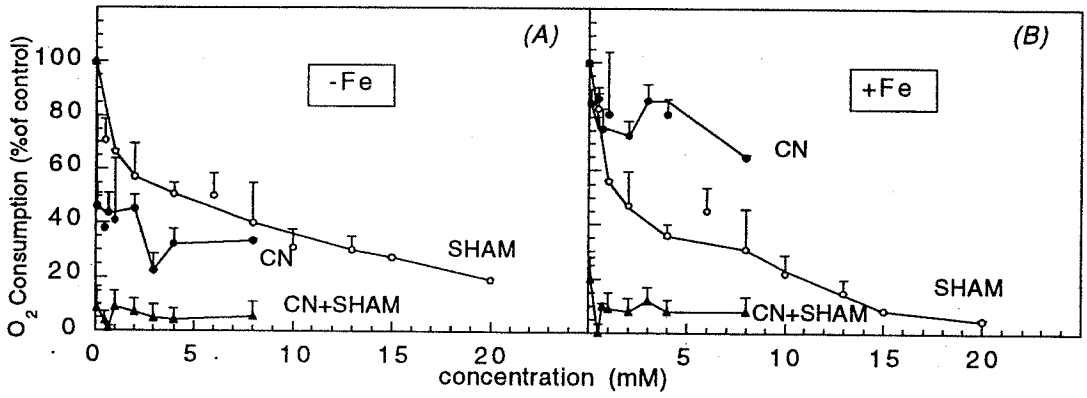

Figure 8. 2. Changes in O_2 consumption rates in Fe-deficient (A) and Fe-sufficient root tips (B) with different concentrations of SHAM (open circles), CN^- (solid circles) and CN^- + SHAM (solid triangles). Data are means ± SE of three different replications.

8. 4. 6. Changes in Ferric Chelate Reductase Activity with Iron Deficiency

Iron deficiency caused a 2.4-fold increase in FC-R activity in tomato root tips, from 22.1 ± 4.4 to 52.7 ± 6.2 nmol min^{-1} g^{-1} FW. The FC-R activity in the whole root system increased 7.0-fold, from 2.9 ± 0.7 nmol min^{-1} g^{-1} FW in control roots to 20.3 ± 5.0 nmol min^{-1} mg^{-1} FW in Fe-deficient roots.

8. 5. Discussion

In the present work we have made a comprehensive study of the biochemical changes induced by Fe deficiency in roots, xylem sap and leaves of tomato plants. Parameters measured included leaf apoplastic pH, root ferric

chelate reductase activity, root O_2 consumption rates, concentrations of organic anions, enzymatic activities related to the organic acid metabolism and redox poises of the pyridine nucleotides in both roots and leaves. The results found provide support for the view that Fe deficiency leads to increases in root C fixation.

Iron-deficient root tips had an enhanced capacity to fix C from bicarbonate. This was associated to a large (10-fold) increase in PEPC activity over the control values. Increases with Fe deficiency in C fixation (Landsberg, 1986; Bienfait et al., 1988, 1989; Miller et al., 1990; Fourier et al., 1992; Alhendawi et al., 1997) and/or PEPC activity (Hiatt, 1966; Miller et al., 1990; Rabotti et al., 1995; López-Millán, chapter 3) have been reported previously to occur in roots of other plant species. PEPC catalyses the carboxylation of PEP to oxalacetate, which could subsequently be reduced to malate via cytosolic MDH. Malate could then be transported to the mitochondria via the malate-oxalacetate shuttle and converted to citrate by CS. The increase in PEPC activity in Fe-deficient roots was also accompanied by increases in MDH (5-fold) and CS (2.5-fold) enzymatic activities, supporting the significance of C fixation by PEPC.

Fe-deficient root tips did not have signs of enhanced mitochondrial activity. This contrasts with data found in sugar beet root tips, where C fixation was enhanced 50-fold and this was associated to a 5-fold increase in mitochondrial activity (López-Millán, chapter 3). Oxygen consumption rates and pyridine nucleotide pool sizes were similar in Fe-sufficient and deficient tomato root tips. Also, the increases in organic anion pools and in enzymatic activities involved in organic acid metabolism were less marked in tomato than in sugar beet. Furthermore, the pyridine nucleotide pool was more oxidised in the yellow parts of the Fe-deficient tomato roots than in the controls, conversely to what has been reported previously in bean by Sijmons et al. (1984). In *Plantago lanceolata* the total pyridine nucleotide pool was more oxidised with Fe deficiency, although the NADPH/$NADP^+$ pool was slightly more reduced (Schmidt and Schuck, 1996). The increased activities of several NADH consuming enzymes, such as LDH, PDC and MDH, could contribute to oxidise the nucleotide pool in tomato.

Iron deficiency caused increases in the root FC-R activity of tomato under the conditions used in the present study. The increase in FC-R was 2.4-fold when

measured with root tips and 7-fold when measured with intact root systems. This induction is within the range found before in tomato by several authors (see Zouari et al., 2000) but it was lower than that found in sugar beet (Susín et al., 1996; López-Millán, chapters 3,4). This further confirms that the responses of tomato roots to Fe deficiency are less marked than those found in other more Fe-efficient plant species such as sugar beet.

Iron deficiency caused a marked increase in the apoplastic pH of tomato leaves as judged by 5-CF fluorescence. The increase in pH was almost one unit, from 5.4 to 6.4. This alkalinization was similar to the increase reported in apoplastic pH of pear leaves (López-Millán, chapter 6) but contrasts with the significant decrease (0.4 pH units) found recently with Fe deficiency in the apoplastic fluid of sugar beet (López-Millán, chapter 5). It has been hypothesised that an increase in apoplastic pH could affect the FC-R of the plasma membrane of mesophyll cells, leading to Fe immobilisation in the apoplastic space (Mengel, 1995). The one-unit pH change found in tomato leaf apoplast with Fe deficiency would lead to 30-50% decreases in FC-R enzyme activity of sugar beet leaf protoplasts (González-Vallejo et al., 2000) and to approximately 20% increases in the FC-R enzyme activity of sugar beet leaf disks (Larbi, 1999). Therefore, these data indicate that the pH increase in the apoplast with Fe deficiency is not likely to cause major problems in Fe acquisition by mesophyll cells.

The increases found in organic acids, especially citrate and malate, with Fe deficiency confirm data obtained previously for roots, leaves and xylem of tomato (Tiffin, 1966; White et al., 1981a,b; Miller et al., 1990; Bialczyk and Lechowsky, 1992). Citrate accumulation in the roots of Fe-deficient plants is not associated to large activity decreases of the Fe-containing enzyme aconitase (de Vos et al., 1986; López-Millán, chapter 3) as formely thought (Bacon et al., 1961). In agreement with the results reported by Pich in tomato (Pich and Scholz, 1993), aconitase activity increased both in roots and leaves of tomato when compared to the controls. This agrees with other observations in roots of bean (Landsberg, 1981), red pepper (de Vos et al., 1986) and sugar beet (López-Millán, chapter 3) and leaves of sugar beet (López-Millán, chapter 7).

The organic acid increases found in the whole leaf homogenates are unlikely to be caused by an increase of the rate of C fixation in leaves, because

leaf photosynthetic rates are markedly decreased by Fe deficiency (Terry, 1980). The activity of leaf PEPC, that might fix bicarbonate in leaves, increased only 20% with Fe deficiency. Moreover, the concentrations of organic anions in the xylem sap increased markedly with Fe deficiency. These data suggest the existence of a flux of organic acids from the roots via xylem (White et al., 1981a, 1981b; Bialczyk and Lechowsky, 1992; López-Millán, chapter 5). The increase in leaf organic anion concentrations with Fe deficiency may be the cause underlying other leaf biochemical changes, such as the slight increases in leaf TCA cycle enzyme activities and the marked changes in the redox poise of the leaf pyridine nucleotide pools.

The activities of two enzymes typical of anaerobic metabolism, LDH and PDC, increased in root tips of Fe-deficient plants when compared to the controls. The induction of enzymes associated to responses to hypoxia has been reported in roots of Fe-deficient barley (Suzuki et al. 1998), tomato (Herbik et al; 1996) and sugar beet (López-Millán, chapter 3). The increased activities of LDH and PDC could account for part of the NADH consumption in Fe-deficient root tips, but their contribution to the energy requirements of Fe-deficient plants is still unclear. These enzymes use pyruvate as substrate and form lactate and ethanol, respectively, suggesting that Fe-deficient roots may increase the amount of pyruvate and in turn increase the activities of these enzymes. Glycolisis may be possibly involved in the supply of pyruvate and PEP for C fixation in roots. In fact, sugar concentrations have been reported recently to increase in Fe-deficient roots, probably associated to an enhanced expression of several genes related to carbohydrate biosynthesis (Thoiron and Briat, 1999). The activity of G3PDH, an enzyme involved in the glycolitic pathway, also increases in Fe-deficient cucumber roots (Rabotti et al., 1995). Furthermore, the total amount of G3PDH in tomato roots has been shown to increase markedly with Fe deficiency (Schmidt and Buckhout, 1997).

In summary, iron deficiency in tomato was associated to organic anion accumulation in root tissues, xylem sap and leaves. In Fe-deficient root tips there was a large increase in C fixation, associated to an increase in PEPC activity. Part of this C could be used to produce energy for the basic maintenance processes through normal mitochondrial activity, whereas another part could be

exported to the shoot via xylem. The increases found in the concentrations of organic acids and in the activities of PEPC and several TCA cycle enzymes in leaves could be possibly a consequence of the large influx of organic acids from the roots to the leaves via xylem. The production and accumulation of organic acids could have two significant advantages. First, it would improve organic anion release to the rhizosphere, thus improving Fe-chelation and microbial growth. Second, it would provide substrates for respiratory and other maintenance processes in leaves with very low photosynthetic activity.

CAPÍTULO 9

DISCUSIÓN GENERAL

9. Discusión General

9. Discusión General

Este trabajo de investigación ha consistido en la caracterización de diversas respuestas bioquímicas ante la deficiencia de Fe desarrolladas por las plantas pertenecientes a la estrategia I, utilizando como especie modelo la remolacha, aunque también se han realizado experimentos sobre tomate y peral. El estudio se ha realizado a nivel de raíz, xilema, apoplasto de hoja y hoja, centrándonos principalmente en el metabolismo de los ácidos orgánicos y en el transporte de Fe.

9. 1. Raíz

Se ha realizado un estudio exhaustivo de las repuestas bioquímicas que desarrollan las puntas de raíz de remolacha y tomate ante la deficiencia de Fe. Las puntas de raíz de remolacha deficientes en Fe se dividieron en dos secciones, la primera de 0 a 5 mm del ápice, engrosada, con un aumento de pelos radiculares y de color amarillo debido a la presencia de flavinas y una segunda sección de 5 a 10 mm del ápice de color blanco (Fig. 3.1). La puntas de tomate deficientes en Fe utilizadas en los ensayos comprendieron la sección apical engrosada existente entre 0 y 5 mm aproximadamente.

9. 1. 1. Metabolismo del C

9. 1. 1. 1. Actividad PEPC

Remolacha

El marcado aumento de la actividad de la PEPC en los extractos de las zonas amarillas de las puntas deficientes en Fe (60 veces a pH 8,5 y 40 veces a pH 7,3 sobre los valores control) indica que estas puntas poseen gran capacidad para fijar C a partir de bicarbonato. Este resultado está en consonancia con datos previos que describen un aumento tanto en la fijación de CO_2 como en la actividad de la PEPC (Rhoads y Wallace, 1960; Hiatt et al., 1966; van Egmond et al., 1977; Landsberg, 1986; Bienfait et al., 1988, 1989; Fournier et al., 1992; Rabotti et al., 1995; Alhendawi et al., 1997). Además, Miller et al. (1990) demostraron la existencia de un aumento en la incorporación de CO_2 marcado radioactivamente a ácidos orgánicos en las raíces de plantas de tomate deficientes en Fe, y sugirieron que la actividad de la PEPC podría estar produciendo substratos que alimentasen el ciclo de los ácidos tricarboxílicos (Welkie y Miller, 1993).

La cantidad de PEP necesaria para mantener la actividad de la PEPC en las raíces deficientes en Fe podría provenir de los azúcares mediante la ruta glicolítica. De hecho, recientemente se ha descrito que las concentraciones de azúcares aumentan en las raíces deficientes en Fe, probablemente como consecuencia de un aumento de la expresión de varios genes relacionados con la síntesis de carbohidratos (Thoiron y Briat, 1999). Por otro lado, la actividad de la G3PDH, enzima perteneciente a la ruta glicolítica, aumenta en raíces de pepino deficientes en Fe (Rabotti et al., 1995). Más aún, la cantidad total de la G3PDH en raíces de tomate aumenta significativamente en condiciones de deficiencia de Fe (Herbik et al., 1996).

El aumento en la actividad de la PEPC en los extractos de las partes amarillas de las puntas de raíz de remolacha deficientes en Fe está asociado a un aumento en la cantidad de proteína. Asimismo, trabajos previos mostraron que una subunidad de la PEPC, separada en geles de 2-D, aumentaba 6 veces su

expresión en condiciones de deficiencia de Fe (González-Vallejo, 1999b). Por otro lado, la sensibilidad de la PEPC a malato, medida a pH 7,3 en presencia de malato 500 µM, fue menor en los extractos de raíces deficientes en Fe que en los extractos de raíces control, suponiendo un 41 y un 58% de la actividad inicial respectivamente. Estos resultados sugieren que el aumento en la actividad de la PEPC también podría estar asociado con una regulación post-transcripcional a través de fosforilación, como ocurre en hojas de plantas C4 y CAM y en las raíces de plantas deficientes en P (ver revisión de Chollet et al., 1996). También conviene mencionar que el aumento en la actividad de la PEPC en raíces de remolacha no fue debido exclusivamente a la presencia de elevadas cantidades de bicarbonato en la solución nutritiva, puesto que las puntas de raíz deficientes en Fe y en ausencia de $CaCO_3$ presentaron un aumento de 40 veces en la actividad de la PEPC (a pH 8,5) con respecto a los valores control.

Los resultados obtenidos en el experimento de aporte de Fe a plantas deficientes en Fe, mostraron que uno de los mayores cambios, entre todos los encontrados en los parámetros fisiológicos medidos tras 24 h, fue una disminución del 50% en la actividad de la PEPC con respecto a los valores iniciales. Después de 96 h del aporte de Fe, la disminución en la actividad de la PEPC fue muy marcada (90%). Ambos resultados confirman la importancia del aumento de la actividad enzimática de la PEPC en el desarrollo de las respuestas a la deficiencia de Fe en remolacha.

Tomate

En las puntas de raíz de tomate deficientes en Fe, la actividad PEPC tan sólo aumentó 10 veces en comparación con los valores obtenidos en puntas control. Esto indica que esta respuesta ante la deficiencia de Fe podría ser más atenuada en tomate que en remolacha.

9. 1. 1. 2. Ácidos Orgánicos

Remolacha

Los aumentos observados en las concentraciones de ácidos orgánicos en condiciones de deficiencia de Fe coinciden con los resultados obtenidos previamente

en raíces de plantas de distintas especies (Iljin, 1951; de Kock y Morrison, 1958; Brown, 1966; Venkat Raju et al., 1972; Landsberg, 1981; de Vos et al., 1986; Sijmons y Bienfait, 1986). Nuestros resultados y los presentes en la bibliografía indican que el citrato es el ácido orgánico que más aumenta en condiciones de deficiencia de Fe. Inicialmente se propuso que el aumento de citrato era debido a una disminución en la actividad de la aconitasa, enzima que cataliza la conversión de isocitrato en citrato y contiene Fe en su estructura (de Kock y Morrison, 1958; Bacon et al., 1961). Sin embargo, en extractos de las partes amarillas de las puntas de raíz de remolacha deficientes en Fe, la actividad aconitasa aumentó en comparación con los controles. La ausencia de disminuciones en la actividad aconitasa en deficiencia de Fe también se ha observado en judía (Landsberg, 1981), pimiento rojo (de Vos et al., 1986) y tomate (Pich y Scholz, 1993).

Existen dos hipótesis que intentan explicar el aumento en la concentración de ácidos orgánicos en las raíces de plantas deficientes en Fe. La primera, propuesta por Landsberg et al. (1986), postula que el aumento en la extrusión de protones que tiene lugar en condiciones de deficiencia de Fe en plantas pertenecientes a la estrategia I produce una alcalinización del citoplasma. Este aumento del pH citoplasmático activaría a la PEPC y ésta sería la responsable del incremento en la cantidad de ácidos orgánicos, cuya función sería mantener el balance iónico en el citoplasma (teoría del "pH-stat"; Davies, 1973). La segunda hipótesis (de Vos et al., 1986) propone que la deficiencia de Fe produciría alteraciones en la ruta glicolítica, como ocurre en algunos hongos (Habison et al., 1979). En condiciones de deficiencia de Fe, la fosfofructoquinasa perdería la sensibilidad al citrato, mientras que la piruvato quinasa la mantendría, produciéndose de este modo una acumulación de PEP, que produciría vía PEPC un aumento en la concentración de ácidos orgánicos. Los resultados obtenidos en este trabajo no permiten excluir ninguna de estas hipótesis.

Los experimentos de aporte de Fe a remolachas deficientes en Fe mostraron que las concentraciones de malato y citrato en las puntas amarillas deficientes en Fe disminuyeron un 20 y un 30%, respectivamente, tras 24 h del aporte de Fe. Tras 96 h, las concentraciones de citrato y malato disminuyeron un 70 y un 50%, respectivamente. Los valores fueron, todavía, entre 4 y 6 veces mayores que los encontrados en las puntas de remolacha control. Estos resultados nos indican que la cinética de desactivación de la síntesis de ácidos orgánicos es relativamente

lenta y que el acervo total de ácidos orgánicos se mantiene relativamente alto durante varios días tras el aporte de Fe.

Por otro lado, la extrusión de protones en las puntas amarillas disminuyó un 15 y un 70% tras 24 y 96 h del aporte de Fe, manteniéndose a 96 h en niveles varias veces más altos que los hallados para las puntas control. La cinética de descenso fue similar a la de los ácidos orgánicos.

Tomate

En las puntas de tomate deficientes en Fe la concentración de citrato aumentó 20 veces mientras que a diferencia de lo que ocurre en remolacha la concentración de malato disminuyó un 70%. Este descenso en la concentración de malato en la raíz podría ser debido a que está siendo exportado, vía xilema, hacia la hoja. El aumento de malato en el xilema de plantas de tomate deficientes en Fe apoyaría la existencia de dicho flujo de malato.

9. 1. 1. 3. Aumento de la Fijación de C

Remolacha

Los aumentos en la actividad de la PEPC, en las concentraciones de ácidos orgánicos, así como en las actividades de diversos enzimas relacionados con su metabolismo como la CS, MDH, ICDH, fumarasa y aconitasa, indicaron una mayor capacidad de fijación de C anaplerótica en las raíces deficientes en Fe, similar a la descrita en raíces de *Lupinus* deficientes en P (Johnson et al., 1994).

De acuerdo con los resultados obtenidos proponemos una ruta de fijación de C, cuya actividad aumenta en condiciones de deficiencia de Fe (Fig. 3.4). La PEPC cataliza la carboxilación de PEP para dar oxalacetato, el cual es transformado en malato vía MDH citosólica. Posteriormente, el malato entra en la mitocondria y se incorpora al ciclo TCA, para dar citrato vía CS. El aumento en la actividad de la PEPC en las partes amarillas de las puntas de raíz de remolacha deficientes en Fe ocurre de forma paralela y es aproximadamente del mismo orden de magnitud que el aumento en las actividades MDH y CS, lo que apoya la importancia de esta ruta en la fijación de C en las puntas de raíz de remolacha deficientes en Fe. Además, la

producción de malato vía PEPC y MDH es más favorable termodinámicamente que a partir de piruvato vía piruvato quinasa (Lance y Rustin, 1984).

En general la asimilación de CO_2 por las raíces es poco significativa (Farmer et al., 1991). Sin embargo, se ha descrito que dicha asimilación puede ser de gran importancia en el balance de C tanto en determinadas raíces (Vuorinen et al., 1992) como en nódulos (Vance et al., 1994). El C fijado en forma de ácidos orgánicos por las raíces podría ser exportado vía xilema hacia las hojas (Bialzyk et al., 1992), ser exudado por las mismas a la rizosfera (Jones, 1998) o bien ser utilizado para la obtención de energía y poder reductor en las mismas raíces. Cualquiera de los destinos finales de los ácidos orgánicos supondría una ventaja desde el punto de vista fisiológico para las plantas deficientes en Fe. La exudación de ácidos a la rizosfera facilita la solubilización del Fe debido a la formación de quelatos Fe-ácido orgánico y también favorecería el crecimiento microbiano. La utilización de los ácidos como fuente de poder reductor en las propias raíces permitiría que éstas mantuviesen una actividad FC-R elevada, facilitando la adquisición del Fe presente en la rizosfera. Por último, el flujo de ácidos hacia las hojas por el xilema supone un aporte de esqueletos carbonados para éstas, que en condiciones de deficiencia de Fe presentan una capacidad de fijación fotosintética de C fuertemente disminuida (Terry, 1980).

Los experimentos de aporte de Fe mostraron un marcado descenso de la actividad de la PEPC después de 96 h, asociado a descensos en las actividades de la MDH (50%) y la CS (80%). Además, se encontró un descenso generalizado en las concentraciones de citrato y malato (70-80%). De acuerdo con estos resultados podemos concluir que la cinética de desactivación de la ruta metabólica de fijación de C en las raíces deficientes en Fe es un proceso relativamente lento, que tiene lugar a lo largo de varios días tras el aporte de Fe. La actividad de la PEPC podría estar regulada por las concentraciones de Fe en el medio de cultivo, indicando, por tanto, su importancia en el desarrollo de las respuestas de las raíces a la deficiencia de Fe.

Por otro lado, los experimentos de aporte de Fe se realizaron sustituyendo la solución nutritiva de crecimiento por otra con Fe, pH 5,5 y sin CO_3Ca. La ausencia de CO_3Ca podría contribuir al descenso en la actividad de la PEPC tras el aporte de Fe debido a una disminución del substrato.

Tomate

Las actividades de la PEPC, CS y MDH aumentaron 10, 2,5 y 5 veces respectivamente con la deficiencia de Fe. Estos resultados indican que la fijación de C aumenta en las puntas de raíz de tomate deficientes en Fe en menor medida que en remolacha. Parte del malato fijado por la PEPC se utilizaría, mediante la actividad mitocondrial normal, para producir citrato, poder reductor y energía para los procesos metabólicos básicos de la raíz, mientras que otra parte estaría siendo exportado al xilema. Este hecho podría explicar el descenso en la concentración de malato observado en las raíces de tomate deficientes en Fe.

9. 1. 2. Actividad Mitocondrial

Remolacha

Las partes amarillas de las puntas de raíz de remolacha presentaron signos de una actividad mitocondrial aumentada. En las partes amarillas de las puntas de raíz la tasa de consumo de oxígeno aumentó 4 veces, el acervo de nucleótidos de piridina aumentó 3 veces y el de quinonas 2 veces, respecto a los valores encontrados en las puntas de raíz control. Este aumento de la actividad mitocondrial coincide con la presencia en estas puntas de raíz de un mayor número de células de transferencia, que se caracterizan por su gran contenido en mitocondrias (Landsberg, 1994) y con un aumento en la cantidad de pelos radiculares. Otros resultados que apoyaron la existencia de una mayor actividad mitocondrial fueron los aumentos observados en el acervo de ácidos orgánicos y en las actividades de determinados enzimas pertenecientes al ciclo TCA. Por ejemplo, las concentraciones de citrato y malato aumentaron 27 y 16 veces y las actividades de la CS, MDH, ICDH, aconitasa y fumarasa aumentaron 45, 16, 14, 5 y 9 veces, con respecto a los valores encontrados en las puntas de raíz control.

El acervo de nucleótidos se encontró mucho más oxidado en las puntas de raíz deficientes en Fe, a pesar del gran potencial que presentaron dichas raíces para producir poder reductor. Esta capacidad de producción de poder reductor está asociada al aumento en las concentraciones de ácidos orgánicos, substratos en el ciclo TCA, y en las actividades de diversos enzimas productores de NADH y

NADPH. Un aumento similar en la cantidad total de nucleótidos de piridina en condiciones de deficiencia de Fe se ha descrito previamente en raíces de judía y *Plantago lanceolata* (Sijmons et al., 1984; Schmidt y Schuck, 1996). En las raíces de *Plantago lanceolata* el acervo total de nucleótidos de piridina se encontró más oxidado en condiciones de deficiencia de Fe, al igual que ocurre en las raíces de remolacha. Por el contrario, en las raíces de judía en condiciones de deficiencia de Fe, el acervo de nucleótidos de priridina se encontró más reducido. En *Plantago lanceolata* el cociente NADH/NAD^+ disminuyó en condiciones de deficiencia de Fe, mientras que el cociente NADPH/$NADP^+$ aumentó a diferencia de lo que ocurre en las raíces de remolacha en las que ambos cocientes disminuyen. Estas discrepancias podrían explicarse debido a la presencia de concentraciones extremadamente altas de sulfatos de flavina oxidados (aproximadamente 700 µM) en las puntas de raíz amarillas de remolacha deficiente en Fe. Tanto la riboflavina como los sulfatos de riboflavina pueden oxidar fácilmente tanto NADH como NADPH (González-Vallejo et al., 1998), produciéndose de este modo un acervo de nucleótidos de piridina oxidado. Estos compuestos flavínicos no están presentes ni en las raíces de judía ni en las de *Plantago lanceolata*. Además, las partes blancas de las puntas de raíz de remolacha deficientes en Fe, que no tienen cantidades significativas de flavinas, presentaron cocientes NADPH/$NADP^+$ similares a los encontrados en puntas de raíz control. Otros factores que podrían contribuir al estado redox del acervo de nucleótidos fueron los aumentos en las actividades de diversos enzimas que consumen NADH, como por ejemplo LDH y PDC.

Por otro lado, el acervo de quinonas mitocondriales en las partes amarillas de las puntas de raíz deficientes en Fe se encontró ligeramente más reducido que en las puntas control. Estos resultados sugieren que el estado oxidado del acervo de nucleótidos de piridina no sería reflejo de un estado de oxidación general inducido por la deficiencia de Fe.

Los resultados obtenidos en este trabajo indican que las flavinas podrían ser de crucial importancia en el establecimiento de una relación metabólica entre la acumulación de ácidos orgánicos y la actividad de la FC-R en las raíces de remolacha deficientes en Fe (Fig. 3.5). El marcado aumento en la actividad PEPC en las partes amarillas de las puntas de raíz deficientes en Fe daría lugar a una acumulación de ácidos orgánicos y en consecuencia a aumentos en las actividades de diversos enzimas productores de NAD(P)H pertenecientes al ciclo TCA. Este

proceso causaría un aumento en la capacidad de producción de nucleótidos de piridina reducidos, que actuarían como donadores de electrones a las flavinas (riboflavina y sulfatos de riboflavina). Las flavinas están en forma oxidada y alcanzan concentraciones hasta 30 veces mayores que las concentraciones de nucleótidos de piridina (700 y 20 µM, respectivamente). Los electrones podrían pasar de una molécula de flavina al siguiente aceptor formándose un puente redox (Rawn, 1989), hasta alcanzar el aceptor final a nivel de la membrana plasmática.

El aceptor final de esta cadena redox podría ser una flavoproteína de membrana o directamente el oxígeno. De hecho, el aumento en la concentración de FAD en las partes amarillas de las puntas de raíz deficientes en Fe (7 veces sobre los valores control) es del mismo orden que el aumento en la actividad FC-R (11 veces sobre los valores control). Estos resultados sugieren que el aumento de la actividad FC-R podría estar asociado a aumentos en la cantidad de una enzima FC-R similar a la recientemente caracterizada en *Arabidopsis thaliana*, que contiene un sitio de unión a FAD (Robinson et al., 1999).

El acervo total de quinonas mitocondriales en las puntas amarillas de las raíces deficientes en Fe no mostró cambios significativos cuando se aportó Fe a las plantas deficientes. Al mismo tiempo, el acervo de nucleótidos de piridina aumentó tras 24 h y disminuyó hasta los valores iniciales tras 96 h. Estos resultados indican que la cantidad relativa de mitocondrias no cambia incluso tras varios días después del aporte de Fe. Además, la actividad mitocondrial no cambia mucho 24 h después del aporte de Fe, aunque a las 96 h alcanzó valores similares a los obtenidos en raíces de plantas control, según indican los descensos en ATP, en la tasa de consumo de oxígeno y en las actividades de la aconitasa, fumarasa e ICDH.

Tomate

El acervo total de nucleótidos de piridina fue similar en puntas de raíz control y deficientes en Fe. Este resultado indica que las raíces de tomate deficientes en Fe no presentan un aumento relativo en la actividad mitocondrial como ocurre en remolacha. Dicho acervo se encontró más oxidado en las raíces deficientes. El cambio a un estado más oxidado no es debido a la presencia de flavinas como ocurre en remolacha. En tomate parece que el aumento del poder

reductor creado en el ciclo TCA estaría siendo utilizado en la cadena respiratoria o por enzimas que consuman NAD(P)H a la misma velocidad en la que es producido, puesto que la actividad mitocondrial no está aumentada en este caso.

9. 1. 3. Consumo de Oxígeno

Remolacha

Las partes amarillas de las puntas de raíz de las remolachas deficientes en Fe presentaron una tasa residual de consumo de O_2, insensible a la presencia de KCN y SHAM, de aproximadamente 130 nmol O_2 g^{-1} PF min^{-1}. Este valor fue del mismo orden que la tasa de reducción de quelatos férricos que presentaron esas mismas puntas de raíz (138 nmol g^{-1} PF min^{-1}). El consumo de O_2 cesó cuando se añadió Fe(III)-EDTA a las puntas de raíz amarillas deficientes en Fe tratadas previamente con KCN más SHAM. Ya que el Fe(III)-EDTA no puede cruzar la membrana plasmática, estos datos apoyarían la posibilidad de que el enzima reductasa férrica de membrana plasmática, que se induce en condiciones de deficiencia de Fe, pueda usar alternativamente O_2 y quelatos férricos. El hecho de que el O_2 pueda actuar como aceptor de la PM FC-R se ha sugerido previamente debido a los aumentos observados, en condiciones de anaerobiosis, en la actividad del enzima presente en preparaciones purificadas de membrana plasmática de raíces de remolacha deficientes en Fe (González-Vallejo et al., 1998, 1999a).

La posibilidad de que el enzima FC-R de la membrana plasmática pueda ceder electrones al oxígeno en condiciones fisiológicas y en ausencia de quelatos férricos podría suponer una ventaja ecológica importante para las raíces deficientes en Fe. El oxígeno es fácilmente asequible en la rizosfera de suelos bien aireados, de modo que en ausencia de quelatos férricos la FC-R podría disipar el exceso de poder reductor generado, que, de acumularse, tendría consecuencias adversas para las raíces.

El consumo de O_2 disminuyó un 15%, 24 h después del aporte de Fe a las remolachas deficientes. Probablemente este hecho es debido a que la presencia de Fe(III) en el medio de cultivo hace que la FC-R lo utilice preferentemente sobre el oxígeno.

Las actividades de dos enzimas característicos del metabolismo anaeróbico, LDH y PDC, aumentaron en los extractos de las puntas de raíz deficientes en Fe. Estos enzimas usan piruvato como sustrato y forman lactato y etanol, respectivamente. Estos aumentos sugieren que las raíces deficientes en Fe pueden sufrir un cierto grado de hipoxia, posiblemente como consecuencia del aumento en el consumo de O_2 observado en estas raíces. La inducción de enzimas relacionados con hipoxia, como por ejemplo la FDH, se ha descrito previamente en raíces deficientes en Fe de cebada (Suzuki et al., 1998) y tomate (Herbik et al., 1996). Por otro lado, los aumentos en las actividades de estos enzimas podrían ser responsables de parte del consumo de NADH observado en las raíces deficientes en Fe, aunque su contribución exacta a los mecanismos de adaptación a la deficiencia de Fe de las raíces no se conoce con exactitud.

Experimentos de aporte de Fe a remolachas deficientes mostraron una disminución de aproximadamente un 70% en las actividades de la PDC y de la LDH después de 24 h del aporte. Esta rápida desactivación tras el aporte de Fe podría ser debida al descenso en el consumo de O_2 observado en estas mismas condiciones, que supondría una mayor disponibilidad del mismo para las células de la raíz. Una explicación alternativa para esta desactivación podría ser un descenso en la actividad del enzima málico, causada por la disminución en la concentración de malato que tiene lugar tras el aporte de Fe. Este fenómeno causaría, en consecuencia, un descenso en la síntesis de piruvato, sustrato tanto para la enzima LDH como para la PDC.

Tomate

El consumo de O_2 fue similar en las puntas de raíz control y deficientes en Fe. Estos datos sugieren que los procesos mitocondriales en las puntas de raíz de tomate no estarían tan aumentados como en remolacha. La presencia de flavinas (ausentes en tomate) podría ser necesaria para crear un puente redox entre los nucleótidos de piridina y el oxígeno.

Las actividades de la LDH y PDC aumentaron 6 y 3 veces en comparación con los controles. En este caso no se puede atribuir estos incrementos al elevado consumo de oxígeno, como ocurre en remolacha. Una posible explicación para el

incremento en las actividades de estos enzimas sería un incremento en la concentración de piruvato, sustrato de ambas enzimas.

9. 1. 4. Actividad FC-R, ATPasa y Cambios Morfológicos

Los cambios observados en las actividades metabólicas discutidas hasta el momento coinciden, espacial y temporalmente, con aumentos en las actividades reductora de Fe(III) y actividad ATPasa, así como con cambios a nivel morfológico en las puntas de raíz de remolacha deficiente en Fe. Los experimentos de aporte de Fe a remolachas deficientes en Fe causaron una desactivación progresiva de las actividades reductora de Fe(III) y de extrusión de protones, dos de las respuestas más características de las plantas pertenecientes a la estrategia I. La cinética de esta desactivación fue bastante lenta, siendo moderada a 24 h y muy importante a 96 h.

En condiciones de deficiencia de Fe, las puntas de remolacha se encuentran engrosadas y presentan un color amarillo debido a un aumento en la concentración de flavinas (Fig. 3.1). Experimentos de aporte de Fe a las plantas deficientes mostraron que, tras 24 h, las puntas amarillas presentaban tan sólo un ligero crecimiento (aproximadamente 1 mm) mientras que las concentraciones de Fe ya se habían incrementado en cinco veces. En estas condiciones, la concentración de Fe en la hoja también había aumentado, presentando una concentración de aproximadamente la mitad de la encontrada en hojas control. Asimismo, las actividades reductoras de Fe y de extrusión de protones, tras 24 h del aporte de Fe, disminuyeron un 20 y un 30%, respectivamente. Estos resultados nos permiten afirmar que la desactivación de ambas respuestas tras 24 h del aporte de Fe fue significativa, aunque no muy marcada.

Tras 96 h del aporte de Fe a las plantas deficientes, las puntas de raíz mostraron un crecimiento apical de aproximadamente 5 mm. La parte nueva de las puntas de raíz fue blanca, no presentó pelos radiculares y tanto las concentraciones de ácidos orgánicos como las actividades enzimáticas medidas no fueron muy distintas de los valores encontrados para las puntas de raíz de plantas controles. Las secciones amarillas todavía mostraron concentraciones altas de flavinas, sin embargo las actividades FC-R y de extrusión de protones fueron un

70-80% menores de los valores encontrados antes del aporte de Fe, valores aún ligeramente superiores a los presentados por las puntas de raíz control. La desactivación de estos procesos tras el aporte de Fe nos permite concluir que su activación depende de la presencia de Fe en el medio. Sin embargo, los resultados obtenidos no nos han permitido determinar si alguno de estos procesos es el desencadenante de las respuestas ante la deficiencia de Fe. Por ejemplo, tanto la actividad reductora como la extrusión de protones y las concentraciones de ácidos orgánicos presentaron, tras 96 h del aporte de Fe, valores varias veces superiores a los observados en las plantas suficientes en Fe y sus cinéticas de desactivación fueron parecidas, por lo que no se puede establecer si alguna de estas tres respuestas es determinante en la puesta en marcha de los mecanismos de eficiencia.

9. 2. Xilema de Remolacha

En este trabajo hemos caracterizado los cambios que se producen en la composición de la savia del xilema de remolacha en condiciones de deficiencia de Fe. El mayor cambio inducido por la deficiencia de Fe en este compartimento es un aumento en la concentración de ácidos orgánicos, fundamentalmente citrato y malato. Dicho aumento fue acompañado por un ligero incremento en la concentración de cationes y una disminución en la concentración de aniones inorgánicos, probablemente para mantener la electroneutralidad en la savia.

9. 2. 1. Transporte de Fe en el Xilema

La concentración de Fe en el xilema disminuyó linealmente con la deficiencia de Fe desde 5,5 hasta 2,5 μM. El Fe en el xilema de remolacha parece encontrarse principalmente formando complejos con citrato, a pesar de que el malato fue el ácido orgánico mayoritario, tanto en remolacha control como deficiente en Fe. Estos resultados coinciden con los obtenidos en exudados de tomate (White et al., 1981a, b), en los que el Fe se encuentra acomplejado únicamente por citrato. Asimismo, confirman la importancia del citrato en el transporte de Fe a larga distancia, propuesta inicialmente por Tiffin (1966). En condiciones control, el Fe se

encontró mayoritariamente en forma de $FeCitOH^-$, que supondría un 66% de la cantidad total de Fe. La deficiencia de Fe produjo en el xilema de remolacha un cambio en la distribución de las formas de Fe, siendo $FeCit_2^{3-}$ la especie mayoritaria (un 96% del Fe total) en estas condiciones. La formación de una especie como el $FeCit_2^{3-}$ puede alterar la movilidad del Fe en el xilema. En primer lugar la formación del quelato citrato-Fe(III) evitaría la precipitación del Fe en forma de hidróxidos o fosfatos férricos. En segundo lugar, el Fe(III) libre tendría poca movilidad en el xilema debido a que las paredes están cargadas negativamente y se comportan como una columna de intercambio iónico, en la que el Fe(III) libre, con una carga positiva tan alta, vería retrasado su desplazamiento hacia la hoja. De este modo, la formación del complejo $FeCit_2^{3-}$ evitaría este inconveniente favoreciendo el desplazamiento del Fe en el xilema hacia las hojas. El ligero descenso en el pH de la savia inducido por la deficiencia de Fe no parece tener mayor repercusión en la distribución del Fe entre las distintas especies Fe-citrato.

9. 2. 2. Transporte de C en el Xilema

Remolacha

Las elevadas concentraciones de ácidos orgánicos en el xilema sugieren la existencia de un flujo de C desde las raíces hasta las hojas, por los vasos del xilema. Como se ha discutido en el apartado 9. 1. 1, las puntas de raíz de remolacha deficientes en Fe presentan un aumento en su capacidad de fijar carbono, a través de un incremento en las actividades de la PEPC y de varios enzimas relacionados con el metabolismo de los ácidos orgánicos como la CS y MDH. En el xilema de remolacha deficiente en Fe, las concentraciones de malato, succinato y citrato fueron 30 mM, 7 mM y 5 mM. Estas concentraciones serían equivalentes a un flujo de C de 4,7 $\mu mol\ C\ m^{-2}\ s^{-1}$, teniendo en cuenta que la tasa de transpiración de agua medida en esas mismas hojas es de 2 $mmol\ m^{-2}\ h^{-1}$. Este flujo de C podría llegar a ser mucho mayor que la tasa de fijación fotosintética de CO_2 en las hojas deficientes en Fe, que alcanza valores de aproximadamente 3 $\mu mol\ C\ m^{-2}\ s^{-1}$ en condiciones de saturación de luz y valores menores de 1 $\mu mol\ C\ m^{-2}\ s^{-1}$ en las condiciones de intensidad luminosa que normalmente hay en la cámara de cultivo

(Terry, 1983). En las plantas control, el flujo de C sería menor de 1 μmol C m^{-2} s^{-1}, lo cual supondría menos de un 1% de la tasa fotosintética máxima en las hojas control, confirmando de este modo, que la fijación de C por las raíces es muy baja en condiciones nutricionales normales (Farmer y Adams, 1991). La existencia de una fijación de C no fotosintética en las raíces y del flujo de este C vía xilema explicaría el poco efecto de la deficiencia de Fe en el crecimiento de las hojas en condiciones de cultivo controladas (Terry, 1979), a pesar de la gran disminución observada en las tasas de fotosíntesis en esas mismas hojas (Terry, 1980).

Por otro lado, el aumento en la concentración de aminoácidos en el xilema de remolachas deficientes en Fe nos indica que parte del C es fijado en la raíz en forma de aminoácidos que se transportan vía xilema hacia la parte aérea de la planta.

Tomate

En el xilema de tomate, tanto control como deficiente en Fe, el ácido mayoritario fue malato y en segundo lugar citrato. El citrato fue el ácido que más aumentó en condiciones de deficiencia de Fe (20 veces) y en segundo lugar el malato (6 veces). Estos datos confirman la existencia de un flujo de C desde la raíz hasta la parte aérea de la planta. Sin embargo, la distribución del C fijado entre los distintos ácidos orgánicos fue distinta, sugiriendo que el tipo de ácido podría depender de la especie o de la existencia de diferencias en la ruta de fijación de C en las raíces.

9. 3. Apoplasto de Hoja

En este trabajo hemos determinado los cambios producidos por la deficiencia de Fe en la composición del fluido apoplástico de hojas de remolacha y peral. Las concentraciones de iones orgánicos e inorgánicos en el apoplasto de hojas de peral y remolacha control se encuentran en el mismo rango que las descritas hasta el momento en hojas de *Quercus*, girasol, guisante, espinaca y judía (Bowling et al., 1987; Speer et al., 1991; Dannel et al., 1995; Hermann et al., 1995; Gabriel et al., 1999). Las concentraciones iónicas en el apoplasto de remolacha fueron mayores que en el de peral, probablemente debido a que las remolachas fueron crecidas en un cultivo hidropónico y los perales en campo. En cultivo

hidropónico existen menos limitaciones que en el suelo para la difusión de los nutrientes hasta las raíces. Las relaciones aniones : cationes en apoplasto de hojas de remolacha controles y deficientes en Fe fueron aproximadamente 1 y en el apoplasto de hojas de peral controles y deficientes en Fe fueron 0,64 y 0,63. Un exceso en la concentración de cationes en relación con la de aniones es normal en el fluido apoplástico y es debido a las cargas negativas de la pared celular y de la membrana plasmática (Dietz et al., 1997).

9. 3. 1. Transporte de Fe

El transporte de Fe al interior de la célula del mesófilo desde el apoplasto requiere un paso previo de reducción que probablemente está mediado por una reductasa férrica situada en la membrana plasmática de las células del mesófilo. La deficiencia de Fe *per se* causa un descenso importante en la actividad de la FC-R tanto en protoplastos como en discos de mesófilo de hojas de remolacha (Larbi et al., 1999; González-Vallejo et al., 2000). Sin embargo, los cambios en la composición del apoplasto inducidos por la deficiencia de Fe podrían influir también en la actividad de esta reductasa férrica de la membrana plasmática.

9. 3. 1. 1. Concentración de Fe en el Apoplasto

Remolacha

La concentración de Fe en el fluido apoplástico fue de aproximadamente 5,6-5,9 µM en hojas extremadamente cloróticas (30 µmol Chl m^{-2}) y en hojas control, mientras que en hojas moderadamente deficientes (100 µmol Chl m^{-2}) fue de aproximadamente 2,2 µM. La concentración de Fe en el xilema disminuyó linealmente con la deficiencia de Fe, siendo similar a la concentración apoplástica en remolachas control y moderadamente deficientes en Fe. La deficiencia de Fe produjo una disminución de 2,5 veces en la concentración de Fe en el apoplasto de hojas moderadamente cloróticas, indicando que la causa más probable de la clorosis de la hoja sería la ausencia de Fe y no su inmovilización. Sin embargo, estos resultados también sugieren la existencia de una dificultad progresiva en la adquisición de Fe por las células del mesófilo de remolacha, de modo que en

condiciones de deficiencia extrema la FC-R sería incapaz de utilizar el Fe presente en el apoplasto.

Peral

La deficiencia de Fe produjo sólo un 50% de disminución en la concentración de Fe en el apoplasto de peral pudiendo indicar que la actividad de la FC-R del mesófilo no aumenta en condiciones de deficiencia de Fe. La ausencia de un aumento en la actividad de la FC-R ha sido descrita previamente en protoplastos de remolacha deficientes en Fe (González-Vallejo et al., 2000) y en discos de hoja (Brüggemann et al., 1993; de la Guardia y Alcántara, 1996; Larbi et al., 1999). El descenso en la concentración apoplástica de Fe fue similar al observado en la cantidad total de Fe en hojas de peral cuando la concentración se expresa en función del volumen de hoja (Morales et al., 1998). Estos resultados indican que en las células del mesóflo de peral existen otros factores limitantes para la adquisición de Fe además de una concentración insuficiente de Fe en el apoplasto.

9. 3. 1. 2. pH del Apoplasto

Remolacha

En condiciones de deficiencia de Fe, el pH apoplástico de las hojas de remolacha disminuyó aproximadamente 0,4 unidades. Esta acidificación causaría un aumento en la actividad FC-R, la cual muestra un máximo de actividad a pH 5,5 en protoplastos aislados a partir de remolachas deficientes en Fe (González-Vallejo et al., 2000). Estos datos no apoyarían la hipótesis previa de Mengel (1995) que sugiere que la deficiencia de Fe en plantas crecidas en campo causaría un aumento del pH apoplástico de hoja, siendo éste, a su vez, el responsable de la disminución en la capacidad de adquisición de Fe por las células del mesófilo, bien por precipitación del Fe o bien por su efecto en la actividad de la FC-R. La disminución del pH apoplástico observada podría ser debida a un aumento en la extrusión de protones causado por la activación de una ATPasa de membrana plasmática de hoja en condiciones de deficiencia de Fe.

Peral

La deficiencia de Fe causó un aumento en el pH de aproximadamente una unidad en el apoplasto de las hojas de peral. Este aumento conduciría a una disminución de un 30-50% en la actividad de la enzima FC-R según la dependencia de la actividad de este enzima con el pH descrita en protoplastos de remolacha (González-Vallejo et al., 2000) y a un 20% de aumento en la actividad de este enzima si se utiliza como material de ensayo discos de hoja de remolacha (Larbi et al., 1999). Este aumento del pH apoplástico en hojas de peral contrasta con la disminución observada en el pH apoplástico (0,4 unidades de pH) de las hojas de remolacha deficientes en Fe. Probablemente esta diferencia sea debida a que la remolacha se cultiva en un medio hidropónico en ausencia total de Fe, mientras que el peral se cultiva en suelos, en los cuales existe Fe pero no es disponible. En cualquier caso los cambios en el pH apoplástico tanto de remolacha como de peral no parecen ser determinantes en la capacidad de adquisición de Fe en las plantas deficientes, al contrario de lo que ha sido sugerido previamente (Mengel, 1995; Kosegarten et al., 1999).

9. 3. 1. 3. Especie Química del Fe

Remolacha

El Fe en el fluido apoplástico de remolacha, como ocurre en el xilema, se encontró principalmente formando complejos con citrato, según los resultados obtenidos mediante especiación química. La deficiencia de Fe produjo un cambio en la distribución del Fe entre los complejos de citrato, siendo $FeCitOH^-$ la especie mayoritaria en condiciones control (50% del Fe total) y $FeCit_2^{3-}$ la mayoritaria en condiciones de deficiencia de Fe (76% del Fe total). La adquisición de Fe por las células del mesófilo en deficiencia de Fe podría estar desfavorecida, entre otras causas, debido a que la especie mayoritaria fue $FeCit_2^{3-}$. Esta especie tiene una carga negativa muy alta, por lo que podría experimentar fuertes repulsiones electrostáticas con la membrana plasmática que se encuentra cargada negativamente.

Peral

En el apoplasto de peral, tanto de hojas controles como deficientes en Fe, el Fe se encontró acomplejado principalmente por citrato, siendo $FeCitOH^-$ y $FeCit_2^{3-}$ las especies mayoritarias. La deficiencia de Fe causó un cambio en la distribución del Fe entre ambas especies de Fe-citrato, de modo que la fracción de Fe total presente en la forma monomérica $FeCitOH^-$ aumentó de un 60 a un 85% con la deficiencia de Fe. Esta especie química puede favorecer la adquisición de Fe por las células del mesófilo, puesto que al estar menos cargada que $FeCit_2^{3-}$, experimentaría menor repulsión electrostática con la membrana plasmática que se encuentra cargada negativamente. Sin embargo, la influencia de la especie química en la que se encuentra el Fe no parece ser muy importante en peral puesto que es la misma en condiciones control y de deficiencia de Fe.

9. 3. 1. 4. Relación Citrato:Fe

Remolacha

Los altos cocientes citrato:Fe que se encontraron en el fluido apoplástico de remolacha podrían disminuir significativamente la capacidad de adquisición de Fe por las células del mesófilo, puesto que la actividad de la FC-R de la membrana plasmática de las hojas desciende marcadamente cuando el cociente citrato:Fe aumenta. Las actividades de la FC-R de la membrana plasmática disminuyen 5 veces cuando el cociente citrato:Fe aumenta de 100 a 500 (González-Vallejo et al., 1999). Por otro lado, los cocientes malato:Fe elevados favorecerían la adquisición de Fe por las células del mesófilo, puesto que la actividad de la enzima FC-R aumenta cuando el cociente malato:Fe aumenta (González-Vallejo et al., 1999). La especiación química, sin embargo, indicó que no existe una formación significativa de complejos malato:Fe que pudiesen servir de substrato para la FC-R.

Por otro lado, se ha propuesto que en las partes aéreas de las plantas se pueden formar polímeros de Fe-citrato (Spiro, 1961a; Bienfait y Scheffers, 1991; Moog y Brüggemann, 1994; Schmidt, 1999) que harían que el Fe no fuese asequible para las células del mesófilo. Sin embargo, no existen evidencias experimentales que apoyen esta teoría. Además, el exceso de citrato compite efectivamente con la

formación de polímeros Fe-citrato, inhibiendo su polimerización (Spiro et al., 1961b). Debido al alto cociente citrato:Fe en el apoplasto de hoja deficiente en Fe, la formación de polímeros Fe-citrato es muy poco probable.

Peral

Las relaciones malato:citrato:Fe en el fluido apoplástico de hoja de peral aumentaron con la deficiencia de Fe desde 300:150:1 hasta 1750:900:1. Este marcado aumento en las relaciones ácido orgánico:Fe con la deficiencia de Fe en hojas de peral es similar al observado en el apoplasto de remolacha y podría ser un factor decisivo que causase una disminución en la disponibilidad de Fe por las células del mesófilo, tal y como se ha discutido en el apartado anterior.

9. 3. 2. Transporte de C en el Apoplasto

Remolacha

Uno de los mayores cambios inducidos por la deficiencia de Fe en el apoplasto de remolacha es un aumento en la concentración de ácidos orgánicos, fundamentalmente citrato y malato. Este aumento fue acompañado por un ligero incremento en las concentraciones de cationes y de aniones inorgánicos probablemente para mantener la electroneutralidad.

Tanto el aumento en la fijación de C observado en las raíces como el aumento en la concentración de ácidos orgánicos en el xilema de remolachas deficientes en Fe, indican que la concentración de ácidos orgánicos en el apoplasto de hojas de remolacha deficientes en Fe es debida a un incremento en el transporte de C desde la raíz hasta la hoja. El exceso de C fijado en las raíces por la PEPC sería exportado en forma de ácidos orgánicos al xilema y transportado por el mismo hasta el apoplasto. Este flujo de C explicaría los aumentos en las concentraciones de ácidos orgánicos encontrados tanto en el apoplasto, como en las hojas.

En el fluido apoplástico la concentración de malato fue mucho menor que en xilema, mientras que la concentración de citrato fue similar en ambos compartimentos. Este hecho sugiere que el malato podría ser retirado del

apoplasto por un transportador específico situado en la membrana plasmática de la células del mesófilo. Se han descrito hasta el momento varios sistemas de transporte de malato en los diferentes orgánulos celulares como mitocondrias y cloroplastos, entre ellos se encuentra el transportador malato-oxalacetato y varios sistemas de antiporte con fosfato, tricarboxilatos y 2-oxoglutarato (Martinoia y Rentsch, 1994). Sin embargo, existen pocos datos en la bibliografía sobre el transporte a nivel de membrana plasmática. El sistema de transporte descrito hasta el momento consiste en un canal de aniones (Martinoia y Rentsch, 1994), que podría ser parte de un sensor de CO_2 (Hedrich y Marten, 1993).

Los aumentos observados en la concentración total de aminoácidos en el apoplasto de hojas deficientes en Fe sugieren que parte del C fijado por las raíces vía PEPC estaría siendo incorporado en forma de aminoácidos por transaminación del oxalacetato. Los aminoácidos mayoritarios que resultan de esta transaminación son glutamato y aspartato (Cramer et al., 1993). Apoyando esta teoría, aspartato y glutamato se encontraron entre los aminoácidos mayoritarios en condiciones de deficiencia de Fe.

Peral

En el apoplasto de peral, al igual que en el de remolacha, la concentración de ácidos orgánicos aumentó con la deficiencia de Fe, siendo citrato y malato los ácidos orgánicos mayoritarios. Estos resultados sugieren la existencia de un flujo de C desde las raíces hasta las hojas, tal y como se ha discutido previamente en el caso de la remolacha.

9. 4. Hoja

9. 4. 1. Concentración de Fe

Remolacha

La concentración de Fe en las hojas disminuyó marcadamente en condiciones de deficiencia de Fe. Este hecho confirmó que la clorosis férrica en remolacha es debida principalmente a una limitación en la cantidad de Fe y no a una inmovilización del mismo en acervos inactivos, como ocurre en algunos casos

en plantas crecidas en el campo (Morales et al., 1998). El aporte de Fe a remolachas deficientes causó un aumento rápido en la concentración de Fe en las hojas alcanzando tras 24 h del aporte valores de aproximadamente la mitad de los encontrados en las hojas de remolachas controles. Tras 96 h se obtuvieron valores similares a los controles. Un aumento similar en la concentración de hierro tras el aporte de dicho elemento se ha descrito previamente tanto en remolacha (Young y Terry, 1982) como en maíz (Thoiron et al., 1997).

9. 4. 2. Metabolismo del C

Remolacha

Nuestros resultados mostraron que la cantidad total de ácidos orgánicos, principalmente de citrato y malato, fue mucho mayor en las hojas deficientes en Fe que en las controles, confirmando resultados previamente descritos en otras especies (Iljin, 1951; de Kock y Morrison, 1958; Palmer et al., 1963; Landsberg, 1981). El aumento de ácidos orgánicos en hoja inducido por la deficiencia en Fe coincidió con aumentos en las concentraciones de Ca^{2+} y Mg^{2+} en las hojas, aumento descrito previamente en remolacha (Nagarajah y Ulrich, 1965). El aumento en Ca^{2+} y Mg^{2+} podría mantener el balance de cargas, contrarrestando el aumento en la concentración de ácidos orgánicos.

Las hojas de remolacha deficientes en Fe presentaron grandes aumentos en las concentraciones de ácidos orgánicos y moderados en las actividades de la PEPC y otros enzimas relacionados con el metabolismo de los ácidos orgánicos como por ejemplo, la MDH y CS. Diversos resultados nos indican que el aumento en el acervo de ácidos orgánicos en las hojas deficientes en Fe no es debido a un aumento en la fijación de C en la hoja, sino a un flujo de C desde las raíces hasta las hojas vía xilema:

a) El aumento en la concentración de ácidos orgánicos tuvo lugar en hojas deficientes en Fe con concentraciones de clorofila bajas y por lo tanto baja tasa fotosintética (Terry, 1980; Abadía, 1992) y bajo contenido en azúcares.

b) Las actividades enzimáticas de la MDH y la CS, enzimas relacionados con la síntesis de citrato y malato, no aumentaron en hojas moderadamente

deficientes en Fe, mientras que en las mismas hojas las concentraciones de citrato y malato aumentaron de 7 a 12 veces.

c) La ruta alternativa de fijación de C descrita en raíces y en la que participan la PEPC, MDH y CS, no parece estar activada en hojas, puesto que los aumentos en las actividades de dichos enzimas fueron pequeños. Además, esta ruta depende de la existencia de una fuente de PEP en la hoja deficiente en Fe, hecho que es poco probable debido al bajo contenido en azúcares de las hojas deficientes en Fe.

d) Los aumentos máximos en las actividades enzimáticas y en la concentración de ácidos orgánicos tienen lugar en hojas con distinto contenido en clorofila. Las concentraciones máximas de ácidos orgánicos tuvieron lugar en hojas moderadamente deficientes en Fe (200 μmol Chl m^{-2}), mientras que las actividades enzimáticas máximas se encontraron en hojas deficientes en Fe con concentraciones de clorofila entre 50 y 100 μmol Chl m^{-2}. Estos resultados indican que la relación entre ambos aumentos no es directa.

e) Tras un aporte de Fe a remolachas deficientes, el acervo de ácidos orgánicos disminuyó gradualmente, siendo la mitad del inicial tras 24 h y similar al medido en hojas control tras 96 h del aporte de Fe. Sin embargo, experimentos de aporte de Fe a las plantas deficientes causaron una disminución rápida en las actividades de estos enzimas, presentando en 24 h valores similares o incluso menores a los observados en los extractos de hojas controles. El descenso relativamente lento en las concentraciones de ácidos orgánicos podría ser debido a la existencia de un aporte externo vía xilema o bien, a dificultades en la utilización de la gran cantidad de C almacenado en la hoja en forma de ácidos orgánicos.

f) El aumento en la fijación de C observado en las raíces deficientes en Fe, así como en el aumento en la concentración de ácidos orgánicos tanto en xilema como en apoplasto en condiciones de deficiencia de Fe indican que al menos parte del aumento en la concentración de ácidos en hojas deficientes en Fe sería debido a un flujo de C desde la raíz.

Como se ha comentado anteriormente este aporte de C a las hojas deficientes en Fe supondría importantes ventajas desde un punto de vista ecológico. En primer lugar, el C fijado en la raíz proporcionaría energía para la respiración y el mantenimiento de los procesos metabólicos básicos en las hojas

deficientes en Fe. Por otro lado, los ácidos orgánicos facilitarían el transporte de Fe en forma de quelatos en el xilema y apoplasto, dificultando así la precipitación del Fe en forma de hidróxidos y fosfatos en estos compartimentos (Tiffin, 1966 a, b; Brown et al., 1971; White et al., 1981; Cataldo et al., 1988).

Por otro lado, el flujo de ácidos orgánicos podría estar regulado por auxina, como sugirió Landsberg (1982). La auxina es capaz de estimular tanto la síntesis de malato como la actividad de la CS en puntas de raíz de judía (Sarkissian, 1972; Haschke et al., 1975).

Peral

En hojas de peral deficientes en Fe también se observó un aumento en la concentración de ácidos orgánicos. En árboles frutales la tasa de fotosíntesis está muy afectada por la deficiencia de Fe (Hurley et al., 1986). Así mismo, la actividad de la PEPC no aumentó en condiciones de deficiencia de Fe. Estos resultados nos permiten sugerir que en peral el aumento de ácidos sería también debido a un flujo de C desde las raíces como ocurre en remolacha.

El aumento en la concentración de ácidos en las hojas de peral deficientes en Fe puede ser el causante de otros cambios metabólicos observados en las hojas de peral deficientes en Fe. Entre estos cambios se encontrarían el ligero aumento observado en las actividades de diversos enzimas pertenecientes al ciclo de Krebs, el aumento en el acervo de nucleótidos y en los cocientes NADPH/$NADP^+$ y NADH/NAD^+. En general estos cambios parecen favorecer las condiciones para un aumento en la actividad FC-R de la membrana plasmática de la hoja, aunque ésta no parece estar aumentada en condiciones de deficiencia de Fe, según los resultados discutidos previamente en el apartado 9. 3. 1. Estas observaciones nos indicarían que en las células del mesófilo de hojas de peral deficientes en Fe existen otros pasos limitantes para la adquisición de Fe.

CAPÍTULO 10

CONCLUSIONES

10. Conclusiones

1- Las puntas amarillas de raíz de remolacha deficiente en Fe presentan un aumento en la capacidad de fijar C, asociado a un incremento en la actividad de la fosfoenol piruvato carboxilasa.

2- La actividad mitocondrial en las puntas amarillas de remolacha deficiente en Fe aumenta en relación con la actividad observada en las raíces de remolacha control.

3.- Las flavinas en las puntas de remolacha deficiente en Fe pueden actuar como puente redox entre los nucleótidos de piridina y la reductasa férrica de la membrana plasmática.

4- La reductasa férrica de la membrana plasmática de las raíces de remolacha deficientes en Fe parece ser capaz de utilizar oxígeno en ausencia de quelatos férricos en el medio.

5- En remolachas cultivadas en condiciones de deficiencia de Fe existe un flujo de C, en forma de ácidos orgánicos, desde la raíz hasta la hoja, vía xilema.

6- El aumento de la concentración de ácidos orgánicos en las hojas deficientes en Fe parece ser debido a un aporte externo de C vía xilema.

7- El Fe en el xilema de remolacha y en el apoplasto tanto de remolacha como de peral se encuentra en forma de complejos Fe(III)-citrato.

8- La acumulación de ácidos orgánicos en apoplasto de peral y remolacha no parece suponer una ventaja para los procesos de adquisición de Fe por las células del mesófilo, sino que su importancia podría radicar en el aporte de C a la hoja deficiente en Fe.

9- Las cinéticas de desactivación de las respuestas inducidas por la deficiencia de Fe en raíces de remolacha deficientes en Fe son relativamente lentas, siendo la actividad de la fosfoenol piruvato carboxilasa y las actividades de la lactato deshidrogenasa y piruvato decarboxilasa las primeras en disminuir.

CAPÍTULO 11

BIBLIOGRAFÍA

11. Bibliografía

Abadía A, Sanz M, de las Rivas J, Abadía J (1989) Pear yellowness: an atypical iron chlorosis? Acta Hortic **256**: 177-181.

Abadía J (1992) Leaf responses to Fe deficiency: a review. J. Plant Nutr. **15**: 1699-1713.

Abadía J, Abadía A (1993) Iron and plant pigments. *En* LL Barton, BC Hemming, eds, Iron Chelation in Plants and Soil Microorganisms. Academic Press, New York, pp 327-343.

Abadía J, Nishio JN, Monge E, Montañés L, Heras L (1985) Mineral composition of peach tree leaves affected by iron chlorosis. J. Plant Nutr. **8**: 697-708.

Abbott AJ (1967) Physiological effects on micronutrient deficiencies in isolated roots of *Lycopersicon esculentum*. New Phytol. **66**: 419-437.

Alcántara E, de la Guardia MD, Romera FJ (1991) Plasmmalemma redox activity and H^+ extrusion in roots of Fe-deficient cucumber plants. Plant Physiol. **96**: 1034-1037.

Alcántara E, Romera FJ, Canete M, de la Guardia MD (1994) Effects of heavy metals on both induction and function of root Fe(III) reductase in Fe-deficient cucumber (*Cucumis sativus* L.) plants. J. Exp. Bot. **45**: 1893-1898.

Alhendawi RA, Römheld V, Kirby EA, Marschner H (1997) Influence of increasing bicarbonate concentrations on plant growth, organic acid accumulation in roots and iron uptake by barley, sorghum and maize. J. Plant Nutr. **20**: 1731-1753.

Allison JD, Brown DS, Novo-Gradak KJ (1991) A geochemical assessment model for environmental systems v. 3.0. Washintong D. C., Environmental Research Laboratory.United States Environmental Protection Agency

Alloush GA, Le Bot J, Sanders FE, Kirby EA (1990) Mineral nutrition of chickpea plants supplied with NO_3^- or NH_4-N. Ionic balance in relation to iron stress. J. Plant Nutr. **13**: 1575-1590.

Anderson W (1976) Transport through roots. In U Luttge, M Pitman, eds, Encyclopedia of plant physiology, transport in plants. Springer-Verlag, Berlin, pp 129-156.

Ao TY, Fan F, Korcak RF, Faust M (1985) Iron reduction by apple roots. J. Plant Nutr. **8**: 629-644.

Arulanathan AR, Rao IM, Terry N (1990) Limiting factors in photosynthesis. IV. Regeneration of ribulose 1,5-bisphosphate limits photosynthesis at low photochemical capacity. Plant Physiol. **93**: 1466-1475.

Arvik J, Zimdahl R (1974) The influence of temperature, pH and metabolic inhibitors on uptake of lead by plant roots. J. Environ. Qual. **3**: 374-376.

Award F, Römheld V, Marschner H (1988) Mobilization of ferric iron chlorosis in calcareous soil by plant-borne chelators (phytosiderophores). J. Plant Nutr. **11**: 701-713.

Bacon JSD, Palmer MJ, de Kock PC (1961) The measurements of aconitase activity in the leaves of various normal and variegated plants. Biochem. J. **78**: 198-204.

Bagnaresi P, Pupillo P (1995) Characterization of NADH-dependant Fe^{3+}-chelate reductases of maize roots. J. Exp. Bot. **46**: 1497-1503.

Barret-Lennard EG, Marschner H, Römheld V (1983) Mechanism of short term Fe(III) reduction by roots. Evidence against the role of secreted reductants. Plant Physiol **73**: 893-898.

Beauford W, Barber J, Barringer A (1977) Uptake and distribution of mercury within higher plants. Physiol. Plant. **39**: 261-265.

Belkhodja R, Morales F, Sanz M, Quilez R, López-Millán AF, Abadía A, Abadía J (1998) Iron deficiency causes changes in chlorophyll fluorescence due to the reduction in the dark of the photosystem II acceptor side. Phosynth. Res. **56**: 265-276.

Bell PF, Chaney RL, Angle JS (1988) Staining localization of ferric reduction on roots. J. Plant Nutr. **11**: 1237-1252.

Bergmeyer HU, Gawehn K, Grassl M (1974) Enzymes as biochemical reagents. *En* HU Bergmeyer, eds, Methods of enzymatic analysis. Academic Press, New York, London, Verlag Chemie, Weinheim, pp 425-556.

Bialzyk J, Lechowski L (1992) Absorption of HCO_3^- by roots and its effect on carbon metabolism of tomato. J. Plant Nutr. **15**: 293-312.

Bian Y, Sun D (1994) The effect of exogenous calmodulin on wall regeneration and the first division of *Angelica dahurica* protoplasts. Acta Phytophysiologica Sinica **20**: 293-297.

Bienfait HF (1987) Biochemical basis of iron efficiency reactions in plants. *En* G Winkelmann, Dvd Helm, JB Neilands, eds, Iron Transport in Microbes, Plants and Animals. VCH Verlagsgesellschaft, Weinheim, F. R. G., pp 339-349.

Bienfait HF (1988a) Mechanisms in Fe-efficiency reactions of higher plants. J. Plant Nutr. **11**: 605-629.

Bienfait HF (1988b) Proteins under the control of the gene for Fe efficiency in tomato. Plant Physiol. **88**: 785-787.

Bienfait HF (1989) Prevention of stress in iron metabolism of plants. Acta Bot. Neerlan. **38**: 105-129.

Bienfait HF (1996) Is there a metabolic link between H^+ excretion and ferric reduction by roots of Fe-deficient plants - A viewpoint. J. Plant Nutr. **19**: 1211-1222.

Bienfait HF, Bino RJ, van der Bliek AM, Duivenvoorden JF, Fontaine JM (1983) Characterization of ferric reducing activity in roots of Fe-deficient *Phaseolus vulgaris*. Physiol. Plant. **59**: 196-202.

Bienfait HF, Scheffers MR (1992) Some properties of ferric citrate relevant to the iron nutrition of plants. Plant Soil **143**: 141-144.

Bindra A (1980) Iron chlorosis in horticultural and field crops. Annu. Rev. Plant Sci. **2**: 221-312.

Blamey F, Asher C, Kerven G, Edwards D (1993) Factors affecting aluminum sorption by calcium pectate. Plant Soil **149**: 87-94.

Blatt MR (1985) Extracellular potassium activity in attached leaves and its relation to stomatal function. J. Exp. Bot. **36**: 240-251.

Boddey R, De-Paula-Resende C, Pereira J, Cantarutti R, Alves B, Ferreira E, Richter M, Cadisch G, Urquiaga S, De-Paula-Resende D (1995) Nitrogen cycle in pure grass and grass/legume pastures: evaluation of pasture sustainability. *En* Nuclear techniques in soil plant studies for sustainable agriculture and environmental preservation, Viena, Austria .

Bolle-Jones EW, Notton BA (1953) The relative proportions of the chloroplast pigments as influenced by different levels of iron and potassium supply. Plant Soil **1**: 87-100.

Bottger M, Hilgendorf F (1988) Hormone action on transmembrane electron and H^+ transport. Plant Physiol. **86**: 1038-1043.

Bowles D, Gilmartin P, Knox J, Lunt G (1994) Molecular botany: signals and the environment. *En* Biochemical Society Symposium 60, University of Leeds .

Bowling DJF (1987) Measurement of the apoplastic activity of K^+ and Cl^- in the leaf epidermis of *Commelina communis* in relation to stomatal activity. J. Exp. Bot **38**: 1351-1355.

Brancadoro L, Rabotti G, Scienza A, Zocchi G (1995) Mechanisms of Fe efficiency in roots of Vitis ssp in response to iron deficiency stress. Plant Soil **171**: 229-234.

Briat JF (1996) Roles of ferritin in plants. J. Plant Nutr. **19**: 1331-1342.

Briat JF, Lobréaux S (1997) Iron transport and storage in plants. Trends in Plant Science **2**: 187-193.

Briggs G, Robertson R (1957) Apparent free space. Ann. Rev. Plant Physiol. **8**: 11-30.

Bright G, Fisher G, Rogowska J, Taylor D (1989) Fluorescence ratio imaging microscopy. Meth. Cell Biol. **30**: 157-192.

Briskin DP (1990) The plasma membrane H^+-ATPase of higher plant cells: biochemistry and transport function. Biochem. Biophys. Acta **1019**: 95-109.

Brown JC (1963) Iron chlorosis in soybeans as related to the genotype of rootstock. Soil Sci. **96**: 387-394.

Brown JC (1966) Fe and Ca uptake as related to root-sap and stem-exudate citrate in soybeans. Physiol. Plant. **19**: 968-976.

Brown JC (1978) Mechanism of iron uptake by plants. Plant Cell Environ. **1**: 249-257.

Brown JC, Ambler JE (1974) Iron-stress response in tomato.I. Sites of Fe reduction, absorption and transport. Physiol. Plant. **31**: 221-224.

Brown JC, Chaney RL (1971) Effect of iron on the transport of citrate into the xylem of soybean and tomatoes. Plant Physiol. **47**: 836-840.

Brown JC, Chaney RL, Ambler JE (1971) A new tomato mutant inefficient in the transport of iron. Physiol. Plant. **25**: 48-53.

Brown JC, Jolley VD (1988) Strategy I and strategy II mechanisms affecting iron availability to plants may be established too narrow or limited. J. Plant Nutr. **11**: 1077-1098.

Brown JC, Tiffin LO (1965) Iron stressed as related to the iron and citrate ocurring in stem exudate. Plant. Physiol. **40**: 395-400.

Brüggemann W, Maas-Kantel K, Moog PR (1993) Iron uptake by leaf mesophyll cells: The role of the plasma membrane-bound ferric-chelate reductase. Planta **190**: 151-155.

Brüggemann W, Moog PR, Nakagawa H, Janiesh P, Kuiper PJC (1991) Plasma membrane-bound NADH: Fe^{3+}-EDTA reductase and iron deficiency in tomato (*Lycopersicum esculentum*). Is there a turbo reductase? Physiol. Plant. **79**: 339-346.

Buchenauer H (1990) Physiological reactions in the inhibition of plant pathogenic fungi. *En* W Browers, W Ebing, D Martin, R Wegler, eds, Chemistry of plant protection 6. Controlled release, biochemical effects of pesticides, inhibition of plant pathogenic fungi. Heidelberg, Germany, Springer-Verlag, pp 217-292.

Buckhout TJ, Bell PF, Luster DG, Chaney RL (1989) Iron-stress induced redox activity in tomato (*Lycopersicum esculentum* Mill.) is localized on the plasma membrane. Plant. Physiol. **90**: 151-156.

Bughio N, Takahashi M, Yoshimura E, Nishizawa NK, Mori S (1997a) Light-dependent iron transporter into isolated barley chloroplasts. Plant Cell Physiol. **38**: 101-105.

Bughio N, Takahashi M, Yoshimura E, Nishizawa NK, Mori S (1997b) Characteristics of light regulated iron transport system in barley chloroplsts. Soil Sci. Plant Nutr. **43**: 959-963.

Cakmak I, Van de Wetering DAM, Marschner H, Bienfait HF (1987) Involvement of superoxide radical in extracellular ferric reduction by iron-deficient bean roots. Plant Physiol. **85**: 310-314.

Canny MJ (1995) Apoplasmic water and solute movement: New rules for an old space. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**: 215-236.

Castillo F, Greppin H (1988) Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L leaves after ozone exposure. Environ. Exp. Bot. **28**: 231-238.

Castillo F, Pennel C, Greppin H (1984) Peroxidase release induced by ozone in *Sedum album* leaves. Plant Physiol. **74**: 846-851.

Cataldo DA, McFadden KM, Garland TR, Wildung RE (1988) Organic constituents and complexation of nickel (II), iron (III), cadmium (II), and plutonium (IV) in soybean xylem exudates. Plant Physiol. **50**: 208-213.

Chaney RL (1984) Diagnostic practises to identify iron deficiency in higher plants. J. Plant Nutr. **7**: 47-67.

Chaney RL (1989) Translocation of iron roots to shoot. *En* 5th International Symposium on Iron Nutrition and Interactions in Plants, Jerusalem, Israel.

Chaney RL, Bell PF (1987) The complexity of iron nutrition: lessons for plant-soil interaction research. J. Plant Nutr. **10**: 963-994.

Chaney RL, Brown JC, Tiffin LO (1972) Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol. **50**: 208-213.

Chaney RL, Chen Y, Green CE, Holden MJ, Bell PF, Luster DG, Angle JS (1992) Root hairs on chlorotic tomatoes are an effect of chlorosis rather than part of the adaptative Fe-stress response. J. Plant Nutr. **15**: 1857-1875.

Chaney RL, Coulombe BA, Loyd CA (1984) Bicarbonate induces Fe-chlorosis with normal plant phosphorous levels. Agron. Abstr. **1984**: 201.

Chen Y, Barak P (1982) Iron nutrition of plants in calcareous soils. Adv. Agron. **35**: 217-240.

Chleplik G (1997) Effects of endophytic fungi on the phenotypic plasticity of *Lolium perenne* (Poaceae). Am. J. Bot. **58**: 34-40.

Chollet R, Vidal J, O'Leary MH (1996) Phosphoenolpyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. Annu. Rev. Plant. Physiol. Plant Mol. Biol **47**: 273-298.

Clark C, Holland P, Smith G (1986) Chemical composition of bleeding xylem sap from kiwifruit vines. Ann. Bot. **58**: 353-362.

Clark RB (1983) Plant genotype differences in the uptake, translocation, accumulation and use of mineral elements required for plant growth. Plant Soil **72**: 175-196.

Clarkson D (1967) Interactions between aluminum and phosphorous on root surfaces and cell wall material. Plant Soil **27**: 347-356.

Clarkson D (1991) Root structure and sites of ion uptake. *En* W Y, A Eshel, U Kafkafi, eds, Plant roots-the hidden half. Marcel Dekker Inc, New York, Basel, Hongkong, pp 417-453.

Clarkson DT (1984) Calcium transport between tissues and its distribution in the plant. Plant Cell Environ. **7**: 449-456.

Cline GR, Powell PE, Szaniszlo PJ, Reid PP (1982) Comparison of the abilities of hydroxamic, synthetic and other natural organic acids to chelate iron and other ions in nutrient solution. Am. J. Soil Sci. **46**: 1158-1164.

Cohen CK, Norwell WA, Kochian LV (1997) Induction of the root cell plasma membrane ferric reductase. An exlusive role for Fe and Cu. Plant Physiol. **114**: 1061-1069.

Collins J, Linstead P (1969) Effect of calcium on the potassium flux into the exudate of excised maize roots. Planta **84**: 353-357.

Cramer M, Lewis O, Lips S (1993) Inorganic carbon fixation and metabolism in maize roots as affected by nitrate and ammonium nutrition. Physiol. Plant. **89**: 632-639.

Daeter W, Hartung W (1995) Stress-dependent redistribution of abscisic acid (ABA) in *Hordeum vulgare* L leaves: The role of epidermal ABA metabolism, tonoplastic transport and the cuticule. Plant Cell Environ. **18**: 1367-1376.

Dannel F, Pfeffer H, Marschner H (1995) Isolation of apoplasmic fluid from sunflower leaves and its use for studies on influence of nitrogen supply on apoplasmic pH. J. Plant Physiol. **50**: 208-213.

Davies DD (1973) Control of and by pH. Symp. Soc. Exp. Biol. **27**: 513-529.

de Kock PC, Morrison RI (1958) The metabolism of chlorotic leaves. Biochem. J. **70**: 272-277.

de la Guardia MD, Alcántara E (1996) Ferric chelate reduction by sunflower (*Helianthus annuus* L.) leaves: influence of light, oxygen, iron deficiency and leaf age. J. Exp. Bot. **47**: 669-675.

De Silva D, Hetherington A, Mansfield T (1996) Where does all the Ca go? Evidence of an important regulatory role for trichomes in two calcicoles. Plant Cell Environment **19**: 880-886.

de Vos CR, Lubberding HJ, Bienfait HF (1986) Rhizosphere acidification as a response to iron deficiency in bean plants. Plant Physiol. **81**: 842-846.

Dietz KJ (1997) Functions and responses of the leaf apoplast under stress. *En* HD Behnke, U Lütthje, K Esser, JW Kadereit, M Runge, eds, Progress in botany, structural botany, physiology, genetics, taxonomy, geobotany. Springer Verlag, Berlin, pp 221-254.

Dinkelaker B, Hengeler C, Marschner H (1995) Distribution and function of proteoid roots and other root clusters. Bot. Acta **108**: 183-200.

Drew MC (1997) Oxygen deficiency and root metabolism: Injury and acclimatation under hypoxia and anoxia. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 223-250.

Egilla JN, Byrne DH, Reed DW (1994) Iron stress response of three peach rootstock cultivars: ferric-iron reduction capacity. J. Plant Nutr. **17**: 2079-2103.

Eide D, Brodenius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc. Natl. Acad. Sci. U.S.A. **93**: 5624-5628.

Farmer AM, Adams MS (1991) Carbon uptake by roots. *En* Y Waisel, A Eshel, U Kafkafi, eds, Plant Roots: The Hidden Half. Dekker, M., New York, pp 627-637.

Felle H (1993) Ion-selective microelectrodes: Their use and importance in modern plant cell biology. Bot. Acta **106**: 5-12.

Findlay J, Buthelezi S, Lavoie R, Pena R, Miller J (1995) Bioactive isocoumarins and related metabolites from conifer endophytes. J. Nat. Prod. **58**: 1759-1766.

Fournier JM, Alcántara E, de la Guardia MD (1992) Organic acid accumulation in roots of two sunflower lines with a different response to iron deficiency. J. Plant Nutr. **15**: 1747-1755.

Fox TC, Guerinot ML (1998) Molecular biology of cation transport in plants. Annu. Rev. Plant Physiol. Plant Mol. **49**: 669-676.

Fox TC, Schaff JE, Grusak MA, Norvell WA, Chen Y, Chaney RL, Kochian LV (1996) Direct measurement of Fe^{59}-labeled Fe^{2+} influx in roots of pea using a chelator buffer system to control free Fe^{2+} in solution. Plant Physiol **111**: 93-100.

Franklin R (1969) Effect of absorbed cations on phosphorous uptake by excised roots. Plant Physiol **44**: 697-700.

Franklin R (1971) Cation effects on chloride, sulphate, and phosphate uptake by excised roots. Soil Sci. **112**: 343-347.

Gabriel R, Schäefer L, Gerlach C, Rausch T, Kesselmeier J (1999) Factors controlling the emissions of volatile organic acids from leaves of *Quercus ilex* (Holm Oak). Atm. Environ. **33**: 1347-1355.

Gardner WK, Parbery DG, Barber DA (1982) The adquisition of phosphorous by *Lupinus albus L.* II. The effect of varying phosphorous supply and soil type on some characteristics of the soil/root interface. Plant Soil **68**: 33-41.

Gauch H (1973) Inorganic plant nutrition. Dowden, Huchinson, Ross, eds, Stroudsburg.

Geiger D, Sovonik S, Shock T, Fellows R (1974) Role of the free space in the translocation in sugar beet. Plant Physiol **54**: 892-898.

Gerke J (1997) Aluminum and iron (III) species in the soil solution including organic complexes with citrate and humic substances. Z. für Pflanzenernähr. Bodenk. **160**: 427-432.

Giaquinta R (1977) Phloem loading of sucrose. Plant Physiol. **59**: 750-755.

Giritch A, Herbik A, Balzer HJ, Ganal M, Stephan UW, Bäumlein H (1997) A root specific iron-regulated gene of tomato encodes a lysil-tRNA-synthase-like protein. Eur. J. Biochem. **244**: 310-317.

Gogorcena Y, Abadía J, Abadía A (1998) Induction of Fe(III) chelate reductase activity in vivo in rootstocks of *Prunnus* sp.J. Plant Nutr. **23**: 9-21 .

González-Vallejo EB, Susín S, Abadía A, Abadía J (1998) Changes in sugar beet plasma membrane Fe(III)-chelate reductase activities mediated by Fe-deficiency, assay buffer composition, anaerobiosis and the presence of flavins. Protoplasma **205**: 163-168.

González-Vallejo EB, González-Reyes JA, Abadía A, López-Millán AF, Yunta F, Lucena JJ, Abadía J (1999) Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet. Aust. J. Plant Physiol. **26**: 601-611.

Gónzalez-Vallejo EB, Morales F, Cistué L, Abadía A, Abadía J (2000) Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. Plant Physiol. **122**: 1-8.

Goodwin T, Mercer E (1983) Nitrogen fixation, amino acid biosynthesis and proteins. *En* eds, Introduction to plant biochemistry. Pergamon Press, Oxford New York, Toronto, Sydney, Paris, Frankfurt.

Gries D, Runge M (1995) Responses of calcicole and calcifuge Poaceae species to iron-limiting conditions. Bot. Acta **108**: 482-489.

Grignon C, Sentenac H (1991) pH and ionic conditions in the apoplast. Ann. Rev. Plant Physiol. Plant Mol. Biol. **42**: 103-128.

Grime JP, Hutchinson TC (1967) The incidence of lime-chlorosis in the natural vegetation of England. J. Ecol. **55**: 557-566.

Grusak MA (1995) 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. Planta **197**: 111-117.

Grusak MA, Pezeshgi S (1996) Shoot-to-root signal transmission regulates root Fe (III) reductase activity in the *dgl* mutant of pea. Plant Physiol. **110**: 329-334.

Grusak MA, Welch RM, Kochian LV (1989) A transport mutant of pea for the study of iron absorption by plant root cells. Plant Physiol. **89**: S-152.

Grusak MA, Welch RM, Kochian LV (1990a) Does iron deficiency in *Pisum sativum* enhance the activity of the root plasmalemma iron transport protein? Plant Physiol **94**: 1353-1357.

Grusak MA, Welch RM, Kochian LV (1990b) Physiological characterization of a single-gene mutant of *Pisum sativum* exhibiting excess iron accumulation. I Root iron reduction and iron uptake. Plant Physiol. **93**: 976-981.

Guerinot ML, Yi Y (1994) Iron: nutritious, noxious, and not readily available. Plant Physiol. **104**: 815-820.

Habison A, Kubicek CP, Röhr M (1979) Phosphofructokinase as a regulatory enzyme in citric acid producing *Aspergillus niger*. FEMS Microbiol. Lett. **5**: 39-42.

Harley CP, Linder RC (1945) Observed responses of apple and pear trees to some irrigation waters of north central Washington. Proc. Am. Coc. Hort. Sci. **46**: 35-44.

Hartung W, Weiler EW, Radin JW (1992) Auxin and cytokinins in the apoplasmic solution of dehydrated cotton leaves. J Plant Physiol. **140**: 324-327.

Haschke HP, Lüttge U (1975) stoichiometric correlation of malate accumulation with auxin-dependent K^+-H^+ exchange and growth in *Avena* coleoptile segments. Plant Physiol. **56**: 696-698.

Hedrich R, Marten I (1993) Malate-induced feedback regulation of plasma membrane anion channels could provide a CO_2 sensor to guard cells. EMBO J. **12**: 897-901.

Hedrich R, Marten I, Lohse G, Dietrich P, Winter H, Lohaus G, Heldt HW (1994) Malate-sensitive anion channels enable guard cells to sense changes in the ambient CO_2 concentration. Plant J. **6**: 741-748.

Herbik A, Giritch A, Horstmann C, Becker R, Balzer HJ, Bäumlein H, Stephan UW (1996) Iron and copper nutrition-dependent changes in protein expression in a tomato wild type and the nicotianamine-free mutant *chloronerva*. Plant Physiol. **111**: 533-540.

Hether NH, Olsen RA, Jackson LL (1984) Chemical identification of iron reductants exuded by plant roots. J. Plant Nutr. **7**: 667-676.

Hiatt AJ (1966) Relationship of cell sap pH to organic acid change during ion uptake. Plant Physiol. **42**: 294-298.

Hider RC, von Wiren N, Leigh R, Bansal S, Briat JF (1997) Physicochemical characterization of Fe-nicotianamine complexes and their physiological implications. *En* 9th International symposium on iron nutrition and interactions in plants, Sttutgart, Germany.

Hill J (1980) The remobilization of nutrients from leaves. J. Plant Nutr. **2**: 407-444.

Hoffman B, Plänker R, Mengel J (1992) Measurements of pH in the apoplast of sunflower leaves by means of fluorescence. Physiol Plant. **84**: 146-153.

Hoffmann B, Kossegarten H (1995) FITC-dextran for measuring apoplast pH and apoplastic pH gradients between various cell types in sunflower leaves. Physiol. Plant. **95**: 327-335.

Holden MJ, Luster DG, Chaney RL, Buckout TJ, Robinson C (1991) Fe^{3+}-chelate reductase activity of plasma membranes isolated from tomato (*Lycopersicon esculentum* Mill.) roots. Comparison of enzymes from Fe-deficient and Fe-sufficient roots. Plant Physiol. **97**: 537-544.

Hooymans J (1964) The role of calcium in the absorption of anions and cations by excised barley roots. Acta Bot. Neerl. **13**: 507-540.

Horst W (1995) The role of the apoplast in aluminum toxicity and resistance of higher plants: a review. Z. Planzenernäh. Bodenk. **158**: 174-178.

Hurley AK, Walser RH, Davis TD, Barney DL (1986) Net photosynthesis, chlorophyll, and foliar iron in apple trees after injection with ferrous sulfate. Hort. Science **21**: 1029-1031.

Husted S, Schjoerring K (1995) Apoplastic pH and ammonium concentration in leaves of *Brassica napus* L. Plant Physiol **109**: 1453-1460.

Hutchinson TC (1967) Coralloid root systems in plants showing lime-induced chlorosis. Nature **214**: 943-945.

Iljin WS (1951) Metabolism of plants affected with lime induced chlorosis. II Organic acids and carbohydrates. Plant Soil **3**: 339-351.

Jachetta J, Appleby A, Boersma L (1986) Use of the pressure vessel to measure concentrations of solutes in apoplastic and membrane-filtered symplastic sap in sunflower leaves. Plant Physiol. **82**: 995-999.

Jacobs M, Ray P (1976) Rapid auxin induced decrease in free space pH and its relationship to auxin-induced growth in maize and pea. Plant Physiol **58**: 210-213.

Jacobson L (1955) Carbon dioxide fixation and ion absorption in barley roots. Plant Physiol. **20**: 233-245.

Jarvis S (1978) Copper uptake and accumulation by perennial ryegrass grown in soil and solution culture. J. Sci. Food Agric. **29**: 12-18.

Jarvis S, Jones L, Hopper M (1976) Cadmiun uptake from solution by plants and its transport from roots to shoots. Plant Soil **44**: 179-191.

Jiaaxu L, Liu J, Sun D (1993) Immunoelectron microscopic localization of calmodulin in corn root cells. Cell Research **3**: 11-19.

Johnson JF, Allan DL, Vance CP (1994) Phosphorous stress-induced proteoid roots show altered metabolism in *Lupinus albus*. Plant Physiol **104**: 657-665.

Johnson JF, Allan DL, Vance CP, Weiblen G (1996a) Root carbon dioxide fixation by phosphorous-deficient *Lupinus albus*. Contribution to organic acid exudation by proteoid roots. Plant Physiol. **112**: 19-30.

Johnson JF, Vance CP, Allan DL (1996b) Phosphorous deficiency in *Lupinus albus*. Altered lateral root development and enhanced expression of phosphoenol pyruvate carboxylase. Plant Physiol. **112**: 31-41.

Johnson R, Jackson W (1964) Calcium uptake and transport by wheat seedlings as affected by aluminum. Soil Sci. Soc. Am. Proc. **28**: 381-386.

Jolley VD, Brown JC, Pushnik JC, Miller GW (1987) Influences of ultraviolet (uv)-blue light radiation on the growth of cotton. I. Effect on iron nutrition and iron stress response. J. Plant Nutr. **10**: 333-351.

Jones DL (1998) Organic acids in the rhizosphere-a critical review. Plant Soil **205**: 25-44.

Jones DL, Darrah PR (1995) Influx and efflux of organics acids accross the soil-root interface of *Zea mays* L. and its implications in rhizosphere C flow. Plant Soil **173**: 103-109.

Jones DL, Darrah PR, Kochian LV (1996) Critical-evaluation of organic-acid mediated iron dissolution in the rhizosphere and its potential role in root iron uptake. Plant Soil **180**: 57-66.

Kannan S, Seshadri K (1988) Physiology responses associated with Fe-deficiency stress in different plant species. J. Plant Nutr. **11**: 1185-1192.

Kashirad A, Marschner H (1974) Effect of pH and phosphate on iron nutrition of sunflower and corn plants. Agrochimica **6**: 497-508.

Kesselmeier J, Bode K, Gerlach C, Jork EM (1998) Exchange of atmospheric formic and acetic acid with trees and crop plants under controlled chamber and purified air conditions. Atm. Environ. **32**: 1765-1775.

Kneen BE, LaRue TA, Welch RM, Weeden NF (1990) Pleiotropic effects of *bzr*. A mutation in *Pisum sativum* L. cv. Sparkle conditioning decreased nodulation and increased iron uptake and leaf necrosis. Plant Physiol. **93**: 717-722.

Kochian L (1995) Cellular mechanism of aluminum toxicity and resistance in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. **46**: 1124-1150.

Kolesch H, Höfner W, Schaller K (1987a) Effect of bicarbonate and phosphate on iron chlorosis of grape-vines with special regard to the susceptibility of the rootstocks. I. Field Experiments. J. Plant Nutr. **10**: 207-230.

Kolesch H, Höfner W, Schaller K (1987b) Effect of bicarbonate and phosphate on iron chlorosis of grape-vines with special regard to the susceptibility of the rootstocks. II. Pot experiments. J. Plant Nutr. **10**: 231-249.

Kolesch H, Oktay M, Hofner W (1984) Effect of iron chlorosis-inducing factors on the pH of the cytoplasm of sunflower (*Helianthus annuus*). Plant and Soil **82**: 215-221.

Kösegarten H, Englisch G (1994) Effects of various nitrogen forms on the pH in leaf apoplast and on iron chlorosis of *Glycine max* L. Z. Pflanzenernähr. Bodenk. **157**: 401-405.

Kosegarten H, Hoffmann B, Mengel, K (1999) Apoplastic pH and Fe3+ reduction in intact sunflower leaves. Plant Physiol. **121**: 1-11.

Köseoglu AT (1995) Effect of iron chlorosis on mineral composition of peach leaves. J. Plant Nutr. **18**: 765-776.

Kraffczyk I, Trolldenier G, Beringer H (1984) Soluble root exudates of maize: Influence of potassium supply and rizosphere microorganisms. Soil Biol. Biochem. **16**: 315-322.

Kramer D, Römheld V, Landsberg EC, Marschner H (1980) Induction of transfer-cell formation by iron deficiency in the root epidermis of *Helianthus annuus* L. Planta **147**: 335-339.

Lance C, Rustin P (1984) The central role of malate in plant metabolism. Physiol. Veg. **22**: 625-641.

Landsberg EC (1981) Organic acid synthesis and release of hydrogen ions in response to Fe deficiency stress of mono and dicotyledoneous plant species. J. Plant Nutr. **3**: 579-591.

Landsberg EC (1982) Transfer cell formation in the root epidermis: A prerequisite for Fe-efficiency?. J. Plant Nutr. **5**: 415-432.

Landsberg EC (1984) Regulation of iron-stress-response by whole-plant activity. J. Plant Nutr. **7**: 609-621.

Landsberg EC (1986) Function of rhizodermal transfer cells in the Fe stress response mechanisms of *Capsicum annuum* L. Plant Physiol. **82**: 511-517.

Landsberg EC (1994) Transfer cell formation in sugar beet roots induced by latent Fe deficiency. Plant Soil **165**: 197-205.

Landsberg EC (1996) Hormonal regulation of iron-stress response in sunflower roots: a morphological and cytological investigation. Protoplasma **194**: 69-80.

Langebartels C, Kerner K, Leonardi S, Schraudner M, Trost M, Heller W, Sandermann Jr H (1991) Biochemical plant responses to ozone. I. Differential induction of polyamine and ethylene biosynthesis in tobacco. Plant Physiol. **95**: 882-889.

Larbi A (1999) Efecto de la clorosis férrica sobre la actividad reductasa en el mesófilo de hojas de remolacha. Tesis Master. Instituto Agrónomico del Mediterraneo (IAMZ).

Lass B, Thiel G, Ulrich-Eberius CI (1986) Electron transport across the plasmalemma of *Lemma gibba* G1. Planta **169**: 251-259.

Lauchli A (1976) Apoplasmic transport in tissues. *En* U Luttge, A Pitman, eds, Encyclopedia of plant physiology, transport in plants. Sringer-Verlag, Berlin, pp 3-34.

Li ZC, McClure JW, Hagerman AE (1989) Soluble and bound apoplasmic activity for peroxidase, β-D-glucosidase, malate dehydrogenase and nonspecific arylesterase in barley (*Hordeum vulgare* L) and oat (*Avena sativa* L) primary leaves. Plant Physiol. **190**: 185-190.

Lindsay WL, Schwab AP (1982) The chemistry of iron soils and its availability to plants. J. Plant Nutr. **5**: 821-840.

Liu J, Adler K, Stephan UW (1998) Adventicious rooting in hypocotyls of sunflower (*Helianthus annuus*) seedlings. IV. The role of changes in endogenous free and conjugated indole-3-acetic acid. Phys. Plant. **86**: 285-292.

Lobréaux S, Hardy T, Briat JF (1993) Abscisic acid is involved in the iron-induced synthesis of maize ferritin. The EMBO Journal **12**: 651-657.

Lobréaux S, Massenet O, Briat JF (1992) Iron induces ferritin synthesis in maize plantlets. Plant Mol. Biol. **19**: 563-575.

Loeppert RH (1986) Reactions of iron and carbonates in calcareous soils. J. Plant Nutr. **9**: 195-215.

Long J, Widders I (1990) Quantification of apoplastic potassium content by elution analysis of leaf lamina tissue from pea (*Pisum sativum* L cv. Argenteum). Plant Physiol **94**: 1040-1047.

Longnecker N, Welch RM (1990) Accumulation of apoplastic iron in plant roots. A factor in the resistance of soybeans to iron-deficiency induced chlorosis. Plant Physiol. **92**: 17-22.

Luwe MWF, Takahama V, Heber V (1993) Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L) leaves. Plant Physiol. **101**: 969-976.

Maas FM, van de Wetering DAM, van Beusichem ML, Bienfait HF (1988) Characterization of phloem iron and its possible role in the regulation of Fe-efficiency reactions. Plant Physiol. **87**: 167-171.

Marentes E, Grusak MA (1998) Characterization of a phloem mobile chelator involved in the phloem transport of iron from vegetative tissues to developing seeds of pea. J. Exp. Bot. **49**: 903-911.

Marschner H (1995) Mineral Nutrition of Higher Plants. Academic Press, London.

Marschner H, Römheld V (1994) Strategies of plants for acquisition of iron. Plant Soil **165**: 261-274.

Marschner H, Römheld V, Kissel M (1986) Different strategies in higher plants in mobilization and uptake of iron. J. Plant Nutr. **9**: 695-713.

Marschner H, Römheld V, Kissel M (1987) Localization of phytosiderophore release and of iron uptake along intact barley roots. Physiol. Plant. **71**: 157-162.

Marschner H, Römheld V, Ossenberg-Neuhaus H (1982) Rapid method for measuring changes in pH and reducing processes along roots of intact plants. Z. Pflanzenphysiol. **105**: 407-416.

Marschner H, Treeby M, Römheld V (1989) Role of root-induced changes in the rhizosphere for iron acquisition in higher plants. Z. Pflanzenernähr. Bodenk. **152**: 197-204.

Marten I, Lohse G, Hedrich R (1991) Plant growth hormones control voltage-dependent activity of anion chanels in plasma membrane of guard cells. Nature **353**: 758-762.

Martinoia E, Rentsch D (1994) Malate: Compartimentation-responses to a complex metabolism. Ann. Rev. Plant Physiol. Plant Mol. Biol. **45**: 447-467.

Matsumura H, Miyachi S (1980) Cycling assay for nicotinamide adenine dinucleotides. Meth. Enzymol. **69**: 465-470.

Mengel K (1995) Iron availability in plant tissues-iron chlorosis on calcareous soils. Plant Soil **165**: 275-283.

Mengel K, Geurtzen G (1986) Iron chlorosis on calcareous soils. Alkaline nutritional condition as the cause for the chlorosis. J. Plant Nutr. **9**: 161-173.

Mengel K, Geurtzen G (1988) Relationship between iron chlorosis and alkalinity in *Zea mays*. Physiol. Plant. **72**: 460-465.

Mengel K, Planker R, Hoffmann B (1994) Relationship between leaf apoplast pH and iron chlorosis of sunflower (*Helianthus Annuus* L). J. Plant Nutr. **17**: 1053-1065.

Michelet B, Boutry M (1995) The plasma membrane H^+-ATPase - A highly regulated enzyme with multiple physiological functions. Plant Physiol. **108**: 1-6.

Millar AH, Atkin OK, Menz RI, Henry B, Farquhar G, Day DA (1998) Analysis of respiratory chain regulation in roots of soybean seedlings. Plant Physiol **117**: 1083-1093.

Millenaar FF, Benschop JJ, Wagner AM, Lambers H (1998) The role of the alternative oxidase in stabilizing the *in vivo* reduction state of the ubiquinone pool and the activation state of the alternative oxidase. Plant Physiol **118** 599-607.

Miller GW, Pushink SC, Welhie GW (1984) Iron chlorosis, a world wide problem. The relation of chlorophyll biosynthesis to iron. J. Plant. Nutr. **7**: 1-22.

Miller GW, Shigematsu A, Welkie GW, Motoji N, Szlek M (1990) Potassium effect on iron stress in tomato. II The effects on root CO_2-fixation and organic acid formation. J. Plant Nutr. **13**: 1355-1370.

Moing A, Carbonne F, Zipperlin B, Svanella L, Gaudillere J (1997) Phloem loading in peach: symplastic or apoplastic? Physiol. Plant **101**: 489-496.

Moog PR, Brüggemann W (1994) Iron reductase systems on the plant plasma membrane- A review. Plant Soil **165**: 241-260.

Moog PR, van der Kooij TAW, Brüggemann W, Schiefelbein JW, Kuiper PJC (1995) Responses to iron deficiency in *Arabidopsis thaliana*: The turbo iron reductase does not depend on the formation of root hairs and transfer cells. Planta **195**: 505-513.

Morales F, Abadía A, Abadía J (1990) Characterization of the xanthophyll cycle and other photosynthetic pigment changes induced by iron deficiency in sugar beet (*Beta vulgaris* L.). Plant Physiol. **94**: 607-613.

Morales F, Cerovic ZG, Moya I (1994) Iron deficiency-induced changes in the photosynthetic pigment composition of field-grown pear (*Pyrus communis* L.) leaves. Plant Cell Environ. **17**: 1153-1160.

Morales F, Grasa R, Abadía A, Abadía J (1998) Iron chlorosis paradox in fruit trees. J. Plant Nutr. **21**: 815-825.

Mori S (1998) Iron transport in graminaceous plants. *En* A Siegel, H Sigel, eds, Metal ions in biological systems. Basel, Switzerland, pp 215-238.

Mori S, Nishizawa N (1987) Methionine as a dominant precursor of phytosiderophores in Graminaceae plants. Plant Cell Physiol. **28**: 1081-1092.

Mori S, Nishizawa N, Hayashi H, Chino M, Yoshimura E, J. I (1991) Why are young rice plants highly susceptible to iron deficiency? *En* Y Chen, H Y., eds, Iron nutrition and interactions in plants. Kluwer Academic Publishing, Dordrecht, pp 175-188.

Moustakas A, Nari J, Diamantidis G, Noat G, Crasnier M, Borel M, Ricard J (1986) Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. 2. The role of pectin methyl esterase in the modulation of electrostatic effects in soybean cell walls. Eur. J. Biochem. **155**: 191-198.

Muday GK, Haworth P (1994) Tomato root growth, gravitropism and lateral development: correlations with auxin transport. Plant Physiol. Biochem. **32**: 193-203.

Mühling K, Plieth C, Hansen U, Sattelmacher B (1995) Apoplastic pH of intact leaves of *Vicia faba* as influenced by light. J. Exp. Bot **46**: 377-382.

Mühling K, Sattelmacher B (1995) Apoplastic ion concentration of intact leaves of field bean (*Vicia faba*) as influenced by ammonium and nitrate nutrition. J. Plant Physiol. **147**: 81-86.

Mühling K, Sattelmacher B (1997) Determination of apoplastic K in intact leaves by ratio imaging of PBFI fluorescence. J. Exp. Bot **48**: 1609-1614.

Mühling K, Wimmer M, Goldbach H (1998) Apoplastic and membrane associated Ca in leaves and roots as affected by boron deficiency. Physiol. Plant. **102**: 179-184.

Mullins G, Sommers L, Housley T (1986) Metal speciation in xylem and phloem exudates. Plant Soil **96**: 377-391.

Münch E (1930) Die Stoffbewegung in der pflanze. Fischer, Jena.

Murad E, Fisher WR (1988) Iron in soils and clay minerals. Dordrecht, The Netherlands.

Nagarajah S, Ulrich A (1965) Iron nutrition of the sugar beet plant in relation to growth, mineral balance and riboflavin formation. Soil Sci. **102**: 399-407.

Naik GR (1984) Inactive iron sugarcane leaves and its influence on enzymatic reactionant chloroplast metabolism. J. Plant Nutr. **7**: 785-798.

Nishio JN, Abadía J, Terry N (1985) Chlorophyll-proteins and electron transport during iron nutrition-mediated chloroplast development. Plant Physiol. **78**: 296-299.

Nishio JN, Terry N (1983) Iron nutrition-mediated chloroplast development. Plant Physiol. **71**: 688-691.

Olsen RA, Bennett JH, Blume D, Brown JC (1981) Chemical aspects of the Fe stress response mechanism in tomatoes. J. Plant Nutr. **3**: 905-921.

Palmer MJ, de Kock PC, Bacon JSD (1963) Changes in concentration of malic acid, citric acid, Ca and K in the leaves during the growth of normal and iron deficient mustard plants. Biochem. J. **86**: 484-493.

Parkhurst DF (1982) Stereological methods for measuring internal leaf structural variables. Am. J. Bot. **69**: 31-39.

Petersen W, Böttger M (1991) Contribution of organic acids to the acidification of the rhizosphere of maize seedlings. Plant Soil **132**: 159-163.

Pfanz H, Dietz K (1987) A fluorescence method for the determination of the apoplastic proton concentration in intact leaf tissues. J. Plant. Physiol **129**: 41-48.

Pfanz H, Oppmann B (1991a) The possible role of apoplastic peroxidases in detoxifying the air pollutant sulfur dioxide. *En* J Lobarzewski, H Greppin, C Penel, T Gaspar, eds, Biochemical molecular and physiological aspects of plant peroxidases. University of Lublin, Geneva, pp 402-417.

Pfanz H, Oppmann B (1991b) The possible role of apoplastic peroxidases in detoxifying the air pollutant sulfur dioxide. *En* H Rennenberg, C Brunold, LJ de Kock, I Stulen, eds, Sulfur nutrition and assimilation in higher plants; fundamental, environmental and agricultural aspect. SPB Academic publishers, The Hague, NL, pp 229-233.

Pich A, Hillmer S, Manteuffel R, Scholz G (1997) Nicotianamine-regulator in plant iron metabolism. Putative molecular-biological aspects. J. Exp. Bot. **48**: 759-767.

Pich A, Scholz G (1991) Nicotianamine and the distribution of iron into apoplast and symplast of tomato (*Lycopersicon esculentum* L.) II. Uptake of iron by protoplasts from the variety Bonner Beste and its nicotianamine-less mutant *chloronerva* and the compartmentation of iron in leaves. J. Exp. Bot. **42**: 1517-1523.

Pich A, Scholz G (1993) The relationship between the activity of various iron-containing and iron-free enzymes and the presence of nicotianamine in tomato seedlings. Physiol. Plant. **88**: 172-178.

Pich A, Scholz G, Stephan UW (1995) Iron-dependent changes of heavy metals, nicotianamine, and citrate in different plant organs and in the xylem exudate of two tomato genotypes. Nicotianamine as possible copper translocator. *En* J Abadía, eds, Iron nutrition in soils and plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 51-58.

Pichakashi-Maunsbach K, Harvey D (1992) X-ray microanalytical (EDX) investigation of potassium distribution in mesophyll cells of non-acclimated and cold-acclimated rye leaves. Plant Cell Environ. **15**: 585-591.

Pinedo ML, Segarra C, Conde RD (1993) Occurrence of two endoproteinases in wheat leaf intercellular washing fluid. Physiol Plant. **88**: 287-293.

Pinton R, Cesco S, de Nobili M, Santi S, Varanini Z (1998) Water and pyrophosphate extractable humic substances fractions as a source of iron for Fe-deficient cucumber plants. Biol .Fert. Soils **26**: 23-27.

Polle A, Chakrabartik K, Schümann W, Rennenberg H (1990) Composition and properties of hydrogen peroxide decomposing systems in extracellular and total extracts from needles of Norway Spruce (*Picea abies* L, Karst). Plant Physiol. **94**: 312-319.

Pomper K, Breen P (1995) Levels of apoplastic solutes in developing strawberry fruit. J. Exp. Bot **46**: 743-752.

Poole RJ (1978) Energy coupling for membrane transport. Ann. Rev. Plant Physiol. **29**: 437-460.

Price L (1968) Iron compounds and plant nutrition. Annu. Rev. Plant Phys. **19**: 239-248.

Rabotti G, de Nisi P, Zocchi G (1995) Metabolic implications in the biochemical responses to iron deficiency in cucumber (*Cucumis sativus* L.) roots. Plant Physiol. **107**: 1195-1199.

Rabotti G, Zocchi G (1994) Plasma membrane-bound H^+-ATPase and reductase activities in Fe-deficient cucumber roots. Physiol. Plant. **90**: 779-785.

Rasi-Caldogno F, De Michelis MI, Pugliarello MC (1981) Evidence for an electrogenic ATPase in microsomal vesicles from pea internodes. Biochim. Biophys. Acta **642**: 37-45.

Rawn J (1989) Bioquímica. IMC Graw-Hill, eds, Madrid, Spain.

Rea PA, Sanders D (1987) Tonoplast energization: two H^+ pumps, one membrane. Physiol. Plant. **71**: 131-141.

Rhoads WA, Wallace A (1960) Possible involvement of dark fixation of CO_2 lime-induced chlorosis. Soil Sci. **89**: 248-256.

Ribas-Carbó M, Lennon A, Robinson S, Giles L, Berry J, Siedow J (1997) The regulation of electron partioning between the cytochrome and alternative pathways in soybean cotyledon and root mitochondria. Plant Physiol. **113** 903-911.

Rich PR (1978) Quinol oxidation in *Arum maculatum* mitochondria and its application to the assay, solubilisation and partial purification of the alternative oxidase. FEBS Lett **96**: 252-256.

Robinson N, Procter C, Connolly E, Guerinot M (1999) A ferric-chelate reductase for iron uptake from soils. Nature **397**: 694-697.

Rohringer R, Ebrain-Nesbat F, Wolf G (1983) Proteins in intercellular washing fluids from leaves of barley (*Hordeum vulgare* L). J. Exp. Bot. **34**: 1589-1605.

Rombolà AD, Brüggemann W, Tagliavini M, Moog P (1997) Iron deficiency in kiwifruit: responses of roots and leaves. *En* IX International symposium on iron nutrition and interactions in plants, Stuttgart. Germany.

Romera FJ, Alcántara E (1994) Iron-deficiency stress responses in cucumber (*Cucumis sativus* L.) roots. Plant Physiol. **105**: 1133-1138.

Romera FJ, Alcántara E, de la Guardia MD (1992) Role of roots and shoots in the regulation of the Fe efficiency responses in sunflower and cucumber. Physiol. Plant. **85**: 141-146.

Romera FJ, de la Guardia MD (1991) La nutrición férrica de las plantas. Grupo Gestión Editorial, Córdoba. España.

Romera FJ, Welch RM, Norwell WA, Schaefer SC, Kochian LV (1996a) Ethylene involvement in the over-expression of Fe(III)-chelate reductase by roots of *E107* pea [*Pisum sativum* L. (*brz, brz*)] and *chloronerva* tomato (*Licopersicon esculentum* L.) mutant genotypes. Biometals **9**: 38-44.

Romera FJ, Welch RM, Norwell WA, Schaefer SC (1996b) Iron requirement for and effects of promoters and inhibitors of ethylene action on stimulation of Fe(III)-chelate reductase in roots of strategy I species. Biometals **9**: 45-50.

Römheld V (1997) The chlorosis paradox: Fe inactivation in leaves as a secondary event in Fe deficiency chlorosis. *En* 9th International symposium on iron nutrition and interactions in plants, Hohenheim, Stuttgart. Germany .

Römheld V, Kramer D (1983) Relationship between proton efflux and rhizodermal transfer cells induced by iron deficiency. Z. Pflanzenphysiol. **113**: 73-83.

Römheld V, Marschner H (1981) Iron deficiency stress induced morphological and physiological changes in root tips of sunflower. Physiol. Plant. **53**: 354-360.

Römheld V, Marschner H (1983) Mechanism of iron uptake by peanuts plants. I. Fe(III) reduction, chelate splitting, and release of phenolics. Plant Physiol. **71**: 949-954.

Römheld V, Marschner H (1986a) Evidence for a specific uptake system for iron phytosiderophores in root of grasses. Plant Physiol. **80**: 175-180.

Römheld V, Marschner H (1986b) Mobilization of iron in the rhizhosphere of different plant species. Praeger Scientific, New York, USA.

Römheld V, Müller C, Marschner H (1984) Localization and capacity of proton pumps in roots of intact sunflower plants. Plant Physiol. **76**: 603-606.

Rosenfield CL, Reed DW, Kent MW (1991) Dependency of iron reduction on development of a unique root morphology in *Ficus benjamina* L. Plant Physiol. **95**: 1120-1124.

Rubinstein B, Luster DG (1993) Plasma membrane redox activity: Components and role in plant processes. Ann. Rev. Plant Physiol. Plant Mol. Biol. **44**: 131-155.

Sanz M, Cavero J, Abadía J (1992) Iron chlorosis in the Ebro river basin, Spain. J. Plant Nutr. **15**: 1971-1981.

Sarkissian IV (1972) Modification of enzyme activity, conformation and size by indolacetic acid. *En* DJ Carr, eds, Plant growth substances. Springer, Berlin, pp 265-271.

Sattelmacher B, Mühling K, Pennewiss K (1998) The apoplast-its significance for the nutrition of higher plants. Z. Pflanzenernähr. Bodenk. **161**: 485-498.

Schaller G (1987) pH changes in the rhizosphere in relation to the pH-buffering of soils. Plant Soil **97**: 439-444.

Schmidt A, Buckhout TJ (1997) The response of tomato roots (*Lycopersicon esculentum* Mill.) to iron deficiency stress: alterations in the pattern of protein synthesis. J. Exp. Bot. **48**: 1909-1918.

Schmidt W (1993) Iron stress-induced redox reactions in bean roots. Physiol. Plant. **89**: 448-452.

Schmidt W (1995) Effects of various inhibitors on *in vivo* reduction by *Plantago lanceolata* L. roots. *En* J Abadía, eds, Iron nutrition in soils and plants. Kluwer Academic Publishers, pp 77-82.

Schmidt W (1999) Review: Mechanisms and regulation of reduction-based iron uptake in plants. New Phytol. **141**: 1-26.

Schmidt W, Bartels M (1996) Formation of root epidermal transfer cells in *Plantago*. Plant Physiol. **110**: 217-225.

Schmidt W, Bartels M, Tittel J, Fuhner C (1997) Physiological effects of copper on iron acquisition processes in *plantago*. New Phytol. **135**: 659-666.

Schmidt W, Diers F (1996) Regulation of ferric reduction in iron sufficient Plantago roots. *En* 10th FESPP, Congress, Florence. Italy.

Schmidt W, Schuck C (1996) Pyridine nucleotide pool size changes in iron-deficient *Plantago lanceolata* roots during reduction of external oxidants. Physiol. Plant. **98**: 215-221.

Scholz G, Becker R, Pich A, Stephan UW (1992) Nicotianamine- a common constituent of Strategies I and II of iron acquisition in plants. A review. J. Plant Nutr. **15**: 1649-1665.

Scholz GR, Becker UW, Stephan A, A. R, Pich A (1988) The regulation of iron uptake and possible functions of nicotianamine in higher plants. Biochem. Physiol. Pflanzen. **183**: 257-269.

Schurr U, Schulze ED (1995) The concentration of xylem sap from intact, transpiring castor bean plants (*Ricinus communis* L.). Plant Cell Environ. **18**: 409-420.

Schwertmann U (1991) Solubility and dissolution of iron oxides. Plant Soil **130**: 1-25.

Serrano R (1989) Structure and function of plasma membrane ATPase. Ann. Rev. Plant Physiol. Plant Mol. Biol. **40**: 61-94.

Shinmachi F, Hasegawa I, Yazaki J (1992) Analysis of iron-deficiency response systems on the plant root using the hairy root. Japanese J. Soil Sci. Plant Nutr. **63**: 202-209.

Shinmachi F, Hasegawa I, Yazaki J (1994) The relation between riboflavin secretion as a result of iron deficiency and ferric reduction system on the plant. Japanese J. Soil Sci. Plant Nutr. **65**: 413-418.

Shinmachi F, Hasegawa I, Yazaki J (1995) Difference in riboflavin secretion phenomenon as a result of iron-deficiency among plant species. Japanese J. Soil Sci. Plant Nutr. **66**: 337-341.

Shojima S, Nishizawa N, Fushiya S, Nozoe S, Irifune T, Mori S (1990) Biosynthesis of phytosiderophores. *In vitro* biosynthesis of 2'-deoxymugineic acid from L-methionine and nicotianamine. Plant Physiol. **93**: 1497-1503.

Sijmons PC, Bienfait HF (1984) Mechanism of iron reduction by roots of *Phaseolus vulgaris* L. J. Plant Nutr. **7**: 687-693.

Sijmons PC, Bienfait HF (1986) Development of Fe^{3+} reduction activity and H^+ extrusion during growth of iron-deficient bean plants in a rhizostat. Biochem. Physiol. Pflanzen **181**: 283-299.

Sijmons PC, Kolattukudi PE, Bienfait HF (1985) Iron deficiency decreased suberization in bean roots through a decrease in suberin-specific peroxidase activity. Plant Physiol. **78**: 115-120.

Sijmons PC, Lanfermeijer FC, De Boer AH, Prins HBA, Bienfait HF (1984a) Depolarization of cell membrane potential during trans-plasma membrane electron transfer to extracellular electron acceptors in iron-deficient roots of *Phaseolus vulgaris* L. Plant Physiol. **136**: 51-55.

Sijmons PC, van den Briel W, Bienfait HF (1984b) Cytosolic NADPH is the electron donor for extracellular Fe(III) reduction in iron-deficient bean roots. Plant Physiol **75**: 219-221.

Smith B (1984) Iron in higher plants: storage and metabolic role. J. Plant Nutr. **7**: 759-766.

Snowden RED, Wheeler BD (1993) Iron toxicity to fen species. J. Ecol. **81**: 35-46.

Speer M, Kaisser WM (1991) Ion relations of symplastic and apoplasmic space in leaves from *spinacia oleracea* L and *Pisum sativum* L under salinity. Plant Physiol. **97**: 990-997.

Spiller S, Terry N (1980) Limiting factors in photosynthesis. II. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. Plant Physiol. **65**: 121-125.

Spiro T, Pape L, Saltman P (1967a) The hydrolitic polymerization of ferric citrate. I. The chemistry of the polimer. J. Am. Chem. Soc. **89**: 5555-5559.

Spiro T, Pape L, Saltman P (1967b) The hydrolitic polymerization of ferric citrate. I. The influence of excess citrate. J. Am. Chem. Soc. **89**: 5559-5562.

Srere PA (1967) Citrate synthase. Methods Enzymol. **13**: 3-11.

Starrach N, Mayer W (1989) Changes in the apoplastic pH and K^+ concentration in the *Phaseoulus pulvinus in situ* in relation to rhytmic leaf movements. J. Exp. Bot **40**: 865-873.

Stein W, Kunkel H, Cole R, Spacman D, Moore S (1957) Observations on the aminoacid composition of human hemoglobins. Biochim. Biophys. Acta **24**: 640-642.

Stephan UW, Scholz G (1993) Nicotianamine: mediator of transport of iron and heavy metals in the phloem?. Physiol. Plant. **88**: 522-529.

Steudle E, Smith JAC, Lüttge U (1980) Water-relation parameters of individual mesophyll cells of *Kalanchöe daigremontiana*. Plant Physiol. **66**: 1155-1163.

Sueyoshi K, Hirata O, Oji Y (1997) Characterization of plasma membrana-bound Fe^{3+}-chelate reductase from Fe-deficient and Fe-sufficient cucumber roots. Soil Sci. Plant Nutr. **43**: 149-156.

Sun D, Bian Y, Zhao L, Yu X, Shengjun D (1995) The effects of extracellular calmodulin on cell wall regeneration of protoplasts and cell division. Plant Cell Physiol. **36**: 133-138.

Susín S (1994) Respuestas inducidas por la deficiencia de hierro en el sistema radicular de *Beta vulgaris* L. Tesis Doctoral. Universidad de Zaragoza.

Susín S, Abadía A, González-Reyes JA, Lucena JJ, Abadía J (1996) The pH requeriment for in vivo activity of the iron-deficiency-induced "turbo" ferric chelate reductase. A comparison of the iron-deficiency-induced iron reductase activities of intact plants and isolated plasma membrane fractions in sugar beet. Plant Physiol. **110**: 111-123.

Susín S, Abián J, Peleato ML, Sánchez-Baeza J, Abadía A, Gelpí E, Abadía J (1994) Flavin excretion from iron deficient sugar beet (*Beta vulgaris* L.). Planta **193**: 514-519.

Susín S, Abián J, Sánchez-Baeza F, Peleato ML, Abadía A, Gelpí E, Abadía J (1993a) Riboflavin 3'- and 5'-sulfate, two novel flavins accumulating in the roots of iron-deficient Sugar Beet (*Beta vulgaris*). J. Biol. Chem. **268**: 20958-20956.

Susín S, Quílez R, Abadía A, Abadía J (1993b) Differences in flavin content of leaves from iron deficient and iron sufficient sugar beet (*Beta vulgaris* L.). *En* Seventh international symposium on iron nutrition and interactions, Zaragoza, Spain.

Suzuki K, Itai R, Suzuki K, Nakanishi H, Nishizawa NK, Yoshimura E, Mori S (1998) Formate dehydrogenase, an enzyme of anaerobic metabolism, is induced by iron deficiency in barley roots. Plant Physiol. **116**: 725-732.

Szabó-Nagy A, Erdei L (1993) The effects of iron deficiency on the ATPase and ferrycianide reductase activities of plasma membrane purified by phase partitioning from sunflower roots. J. Plant Physiol. **142**: 579-584.

Sze H (1985) H^+-translocating ATPases: advances using membrane vesicles. Ann. Rev. Plant Physiol. **36**: 175-208.

Taiz L (1984) Plant cell extension: regulation of cell wall mechanical properties. Annu. Rev. Plant Physiol. **35**: 585-657.

Takagi S, Nomoto K, Takemoto T (1984) Physiological aspect of mugineic acid, a possible phytosiderophore of graminaceous plants. J. Plant Nutr. **7**: 469-477.

Terry M, Bonner B (1980) An examination of centrifugation as a method of extracting an extracellular solution from peas and its use for the study of indoleacetic acid-induced growth. Plant Physiol **66**: 321-325.

Terry N (1979) The use of mineral nutrient stress in the study of limiting factors in photosynthesis. *En* R Marcelle, H Clijsters, M Van Poucke, eds, Photosynthesis and plant development. Dr W. Junk Publishers, The Hague, pp 151-160.

Terry N (1980) Limiting factors in photosynthesis. I. Use of iron stress to control photochemical capacity *in vivo*. Plant Physiol. **65**: 114-120.

Terry N (1983) Limiting factors in photosynthesis. IV. Iron stress-mediated changes in light harvesting and electron transport capacity and its effects on photosynthesis *in vivo*. Plant Physiol. **75**: 855-860.

Terry N, Abadía J (1986) Function of iron in chloroplast. J. Plant Nutr. **9**: 609-646.

Terry N, Low G (1982) Leaf chlorophyll content and its relation to the intracellular localization of iron. J. Plant Nutr. **5**: 301-310.

Terry N, Zayed A (1995) Physiology and biochemistry of leaves under iron deficiency. *En* J Abadía, eds, Iron nutrition in soils and plants. Kluwer Academic, Dordrecht, Boston, London, pp 283-294.

Tetlow IJ, Farrar JF (1993) Apoplasmic sugar concentration and pH in barley leaves infected with brown rust. J. Exp. Bot. **44**: 929-936.

Thoiron S, Briat J-F (1999) Differential expression of maize sugar responsive genes in response to iron deficiency. Plant Physiol. Biochem. **37**: 759-766.

Thoiron S, Pascal N, Briat J-F (1997) Impact of iron deficiency and iron re-supply during the early stages of vegetative development in maize (*Zea Mays* L.). Plant Cell Environ. **20**: 1051-1060.

Tiffin LO (1966a) Iron translocation I. Plant culture, exudate sampling, iron-citrate analysis. Plant Physiol. **41**: 510-514.

Tiffin LO (1966b) Iron translocation. II Citrate/iron ratios in plant stem exudates. Plant Physiol. **41**: 515-518.

Tiffin LO (1972) Translocation of micronutrients in plants. *En* eds, Micronutrients in agriculture. Soil Sci. Soc. of America, Madison, USA, pp 199-229.

Tiffin LO, Brown JC (1962) Iron chelates in soybean exudate. Science **19**: 466-480.

Toulon V, Sentenac H, Thibaud JB, Davidian JC, Moulineaz C, Grignon C (1992) Role of apoplast acidification by the H^+ pump. Effect on the sensitivity to pH and CO_2 of iron reduction by roots of *Brassica napus* L. Planta **186**: 212-218.

Turner R (1970) The subcellular distribution of zinc and copper within the roots of metal tolerant clones of *Agrostis tenuis* Sibth. New Phytol. **69**: 725-731.

Turner R, Marshal C (1971) The accumulation of 65-Zn by root homogenates of zinc-tolerant clones of *Agrostis tenuis* Sibth. New Phytol. **70**: 539-545.

Tyler G, Falkengren-Grerup U (1998) Soil chemistry and plant performance ecological considerations. Progr. Bot. **59**: 634-658.

Va H, Kuraishi S, Sakurai N (1994) Aluminum induced rapid root inhibition and changes in cell-wall components of asquash seedlings. Plant Physiol. **106**: 976.

Vakhmistrov DB (1967) Localization of the free space in the barley roots. Fiziol Rasten. **14**: 397-404.

Valenti V, Scalorbi M, Guerrini F (1991) Induction of plasma membrane NADH-ferrycianide reductase following iron stress in tomato roots. Plant Physiol. Biochem. **29**: 249-255.

van Egmond F, Aktas M (1977) Iron-nutritional aspects of the ionic balance of plants. Plant Soil **48**: 685-703.

Vance CP, Gregerson RG, Robinson DL, Miller SS, Gantt JS (1994) Primary assimilation of nitrogen in alfalfa nodules: molecular features of the enzymes involved. Plant Sci. **101**: 51-64.

Vance CP, Stade S, Maxwell CA (1983) Alfalfa root nodule carbon dioxide fixation. I. Association with nitrogen fixation and incorporation into aminoacids. Plant Physiol. **72**: 469-473.

Venkat-Raju K, Marschner H, Römheld V (1972) Effect of iron nutritional status on ion uptake, substrate pH and production and release of organics acids and riboflavin by sunflower plants. Z. Pflanzener. Bodenk. **132**: 177-190.

Viets F (1944) Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol **19**: 466-480.

Voltz M, Jacobson L (1974) A specific Ca requirement for potassium uptake by excised vetch roots. Plant Soil **41**: 647-659.

Vost PB (1982) Iron nutrition in plants. J. Plant Nutr. **5**: 233-249.

Vuorinen AH, Vapaavuori EM, Raatikainen O, Lapinjok SP (1992) Metabolism of inorganic carbon taken up by roots in *Salix* plants. J. Exp. Bot. **43**: 789-795.

Walker D (1987) The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. Oxygraphics Limited, Sheffield, UK.

Wallace A (1982) Historical landmark in progress relating to iron chlorosis in plants. J. Plant Nutr. **5**: 277-288.

Walsh K, Bushby H, Date A, Dart P (1995) Physiology of the legume nodule and its response to stress. *En* 10th Australian nitrogen fixation conference microbial ecology, Australia.

Webb A, Hethrington (1997) Convergence of the abscisic, CO_2, and extracellular calcium signal transduction pathway in stomatal guard cells. Plant Physiol. **114**: 1557-1569.

Wei L, Loeppert R, Ocumpaugh W (1997) Fe-deficiency stress response in Fe-deficiency resistant and susceptible subterranean clover - Importance of induced H^+ release. J. Exp. Bot. **48**: 239-246.

Welbaum G, Meinzer F (1990) Compartimentation of solutes and water in developing sugarcane stalk tissue. Plant Physiol **93**: 1147-1153.

Welch RM, LaRue TA (1990) Physiological characteristics of Fe accumulation in the 'bronze' mutant of *Pisum sativum* L. cv. Sparkle E107 (*brz brz*). Plant Physiol. **93**: 723-729.

Welkie GW (1996) Iron-Deficiency Stress Responses of a chlorosis-susceptible and a chlorosis-resistant cultivar of muskmelon as related to root riboflavin excretion. J. Plant Nutr. **19**: 1157-1169.

Welkie GW, Miller GW (1960) Iron nutrition of *Nicotiana tabaccum* L. in relation to riboflavin, riboflavin-5'-phosphate, and flavin adenine dinucleotide content. Plant Physiol. **35**: 516-520.

Welkie GW, Miller GW (1988) Riboflavin excretion from roots of iron-stressed and reciprocally grafted tobacco and tomato plants. J. Plant Nutr. **11**: 691-700.

Welkie GW, Miller GW (1992) Iron stress and salt stress responses of lettuce (*Lactuca sativa* L.). J. Plant Nutr. **15**: 1757-1764.

Welkie GW, Miller GW (1993) Plant iron uptake physiology by nonsiderophore systems. *En* LL Barton, BC Hemming, eds, Iron chelation in plants and soil microorganisms. Academic Press, San Diego, USA, pp 345-369.

White MC, Decker AM, Chaney RL (1981a) Metal complexation in xylem fluid. I. Chemical composition of tomato and soybean stem exudate. Plant Physiol. **67**: 292-300.

White MC, Baker FD, Chaney RL, Decker AM (1981b) Metal complexation in xylem fluid.II. Theoretical equilibrium model and computational computer program. Plant Physiol. **67**: 301-310.

White PF, Robson AD (1989) Rhizosphere acidification and Fe(III) reduction in lupins and peas: iron deficiency in lupins is not due to a poor ability to reduce Fe(III). Plant Soil **119**: 163-175.

Wilbur KM, Anderson NG (1948) Electrometric and colourimetric determination of carbonic anhydrase. J. Biol. Chem. **176**: 147-154.

Wilson T, Canny M, McCully M (1991) Leaf teeth, transpiration and the retrieval of apoplastic solutes in balsam poplar. Physiol. Plant. **83**: 225-232.

Winder T, Nishio J (1995) Early iron deficiency stress response in leaves of sugar beet. Plant Physiol **108**: 1487-1494.

Wolf O, Munns R, Tonnet ML, Jeschke WD (1990) Concentrations and transport of solutes in xylem and phloem along the leaf axis of NaCl-treated *Hordeum vulgare*. J. Exp. Bot. **41**: 1133-1141.

Yang X, Römheld V, Marschner H (1994) Effect of bicarbonate on root growth and accumulation of organic acids in Zn-inefficient and Zn-efficient rice cultivars *Oryza sativa* L. Plant Soil **164**: 1-7.

Yi Y, Guerinot ML (1996) Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. Plant J. **10**: 835-844.

Young TF, Terry N (1982) Transport of iron into leaves following iron resupply to iron-stressed sugar beet plants. J. Plant Nutr. **5**: 1273-1283.

Zhang FS, Römheld V, Marschner H (1991) Role of the root apoplasm for iron acquisition by wheat plants. Plant Physiol. **97**: 1302-1305.

Zouari M (1996) Reponses radiculaires face a la deficience en fer chez differents genotypes de tomate et de betterave. Tesis Master. Instituto Agrónomico del Mediterraneo (IAMZ).

ANEXO

Publicaciones:

1 **Belkhodja R, Morales R, Quílez R, López-Millán AF, Abadía A, Abadía J.**
Iron deficiency causes changes in chlorophyll fluorescence due to the reduction in the dark of the Photosystem II acceptor side.
Photosynthesis Research (1998) 56: 265-276.

2 **González-Vallejo EB, González Reyes JA, Abadía A, López-Millán AF, Yunta F, Lucena JJ, Abadía J.**
Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet.
Australian Journal of Plant Physiology (1999) 26: 601-611.

3 **López-Millán AF, Morales F, Andaluz S, Gogorcena Y, Abadía A, De Las Rivas J, Abadía J.**
Protective mechanisms in roots of iron deficient sugar beet: Changes in carbon assimilation and oxygen use.
Enviado a Plant Physiology

4 **López-Millán AF, Morales F, Abadía A, Abadía J.**
Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport.
Enviado a Plant Physiology

5 **López-Millán AF, Morales F, Abadía A, Abadía J.**
Changes induced by iron deficiency in the composition of the leaf apoplastic fluid from field-grown pear (*Pyrus communis* L.) trees.
Enviado a Journal of Experimental Botany

6 **López-Millán AF, Morales F, Abadía A, Abadía J.**
Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (*Beta vulgaris* L.) leaves.
Enviado a Physiologia Plantarum

7 **López-Millán AF, Morales F, Gogorcena Y, Abadía A, Abadía J.**
Effects of Fe resupply on the physiology of Fe-deficient sugar beet roots.
Enviado a Australian Journal of Plant Physiology

8 **Larbi A, Morales F, Moog PR, López-Millán AF, Gogorcena Y, Abadía A, Abadía J.**
Reduction of Fe (III) chelates by mesophyll leaf disks of sugar beet affected by Fe deficiency.
Enviado a Planta

9 **López-Millán AF, Morales F, Gogorcena Y, Abadía A, Abadía J.**
Changes induced by iron deficiency in the organic acid metabolism of tomato (*Lycopersicon esculentum* L.)
En preparación

Comunicaciones a congresos

1 **López-Millán AF, Abadía A, Abadía J.**
Organic acid concentrations in the apoplast of iron-sufficient and iron-deficient sugar beet (*Beta vulgaris* L.).
IX International symposium on iron nutrition and interactions in plants. Sttutgart, Alemania. 1997.

2 **López-Millán AF, Morales F, Abadía A, Abadía J.**
Metabolic implications of the biochemical response to Fe deficiency in sugar beet (*Beta vulgaris* L).
VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas. Madrid, 1998.

Photosynthesis Research **56:** 265–276, 1998.

Regular paper

Iron deficiency causes changes in chlorophyll fluorescence due to the reduction in the dark of the Photosystem II acceptor side

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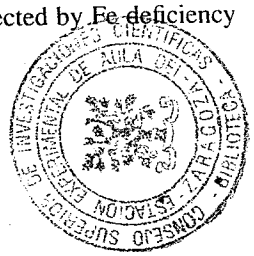
Abstract

Iron deficiency was found to affect the redox state of the Photosystem II acceptor side in dark-adapted, attached leaves of sugar beet (*Beta vulgaris* L.). Dark-adapted iron-deficient leaves exhibited relatively high F_o and F_{pl} levels in the Kautsky chlorophyll fluorescence induction curve when compared to the iron-sufficient controls. However, far-red illumination led to marked decreases in the apparent F_o and F_{pl} levels. Modulated fluorescence showed that far-red light decreased the fluorescence yield to the true F_o levels by increasing photochemical quenching, without inducing changes in the level of non-photochemical quenching. In dark-adapted, iron-deficient leaves, far-red illumination induced a faster fluorescence decay in the μs-ms time domain, indicating an improvement in the electron transport after the primary quinone acceptor in the reducing side of Photosystem II. All these data indicate that in iron-deficient leaves the plastoquinone pool was reduced in the dark. The extent of the plastoquinone reduction in sugar beet depended on the chlorophyll concentration of the leaf, on the time of preillumination and on the duration of dark adaptation. The dark reduction of plastoquinone was observed not only in sugar beet but also in other plant species affected by iron deficiency both in controlled conditions and in the field.

Abbreviations: Chl – chlorophyll; F_o – initial Chl fluorescence level; F'_o – Chl fluorescence after switching off the actinic illumination; F'_m – maximum Chl fluorescence with all PS II reaction centers closed in any light-adapted state; F_{pl} – intermediate Chl fluorescence plateau in the Kautsky Chl fluorescence induction curve; F_p – Chl fluorescence intensity at the peak of the induction curve; FR-far red; F_s – Chl fluorescence at steady-state photosynthesis; F_v – variable part of Chl fluorescence (F_p-F_o); PAR – photosynthetic active radiation; PPFD – photosynthetic photon flux density; PQ – plastoquinone; Q_A – primary quinone electron acceptor in Photosystem II; Q_B – secondary quinone electron acceptor in Photosystem II; SPAD – portable chlorophyll meter

Introduction

Iron deficiency is a common abiotic stress for many photosynthetic organisms on earth (Terry and Abadía 1986; Geider and La Roche 1994; Straus 1994). Iron deficiency markedly affects photosynthesis. Species affected range from high value crops in arid and semi-arid environments (Mortvedt 1991) to phytoplankton living in the sea (Behrenfeld et al. 1996). Leaves of Fe-deficient higher plants have a reduced number of granal and stromal lamellae per chloroplast (Spiller and Terry 1980), and this is accompanied by a decrease in all membrane components, including electron carriers in the photosynthetic electron transport chain (Spiller and Terry 1980; Terry 1980) and the light-harvesting pigments chlorophylls and carotenoids (Morales et al. 1990, 1994; Abadía and Abadía 1993). Since leaves affected by Fe deficiency



show a characteristic greenish-yellow color they are usually called 'chlorotic', and Fe deficiency in higher plants is usually referred to as 'iron chlorosis'.

Spiller and Terry (1980) concluded that Fe deficiency in sugar beet diminished photochemical capacity by reducing the number of photosynthetic units per unit leaf area, having no effect on their efficiency of photosynthetic energy conversion. However, decreases in the apparent efficiency of photosynthetic energy conversion in response to Fe deficiency, estimated by chlorophyll (Chl) fluorescence, have been described in higher plants (Morales et al. 1990, 1991), cyanobacteria (Guikema 1985) and eukaryotic marine algae (Greene et al. 1992; Falkowski et al. 1995). This discrepancy could be explained because Fe deficiency may decrease the efficiency of photosynthetic energy conversion only below a Chl concentration threshold level (Morales et al. 1991).

Iron deficiency has been reported to induce marked changes in the shape of the Chl fluorescence induction curve, including, apart from major increases in F_o, increases in the proportion of variable fluorescence leading to F_{pl} (Guikema 1985; Morales et al. 1991). The shape of the Chl fluorescence induction curve is thought to be controlled primarily by the redox state of Q_A. Upon exposure of a dark-adapted leaf to continuous actinic illumination, the Chl fluorescence rises immediately to an initial level, F_o, when Q_A is still fully oxidized. Q_A is progressively reduced and fluorescence increases to an intermediate level, F_{pl}, and continues increasing to a maximum (F_p or F_m), which is reached when Q_A is fully reduced to the semiquinone anion, Q_A^- (Schreiber et al. 1995). The fluorescence increase to F_m has been used to investigate the characteristics of the PS II acceptor side and the antenna size serving the PS II reaction centers. The initial part of the Chl fluorescence induction curve (i.e. the F_o to F_{pl} rise) has been attributed to PS II units with a reduced antenna size (PS II$_\beta$ centers) (Melis 1985), and to 'slow' PS II units lacking the ability to reduce Q_B (Q_B-nonreducing PS II centers) (Cao and Govindjee 1990). Recently, a model has been proposed for the interpretation of the fluorescence induction curve that includes both donor and acceptor side characteristics (Strasser 1997).

In this paper we have investigated the causes for the changes in Chl fluorescence in Fe-deficient leaves. We have found that these changes are caused in part by a reduction of the PS II acceptor pool in the dark. We have used short-term far-red illumination to test the effect of a preferential PS I excitation on Fe-deficient and control attached leaves by using three different non-intrusive probes: i) Chl fluorescence induction curves; ii) modulated Chl fluorescence, and iii) variable Chl fluorescence decays in the μs-ms time domain after an actinic saturating xenon flash. All these techniques indicate that the PQ pool becomes reduced within a few minutes in the dark in Fe-deficient leaves. We have also examined the changes in the capacity of the PQ pool to undergo dark reduction, and found that they are affected by the leaf Chl concentration and the duration of pre-illumination and dark adaptation. The dark reduction of plastoquinone was observed not only in sugar beet growing in controlled environments but also in several plant species affected by Fe deficiency in the field. We suggest that Fe deficiency may lead to an excess of reducing power that cannot be dissipated after a light-dark transition, causing in turn the dark reduction of PQ.

Materials and methods

Plant material

Sugar beet (*Beta vulgaris* cv. Monohil, Hilleshög, Landskröna, Sweden) was grown in a growth chamber. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for two more weeks in nutrient solution (in 3/8-strength Hoagland's nutrient solution with 22.4 μM Fe and then transplanted (4 plants per bucket) to 20 l plastic buckets lined with polyethylene bags and containing half-strength Hoagland's solution (Young and Terry 1982) with either 0 or 44.8 μM Fe(III)-EDTA. The nutrient solutions of some of the buckets containing no Fe were buffered at an initial pH of approximately 7.7 by adding 1 mM NaOH and 1 g l^{-1} of $CaCO_3$. This treatment simulates conditions usually found in the field which lead to Fe deficiency. Plants were used for measurements one week after transferring the plants to an iron-free nutrient solution. Plants were grown with a PPFD of 350 μmol photons m^{-2} s^{-1} PAR (measured with a Licor sensor placed horizontally at maximum plant height) at a temperature of 25 °C, 80% relative humidity and a photoperiod of 16 h light/8 h dark. Young, rapidly expanding leaves were used for all measurements. All chlorotic leaves sampled had no green veins, and showed a homogeneous color throughout the leaf. Leaves receiving a similar PPFD on their surface (250 μmol photons m^{-2} s^{-1}, measured by placing the sensor at the same angle than the leaf surface) were chosen for analysis.

Pear (*Pyrus communis* L.) and peach (*Prunus persica* L.) trees were grown in calcareous soils orchards in the Aula Dei Campus, located in the Gállego river, in the Ebro river basin in northeastern Spain. Young, expanded leaves were used for all measurements. *Chlamydomonas reinhardtii* cells were grown in a Tris-acetate-phosphate medium as described by Delepelaire and Wollman (1985), with or without Fe. Cultures were grown in 0.50 l culture flasks at room temperature, under laboratory light conditions and bubbled continuously with air.

Pigment analysis

The concentration of Chl per area was estimated in attached leaves by using a SPAD portable apparatus (Minolta Co., Osaka, Japan). To calibrate the SPAD, leaf disks were first measured with the SPAD, then frozen in liquid N_2, extracted with 100% acetone and the extracts analyzed spectrophotometrically (Abadía and Abadía 1993).

Chlorophyll fluorescence transients from attached leaves in the ms-s time domain

Continuous Chl fluorescence measurements in the ms-s time domain were made on intact, attached leaves at 25 °C. The experimental set-up was described elsewhere (Morales et al. 1991). All leaves were first dark-adapted for 30 min. Actinic light intensity was 150 μmol photons m^{-2} s^{-1} at the leaf level. The FR treatment consisted usually in illuminating the leaves for 1 min with FR light (7 μmol photons m^{-2} s^{-1} obtained with a RG715 Schott filter). Other FR intensities and/or periods of illumination were used in some experiments. After FR treatment leaves were maintained in the dark for 1 min before fluorescence measurements were made.

Modulated chlorophyll fluorescence analysis

Modulated Chl fluorescence measurements were made on attached sugar beet leaves in a dark room at 25 °C with a PAM fluorometer (H. Walz, Effeltrich, Germany). F_o was measured after 30 min of dark adaptation by switching on the modulated light at 1.6 kHz (the PPFD of the modulated light was less than 0.1 μmol photons m^{-2} s^{-1} at the leaf surface).

Table 1. Effect of FR light treatment on the F_v/F_p and $(F_{pl} - F_o)/F_v$ ratios in the Chl fluorescence induction curves from control and Fe-deficient sugar beet leaves. Leaf total Chl was approximately 35 and 3 nmol cm^{-2} in control and Fe-deficient plants, respectively. Data are the mean ± SE of 3 replicates

	Dark adapted ($n = 3$)	+ FR ($n = 3$)
Fe-sufficient		
F_v/F_p	0.86 ± 0.01	0.86 ± 0.01
$(F_{pl} - F_o)/F_v$	0.15 ± 0.01	0.14 ± 0.01
Fe-deficient		
F_v/F_p	0.55 ± 0.05	0.70 ± 0.05
$(F_{pl} - F_o)/F_v$	0.73 ± 0.03	0.29 ± 0.02

Chlorophyll fluorescence decays in the µs-ms time domain

The re-oxidation kinetics of Q_A^- was measured in intact, attached leaves from Fe-deficient and Fe-sufficient plants using a PAM fluorometer (H. Walz, Effeltrich, Germany). The high-intensity single-turnover flashes (8 μs half-width) were obtained from a XST 103 xenon discharge lamp (H. Walz, Effeltrich, Germany) connected to the PAM 103 unit. In the following dark period, the variable fluorescence decay, reflecting the re-oxidation of Q_A^-, was detected at 17 μs resolution by means of a weak modulated light. Due to the PAM detector internal gating, measurements started 110 μs after the flash. The decay half-times were determined from plots of the decay of variable Chl fluorescence on a logarithmic time scale using the QA software (QA Data, Turku, Finland).

Pyridine nucleotides

Pyridine nucleotides were extracted from N_2-liquid frozen leaf disks of 0.94 cm^2 in 1 ml of 0.1 M NaOH (for NAD(P)H) or 5% (w/v) TCA (for $NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifugated at 12000 *g* for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 0.1 M bicine, pH 8.0. Nucleotides were quantified by the enzyme-cycling method of Matsumura and Miyachi (1980).

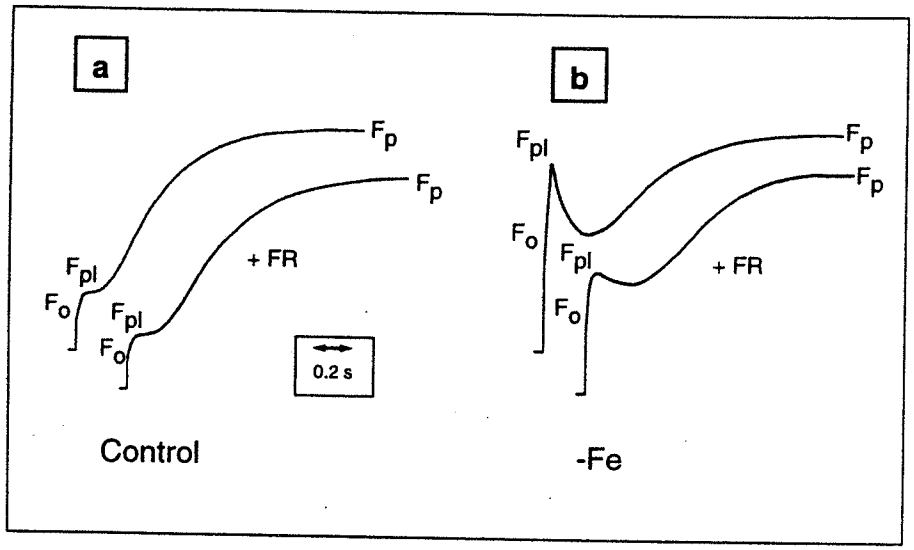


Figure 1. Chlorophyll fluorescence induction kinetics measured *in vivo* from (a) control, iron-sufficient (35 nmol Chl cm^{-2}) and (b) iron-deficient (−Fe; 4 nmol Chl cm^{-2}) attached leaves of sugar beet. Measurements were made after 30 min of dark adaptation or after a treatment consisting of 30 min of dark adaptation, 1 min of FR illumination and 1 min of dark-adaptation (+FR).

Table 2. Effect of FR light treatment on the estimated half-times ($t_{1/2}$; $t_{1/2}$ = ln 2-lifetime) and amplitudes (in parenthesis) of the different phases in the decay of the variable Chl fluorescence in control and Fe-deficient sugar beet leaves. Leaf total Chl was approximately 35 and 3 nmol cm^{-2} in control and Fe-deficient plants, respectively. Data are the mean ± SE of the number of replicates indicated

	$t_{1/2}$ (amplitude) dark adapted (n = 5)	$t_{1/2}$ (amplitude) + FR (n = 3)
Fe-sufficient		
Fast phase	259 ± 1 μs (84 ± 1%)	229 ± 2 μs (89 ± 1%)
Medium phase	12.6 ± 1.2 ms (6 ± 1%)	4.7 ± 1.0 ms (4 ± 1%)
Slow phase	> 100 ms (10 ± 1%)>	100 ms (7 ± 1%)
Fe-deficient		
Fast phase	277 ± 11 μs (34 ± 5%)	309 ± 32 μs (79 ± 4%)
Medium phase	3.9 ± 1.1 ms (20 ± 4%)	6.5 ± 1.8 ms (5 ± 1%)
Slow phase	> 100 ms (46 ± 1%)>	100 ms (16 ± 3%)

Results

Chlorophyll fluorescence induction in dark-adapted, Fe-deficient and control leaves

Sugar beet plants were placed in a dark room after being illuminated for approximately 3–4 h in the growth chamber. After 30 min of dark adaptation Fe-sufficient leaves showed typical Chl fluorescence induction curves, variable fluorescence accounting for 86% of the total fluorescence (F_v/F_p = 0.86; Table 1 and Figure 1a). These values indicate a good PS II photochemical efficiency. In these control leaves the F_{pl} level accounted for 15% of the variable fluorescence ($(F_{pl} - F_o)/F_v$ = 0.15; Table 1).

Severely Fe-deficient leaves (approximately 3 nmol Chl cm^{-2}) showed, after 30 min dark-adaptation, large decreases in the F_v/F_p ratio and large increases in the $(F_{pl} - F_o)/F_v$ ratio when compared to control leaves (Figure 1b). The F_v/F_p and the $(F_{pl} - F_o)/F_v$ ratios were 0.55 and 0.73, respectively (Table 1). These induction curves agree with data published previously for plants illuminated for 2 h (Morales et al. 1991). One possible interpretation for these data would be that Fe-deficient leaves have an impaired PS II photochemical efficiency.

Another possible cause for the increase of F_{pl} in the fluorescence induction curves from the Fe-deficient leaves that had been dark-adapted for 30 min may be the accumulation of electrons in the PS II acceptor side. Since Q_A is thought to exist in a quasi-equilibrium with PQ to which drives electrons via Q_B, any reduction of the PS II acceptor side may affect the F_{pl} level. A simple way of removing an electron accumulation in the PS II acceptor side is to use FR light, that excites preferentially PS I. Dark-adapted, attached leaves from control plants were submitted to a FR light treatment consisting of 1 min of illumination with FR light, plus 1 min of dark adaptation to avoid any possible actinic effect induced by FR. The Chl

fluorescence induction curves of control leaves treated in this manner showed similar characteristics to those of leaves measured after 30 min of dark adaptation (Figure 1a). The absolute magnitude of F_o, F_{pl} and F_p were decreased by FR illumination by 4 ± 1, 10 ± 1 and 5 ± 1%, respectively (n = 3). The F_v/F_p ratio was not changed by this treatment, whereas the $(F_{pl} - F_o)/F_v$ ratio was decreased by 10% (Table 1).

Iron-deficient leaves, however, had a behavior markedly different from that of controls when treated with FR light. The Chl fluorescence curves from these FR light-treated plants showed marked decreases in F_o and F_{pl} (37 and 43%, respectively) and very small decreases in F_p (3%) when compared to dark-adapted Fe-deficient leaves not treated with FR light (Figure 1b). As a consequence of these changes, the relative fluorescence at the F_{pl} point in the induction curve ($(F_{pl} - F_o)/F_v$), was decreased by FR treatment by 61%, down to a level accounting for 29% of F_v (Table 1). The F_v/F_p ratio was also markedly increased (by 28%) as a consequence of FR illumination (Table 1). These data indicate that the treatment with FR light improves significantly the apparent PS II photochemical efficiency of Fe-deficient leaves, as estimated by the F_v/F_p ratio, while reducing the relative fluorescence at point F_{pl}. Both effects are likely to be caused by the preferential excitation of PS I by FR.

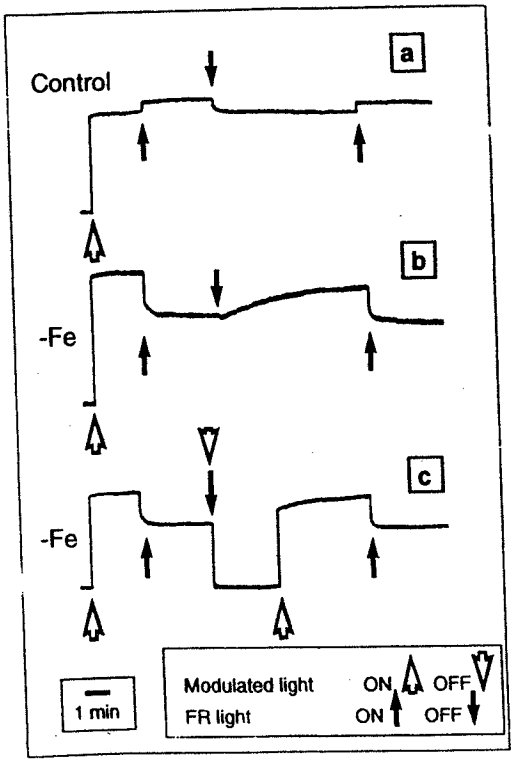


Figure 2. Changes in the modulated dark F_o level in response to FR illumination measured *in vivo* from (a) control, iron-sufficient (35 nmol Chl cm^{-2}) and (b, c) iron-deficient (–Fe; 2–3 nmol Chl cm^{-2}) attached leaves of sugar beet.

Modulated fluorescence in Fe-deficient and control leaves as affected by FR illumination

An assessment of the redox state of PS II can be done by using modulated fluorescence to measure F_o. The F_o level obtained by illumination of plant leaves with a weak modulated light is thought to originate from the excited Chl a PS II antenna molecules before excitons have reached the PS II reaction centers. In these experiments we used sugar beet plants placed in a dark room after being illuminated for approximately 3–4 h in the growth chamber.

The onset of FR illumination resulted in slight (5–10%) F_o increases in control leaves, occurring with a half-time of 1–2 s (Figure 2a). When FR light was switched off, a decay to the original F_o level occurred with a half-time of approximately 7 s. A similar effect of FR light on the modulated F_o level in control plants has been reported by other authors (Asada et al. 1993; Harris and Heber 1993). This increase in the F_o level in control leaves cannot be due to a transition to the high fluorescence state-1 (for a review, see Fork and Satoh 1986), because the time required for the relaxation of the high F_o induced by FR was too fast. On the other hand, saturating white light pulses given before and during FR treatment resulted in nearly identical F_m levels (Figure 3a). This indicates that the increase in F_o induced by FR light in control plants was caused by a decrease in photochemical quenching, non-photochemical quenching being unchanged by FR treatment.

In contrast to control leaves, the onset of FR illumination induced marked F_o decreases in Fe-deficient leaves (Figure 2b). These F_o decreases were larger in severely deficient leaves than in leaves moderately deficient (see below). The decrease in F_o caused by FR light occurred in a multiphasic way, a rapid decrease (half-time of approximately 2 s) being followed by a slower decay.

Upon termination of FR illumination, the F_o level increased slowly, with a half-time of several min (Figure 2b). The dark recovery of F_o was not complete in the time scale measured (up to 15 min); generally, this dark recovery was similar in size to the rapid phase of the F_o decay induced by FR light. Further illumina-

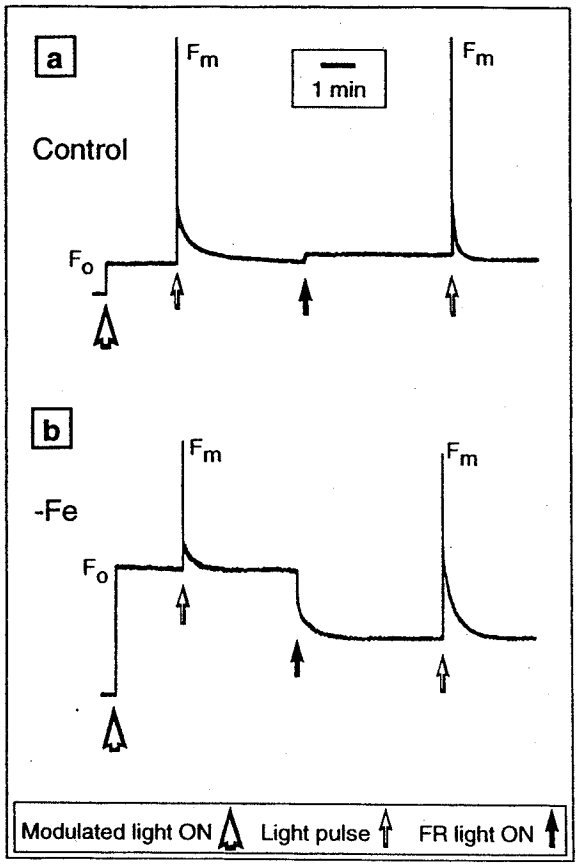


Figure 3. Changes in the modulated dark F_o level and in the F_m level induced by a short saturating flash, in response to FR illumination measured *in vivo* from (a) control, iron-sufficient (35 nmol Chl cm^{-2}) and (b) iron-deficient (−Fe; 4 nmol Chl cm^{-2}) attached leaves of sugar beet.

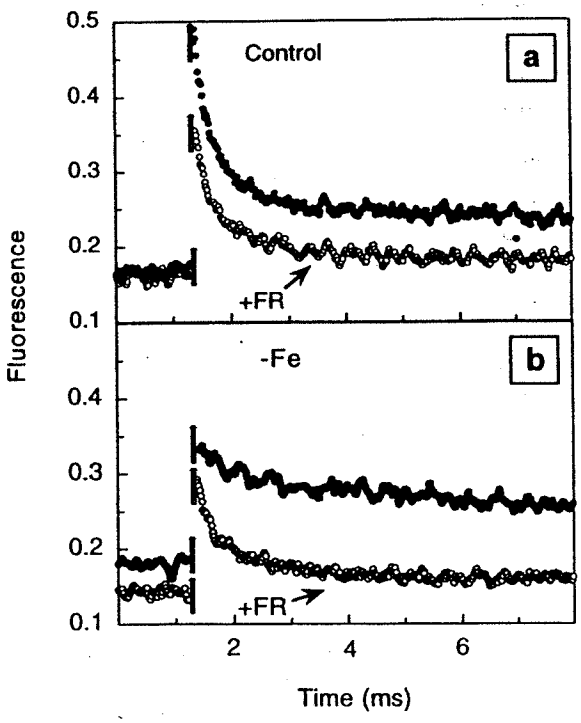


Figure 4. Decays of the variable chlorophyll fluorescence following an actinic flash measured *in vivo* from (a) control, iron-sufficient (35 nmol Chl cm^{-2}) and (b) iron-deficient (−Fe; 3 nmol Chl cm^{-2}) attached leaves of sugar beet. Measurements were made after 30 min dark adaptation (full circles) or in 30 min dark-adapted leaves, 2 s after the end of a treatment with FR light (1 min, 7 μmol photons m^{-2} s^{-1}) (open circles). Vertical bars mark the limits of the instrument time-delay.

tions with FR induced decreases in F_o similar in size to the fast phase resulting from the first FR illumination. The cycles FR-induced decrease in F_o/dark-induced increase in F_o could be repeated many times in the same leaf. The slower decay phase could only be induced by FR after illumination of the leaf with white actinic light (not shown).

Saturating white light pulses given before and during FR treatment in Fe-deficient leaves resulted only in small (2–5%) reductions in the F_m level (Figure 3b). This indicates that non-photochemical quenching was little changed by FR treatment, and that the change in F_o induced by FR light in Fe-deficient leaves resulted mainly from increases in photochemical quenching. These data support that some component of the inter-system electron transport chain in the PS II acceptor side was partially reduced in dark-adapted Fe-deficient leaves.

The possibility that the increase in F_o upon ceasing FR illumination may be induced artifactually by the weak modulated measuring beam was tested by switching off that light for a short period (Figure 2c). The results of this experiment indicate that some increase in F_o occurred also in complete darkness, and with similar kinetics to that found in the presence of the modulated beam. Therefore, in the Fe-deficient leaves a mechanism exists that is apparently able to reduce the PS II acceptor side in the dark.

Decay of the variable chlorophyll fluorescence

Sugar beet plants were placed in a dark room after being illuminated for approximately 3–4 h in the growth chamber. The typical variable fluorescence decay after giving one saturating xenon flash to 30 min dark-adapted control leaves could be resolved into three different decay phases, similar to those found by other authors (Figure 4a). The decay half-time for the two

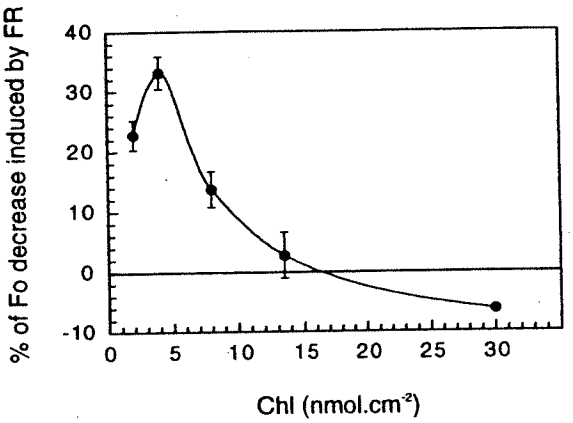


Figure 5. Changes in the extent of the decrease in F_o induced by FR in attached leaves of Fe-deficient sugar beet plants as affected by the Chl concentration. Plants were pre-illuminated for 4–5 h in the growth chamber before the 30 min dark-adaptation period. Data shown are the mean ± SE of 4 replicated measurements.

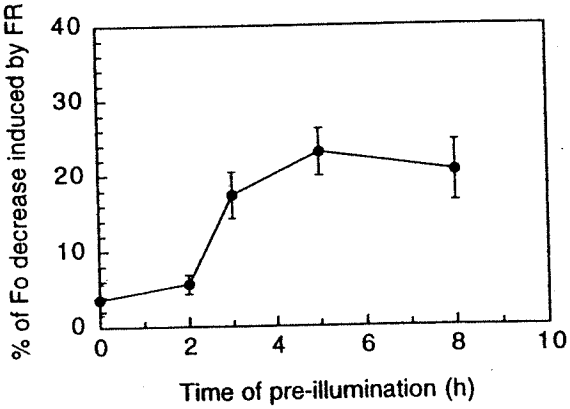


Figure 6. Time-course of the decreases in F_o induced by FR in attached leaves of Fe-deficient sugar beet plants (3 nmol Chl cm^{-2}). Plants were pre-illuminated in the growth chamber for the time indicated in the X-axis before the 30 min dark-adaptation period. Data shown are the mean ± SE of 4 replicates.

fast phases of Q_A^- re-oxidation were 259 μs and 13 ms, accounting for 84 and 6% of the decay (Table 2). A slow phase with a half-time > 100 ms accounted for the remaining 10% of the decay. When the flash was applied 2 s after the 1 min FR treatment, small increases in the rate of Q_A^- re-oxidation were found (Figure 4a). The decay half-time for the three phases were 229 μs, 5 ms and > 100 ms, and they accounted for 89, 4 and 7% of the decay (Table 2).

In the Fe-deficient leaves that had been dark-adapted for 30 min the re-oxidation of Q_A^- was markedly slower than in the controls (Figure 4b). The half-times of the two fast phases were 277 μs and 4 ms, and they accounted for 34 and 20% of the decay, whereas the slow phase of half-time > 100 ms accounted for 46% of the decay (Table 2). When FR light was used for 1 min to remove any remaining electron accumulation in the PQ pool in these leaves the re-oxidation of Q_A^- became significantly faster. This was the result of a major increase in the amplitude of the fastest phase of Q_A^- re-oxidation (Table 2). The half-times of the two fast phases were 309 μs and 7 ms, and they accounted for 79 and 5% of the decay. The slow phase of half-time > 100 ms accounted for 16% of the decay (Table 2).

Factors affecting the extent of the FR-induced decrease in F_o

The capacity of FR light to induce changes in F_o varied with leaf Chl concentration. Control leaves (approximately 30 nmol Chl cm^{-2}) showed net increases in F_o upon FR illumination (Figure 5). Moderately deficient leaves (approximately 13 nmol Chl cm^{-2}) showed minor decreases in F_o, whereas leaves with marked chlorosis (4 nmol Chl cm^{-2}) exhibited the largest decreases. Extremely deficient leaves (2 nmol Chl cm^{-2}) showed decreases in F_o not as large as those found in Fe-deficient leaves with 4 nmol Chl cm^{-2}.

We have also found that the capacity of FR light to induce changes in F_o was also dependent on the time plants had been exposed to the growth chamber light (Figure 6). Leaves from Fe-deficient plants (3 nmol Chl cm^{-2}) dark-adapted overnight (point at zero time in Figure 6) showed only small decreases in F_o when treated with FR. Leaves exposed to the light for 1–2 h also showed small decreases in F_o when illuminated with FR light, whereas plants exposed for longer periods of time exhibited this effect to a larger extent. This effect appeared to saturate at approximately 5 h of illumination.

A third factor affecting the F_o level in Fe-deficient plants was the time plants had been in the dark. The F_o level decreased in an almost linear trend from 0 to 7 h of darkness (Figure 7).

The intensity of FR light needed for producing maximal decreases in F_o was approximately 1 μmol photons m^{-2} s^{-1} (Figure 8). It is necessary to point out that this intensity is higher than that produced by commercial modulated fluorescence systems that include a FR light source (such as the PAM-2000 system), even when the fiber optics is located close to the leaf surface.

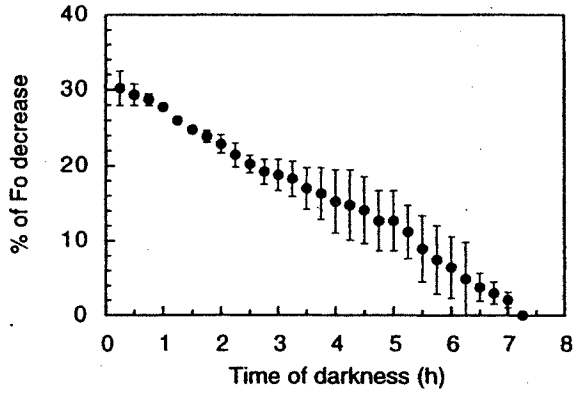


Figure 7. Time-course of the changes in F_o in attached leaves of Fe-deficient sugar beet plants (4 nmol Chl cm^{-2}). Plants were in the dark in the growth chamber for the time indicated in the X-axis after a 5 h pre-illumination period. Data shown are the mean ± SE of 2 replicates.

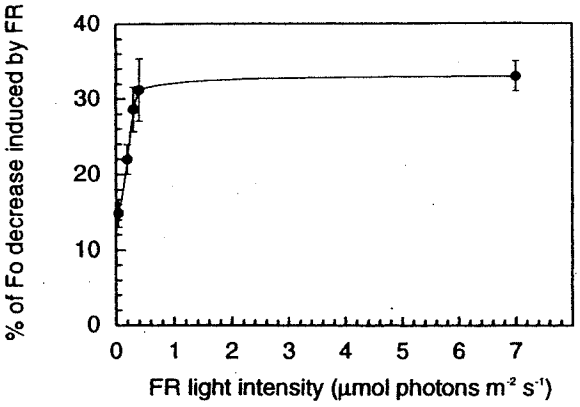


Figure 8. Decreases in F_o induced by different FR intensities in attached leaves of Fe-deficient sugar beet plants (4 nmol Chl cm^{-2}). Plants were pre-illuminated in the growth chamber for 5 h before being treated with FR for 6 min. Data shown are the mean ± SE of 3 replicates.

Oxygen and FR effect

The process reducing PQ in the dark was suppressed partially by flushing the leaf with pure O_2 and completely by removing the leaf epidermis or making small holes in the leaf with dissection needles (data not shown). This effect could be ascribed to the re-oxidation of reductants in the leaf.

Pyridine nucleotide pools in Fe-deficient and control plants

We have measured the leaf pyridine nucleotide pools, and found that at steady state photosynthesis after 5 h of illumination, when the FR effect was maximum, the ratio NADPH/$NADP^+$ increased by 14% in Fe-deficient leaves, when compared to the controls (Table 3). Arulanantham et al. (1990) gave similar values for the control NADP(H) pools in sugar beet leaves. It is clear that the changes in nucleotide pool sizes in response to Fe-deficiency cannot explain *per se* FR effects on Chl fluorescence.

FR-induced decrease in F_o in other species

Far-red light was also able to decrease the apparent F_o in leaves from other Fe-deficient plants. Leaves from pear and peach trees growing in the field showed similar results to those obtained with sugar beet (Table 4). Iron-deficient *Chlamydomonas reinhardtii* cells grown in the laboratory also showed decreases in F_o with FR illumination (Table 4).

Discussion

Iron deficiency is currently believed to induce decreases in PS II photochemical efficiency. This was concluded from the markedly increased F_o and F_{pl} levels in the Chl fluorescence induction curves from Fe-deficient dark-adapted photosynthetic materials, such as higher plants (Morales et al. 1991), cyanobacteria (Guikema 1985) and eukaryotic marine algae (Greene et al. 1992; Falkowski et al. 1995). Slow fluorescence decays were also found in Fe-deficient, eukaryotic marine algae, and interpreted as reflecting a 'sluggish electron transport between Q_A and PQ' (Greene et al. 1992). All these interpretations were based on the apparent similarity of the fluorescence induction curves and decays in Fe-deficient materials with those found in other cases where PS II electron transport is known to be impaired. For instance, similar relative increases in F_{pl} and slow fluorescence decays have been found in herbicide-resistant genotypes (Arntzen et al. 1982; Erickson et al. 1989). However, CO_2 exchange data suggest that Fe-deficiency had little effect on the photochemical efficiency of sugar beet, at least for leaf Chl concentrations above 10 nmol cm^{-2} (Terry 1980).

Our data indicate that the PS II photochemical efficiency of Fe-deficient leaves could be largely underestimated when using Chl fluorescence. A large part of the fluorescence changes previously attributed to low photochemical efficiency are actually caused by the reduction of the pool of electron carriers in the PS II acceptor side that occurs in the leaves of Fe-deficient plants during dark adaptation, i.e. the routine protocol used in fluorescence experiments to avoid

Table 3. Steady-state leaf pyridine nucleotide concentrations (in μmol m^{-2}) in 8 h dark-adapted and 5 h illuminated control and Fe-deficient sugar beet leaves. Leaf Chl concentration was approximately 35 and 4 nmol cm^{-2} in control and Fe-deficient plants, respectively. Data are the mean ± SE of 3 replicates

	NADPH	$NADP^+$	NADH	NAD^+	NADPH/$NADP^+$
Fe-sufficient					
Dark	2.07 ± 0.54	3.56 ± 0.62	0.90 ± 0.20	3.21 ± 0.63	0.58
Light	2.56 ± 0.44	5.23 ± 0.46	1.83 ± 0.32	2.91 ± 0.33	0.49
Fe-deficient					
Dark	2.40 ± 0.58	3.32 ± 1.00	1.10 ± 0.20	3.19 ± 0.70	0.72
Light	1.10 ± 0.17	1.97 ± 0.25	1.24 ± 0.28	2.59 ± 0.30	0.56

Table 4. Extent of the decrease in F_o induced by FR in leaves of several Fe-deficient plant species and in Fe-deficient *Chlamydomonas reinhardtii*. Data shown are the mean ± SE of at least 3 replicates

Species	% of F_o decrease induced by FR
Pear (*Pyrus communis* L.)	
Fe-sufficient (40 nmol Chl cm^{-2})	5 ± 1%
Fe-deficient (5 nmol Chl cm^{-2})	15 ± 3%
Peach (*Prunus persica* L.)	
Fe-sufficient (35 nmol Chl cm^{-2})	2 ± 2%
Fe-deficient (6 nmol Chl cm^{-2})	11 ± 1%
Chlamydomonas reinhardtii	
Fe-sufficient (30 μmol Chl ml^{-1})	−1 ± 1%
Fe-deficient (5 μmol Chl ml^{-1})	30 ± 3%

several types of fluorescence quenching caused by pre-illumination (Schreiber et al. 1995). This process may occur also in the light but cannot be observed except in darkness. FR pre-illumination, exciting preferentially PS I, decreased the high F_o and F_{pl} levels in the fluorescence induction curves from dark-adapted Fe-deficient leaves. Modulated fluorescence techniques indicated that FR pretreatment caused the fluorescence yield to decline to a true F_o level by oxidizing Q_A. Furthermore, the variable fluorescence decays became markedly faster in Fe-deficient leaves after FR illumination. Overall, these data indicate that the primary quinone electron acceptor of PS II, Q_A, was reduced in dark-adapted leaves from Fe-deficient plants. This implies, since Q_A is in quasi-equilibrium with Q_B and PQ, that the PQ pool should be also partially reduced. Since an estimation of the fraction of PS II centers closed can be made from the ratio $(F_s - F'_o)/(F'_m - F'_o)$ (Björkman and Demmig 1994) we have used the ratio $(F_o - F'_o)/(F_m - F'_o)$ in an analogous way to estimate the degree of PS II center closure in the dark. This ratio indicates that between 20 and 40% of the centers in dark-adapted, Fe-deficient leaves were in the closed state. This degree of closure (Groom et al. 1993) would indicate that a large part (more than 80%) of the PQ pool was actually reduced in the dark in these leaves.

Our work indicate that the reduction of the PQ pool following a light to dark transition is a mechanism likely to occur in large crop areas in the world affected by Fe deficiency. This mechanism occurs both in growth chamber- and field-grown plant species and in algae grown in solution culture. Dark reduction of PQ has been previously reported to occur in leaves of

several C_4 species but only in one of five C_3 species tested (*Helianthus annuus*) (Groom et al. 1993). The process causing the dark reduction of the PQ pool causes the underestimation of the true photochemical efficiency and the capacity for electron transport after Q_A, when using Chl fluorescence techniques to study dark-adapted Fe-deficient materials. Our data indicate that a relatively prolonged (at least 1 min) illumination with quite strong (at least 1 μmol photons m^{-2} s^{-1}) FR light must be carried out when studying Fe-deficient photosynthetic organisms with fluorescence techniques. The results obtained with this protocol agree with published CO_2 exchange data indicating that Fe deficiency has little effect on photochemical efficiency (Terry 1980). On the other hand, the extent of the underestimation of the photochemical efficiency would depend on several factors, such as the Chl concentration, the time of the day measurements are carried out and the duration of the dark-adaptation period.

At this moment we can only speculate on the molecular basis of the mechanism causing the reduction of the thylakoid PQ pool in the dark. One possibility is chlororespiration, that may be enhanced in the light in Fe-deficient plants, in turn depleting the O_2 level in the stroma, and that may be subsequently inhibited upon darkening. Pyridine nucleotides are likely the stromal electron donors to the intersystem chain, derived from malate oxidation, by malate dehydrogenase, or via triose phosphates, by glyceraldehyde-phosphate dehydrogenase (Asada et al. 1993). In fact, NADPH is able to reduce PQ in isolated thylakoids (Groom et al. 1993; Harris and Heber 1993). An alternative electron donor is reduced Fd. NADPH is an allosteric inhibitor of the Fd/NADP reductase, permitting electrons to accumulate at the level of Fd, that in turn can donate electrons to PQ, even if the PQ pool is largely reduced (Heber and Walker 1992).

Several lines of evidence suggest that these processes may occur in Fe deficiency. First, leaf malate concentrations have been shown to increase in response to Fe deficiency (Welkie and Miller 1993). An increased chlororespiration was found in low Chl, N-starved *Chlamydomonas* (Peltier and Schmidt 1991). The quantum yield of net O_2 evolution was previously shown to be markedly decreased in severely Fe-deficient plants, and that was attributed to an unidentified process consuming O_2 (Morales et al. 1991). An increase in the O_2 concentration, obtained either by increasing the external O_2 supply or by perforating the leaf, supressed the PQ reducing process in Fe-deficient leaves, possibly by oxidizing NADPH and/or reduced Fd. The PQ-reducing process in Fe-deficient leaves is likely to involve an electron donor close to the PS I acceptor side, because many consecutive cycles FR-induced decrease in F_o/dark-induced increase in F_o could be repeated, each FR treatment possibly regenerating the pool of reductants at the PS I acceptor side. Another process that may affect the redox state of the Fe-deficient chloroplasts is the possible impairment of NO_2^- reduction, which uses reduced Fd. This can be mediated by low cytosolic nitrate reductase activity (Marschner 1986) or by a decrease in the activity of the Fe-containing, chloroplastic nitrite reductase enzyme.

The data presented here are aimed towards understanding what happens in the Fe-deficient leaves in the dark and how this affects fluorescence measurements. From the data presented here is not possible to infer valid conclusions on the physiological status of Fe-deficient, illuminated plants. However, the data indicate that the rate of PQ dark-reduction is much lower than the rate of PQ reduction by photosynthetic processes. In *Amaranthus* the rates of dark PQ reduction and PQ photosynthetic reduction at saturating light were estimated in 0.2 and 250 meq mol Chl^{-1} s^{-1}, respectively (Groom et al. 1993, calculated from an active PQ pool size of 16 meq mol Chl^{-1}). The pool size of PQ in Fe-deficient sugar beet leaves has not been measured yet, but any reasonable value for the PQ pool size (in the range 10–200 meq mol Chl^{-1}), along with the observed reduction times (longer than 200 s, Figure 2) suggest that the rate of dark PQ reduction would be much lower (0.1–1.0 meq mol Chl^{-1} s^{-1}) than the rate of PQ photosynthetic reduction (200 meq mol Chl^{-1} s^{-1} at saturating light; Terry 1983).

In summary, we have shown that FR illumination of Fe-deficient leaves: i) leads to decreases in the F_o and F_{pl} levels in the fluorescence induction curves; ii) causes the modulated fluorescence yield to decline to true F_o levels; and iii) induces in Fe-deficient leaves a faster fluorescence decay. All these data indicate that in Fe-deficient leaves the PQ pool becomes reduced in the dark. The extent of the PQ reduction depends on the degree of Fe chlorosis and on the duration of pre-illumination and dark-adaptation. The existence of this process makes appropriate the re-evaluation of any fluorescence data obtained previously from Fe-deficient plant materials.

Acknowledgements

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References

Abadía J and Abadía A (1993) Iron and plant pigments. In: Barton LL and Hemming L (eds) Iron Chelation in Plants and Soil Microorganisms, pp 327–343. Academic Press, New York

Arntzen CJ, Pfister K and Steinback KE (1982) The mechanism of chloroplast triazine resistance: Alterations in the site of herbicide action. In: Le Baron H and Gressel J (eds) Herbicide Resistance in Plants, pp 185–214. John Wiley & Sons, New York

Arulanantham AR, Rao IM and Terry N (1990) Limiting factors in photosynthesis. VI. Regeneration of ribulose 1,5-bisphosphatase limits photosynthesis at low photochemical capacity. Plant Physiol 93: 1466–1475

Asada K, Heber U and Schreiber U (1993) Electron flow to the intersystem chain from stromal components and cyclic electron flow in maize chloroplasts, as detected in intact leaves by monitoring redox change of P700 and chlorophyll fluorescence. Plant Cell Physiol 34: 39–50

Behrenfeld MJ, Bale AJ, Kolber ZS, Aiken J and Falkowski PG (1996) Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. Nature 383: 508–511

Björkman O and Demmig-Adams B (1994) Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves. In: Schulze E-D and Caldwell MM (eds) Ecophysiology of Photosynthesis, pp 17–47. Springer-Verlag, New York

Cao J and Govindjee (1990) Chlorophyll *a* fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes. Biochim Biophys Acta 1015: 180–188

Delepelaire P and Wollman FA (1985) Correlations between fluorescence and phosphorylation changes in thylakoid membranes of *Chlamydomonas reinhardtii in vivo*: A kinetic analysis. Biochim Biophys Acta 809: 277–283

Erickson JM, Pfister K, Rahire M, Togasaki RK, Mets L and Rochaix J-D (1989) Molecular and biophysical analysis of herbicide-resistant mutants of *Chlamydomonas reinhardtii*: Structure-function relationship of the Photosystem II D1 polypeptide. Plant Cell 1: 361–371

Falkowski P, Behrenfeld M and Kolber Z (1995) Variations in photochemical energy conversion efficiency in oceanic phytoplankton: scaling from reaction center to the global ocean. In: Mathis P (eds) Photosynthesis: From Light to Biosphere, Vol V, pp 755–759. Kluwer Academic Publishers, Dordrecht, The Netherlands

Fork DC and Satoh K (1986) The control by state transitions of the distribution of excitation energy in photosynthesis. Ann Rev Plant Physiol 37: 335–361

Geider RJ and La Roche J (1994) The role of iron in phytoplankton photosynthesis, and the potential for iron-limitation of primary productivity in the sea. Photosynth Res 39: 275–301

Greene RM, Geider RJ, Kolber Z and Falkowski PG (1992) Iron-induced changes in light harvesting and photochemical energy conversion processes in eukaryotic marine algae. Plant Physiol 100: 565–575

Groom QJ, Kramer D, Crofts AR and Ort DR (1993) The non-photochemical reduction of plastoquinone in leaves. Photosynth Res 36: 205–215

Guikema JA (1985) Fluorescence induction characteristics of *Anacystis nidulans* during recovery from iron deficiency. J Plant Nutr 8: 891–908

Harris GC and Heber U (1993) Effects of anaerobiosis on chlorophyll fluorescence yield in spinach (*Spinacia oleracea*) leaf discs. Plant Physiol 101: 1169–1173

Heber U and Walker D (1992) Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiol 100: 1621–1626

Marschner H (1986) Mineral Nutrition of Higher Plants. Academic Press, London, UK

Matsumura H and Miyachi S (1980) Cycling assay for nicotinamide adenine dinucleotides. Meth Enzymol 69: 465–470

Melis A (1985) Functional properties of Photosystem II β in spinach chloroplasts. Biochim Biophys Acta 808: 334–342

Morales F, Abadía A and Abadía J (1990) Characterization of the xanthophyll cycle and other photosynthetic pigment changes induced by iron deficiency in sugar beet (*Beta vulgaris* L.). Plant Physiol 94: 607–613

Morales F, Abadía A and Abadía J (1991) Chlorophyll fluorescence and photon yield of oxygen evolution in iron-deficient sugar beet (*Beta vulgaris* L.) leaves. Plant Physiol 97: 886–893

Morales F, Abadía A, Belkhodja R and Abadía J (1994) Iron deficiency-induced changes in the photosynthetic pigment composition of field-grown pear (*Pyrus communis* L.) leaves. Plant Cell Environ 17: 1153–1160

Mortvedt JJ (1991) Correcting iron deficiencies in annual and perennial plants: Present technologies and future prospects. Plant Soil 130: 273–279

Peltier G and Schmidt GW (1991) Chlororespiration: An adaptation to nitrogen deficiency in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 88: 4791–4795

Schreiber U, Bilger W and Neubauer C (1995) Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of in vivo photosynthesis. In: Schulze E-D and Caldwell MM (eds) Ecophysiology of Photosynthesis, pp 17–47. Springer-Verlag, New York

Spiller S and Terry N (1980) Limiting factors in photosynthesis. II. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. Plant Physiol 65: 121–125

Strasser BJ (1997) Donor side capacity of Photosystem II probed by chlorophyll a fluorescence transients. Photosynth Res 52: 147–155

Straus NA (1994) Iron deprivation: Physiology and gene regulation. In: Bryant DA (eds) The Molecular Biology of Cyanobacteria, pp 731-750. Kluwer Academic Publishers, Dordrecht, The Netherlands

Terry N (1980) Limiting factors in photosynthesis. I. Use of iron stress to control photochemical capacity *in vivo*. Plant Physiol 65: 114–120

Terry N (1983) Limiting factors in photosynthesis. IV. Iron stress-mediated changes in light harvesting and electron transport capacity and its effects on photosynthesis *in vivo*. Plant Physiol 71: 855–860

Terry N and Abadía J (1986) Function of iron in chloroplasts. J Plant Nutr 9: 609–646

Welkie GW and Miller GW (1993) Plant iron uptake physiology by nonsiderophore systems. In: Barton LL and Hemming L (eds) Iron Chelation in Plants and Soil Microorganisms, pp. 345–370. Academic Press, New York

Young TF and Terry N (1982) Transport of iron into leaves following iron resupply to iron-stressed sugar beet plants. J Plant Nutr 5: 1273–1283

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Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet

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Abstract. The ferric chelate reductase activities of leaf plasma membranes isolated from the leaves of Fe-deficient and Fe-sufficient sugar beet have been characterized. Substrates used were the complexes of ferric iron with ethylene diamine tetraacetic acid, citric acid and malic acid. Iron deficiency was associated with 1.5- to 2-fold increases in leaf plasma membrane ferric chelate reductase activity when rates were calculated on a protein basis. The natural complexes of ferric iron with citrate and especially with malate were good substrates for the ferric chelate reductase enzyme present in leaf plasma membrane preparations. The apparent affinities were higher for the ferric malate complex. The optimal pH for the activity of the ferric chelate reductase in sugar beet leaf plasma membranes was in the range 6.5–7.0. The ferric chelate reductase activity decreased by approximately 30% when the assay pH was decreased to 5.8 or increased to 7.5. Therefore, our data provide evidence against the hypothesis that changes in apoplastic pH could decrease markedly the activity of the ferric chelate reductase enzyme in plasma membrane preparations from the leaves of Fe-deficient plants.

Introduction

The roots of dicotyledonous plants are able to reduce Fe(III), present in the root–soil interface, to Fe(II) (Chaney *et al.* 1972). The reduction of Fe(III) in the roots of Fe-deficient plants is carried out by one or several plasma membrane (PM)*-bound ferric chelate reductase (FC-R) enzyme(s) (Bienfait 1985; Cakmak *et al.* 1987; Bienfait 1988; Rubinstein and Luster 1993; Moog and Brüggemann 1994; Susín *et al.* 1996; Schmidt 1999). The increase in the root's capacity to reduce Fe(III) is considered an integral part of the so called Strategy I, that involves a number of mechanisms resulting in an improvement in Fe acquisition in Fe-deficient dicots and non-Poaceae monocots (Marschner *et al.* 1986; Welkie and Miller 1993; Marschner and Römheld 1994).

Iron uptake from the soil is not the only limiting step in Fe acquisition, because Fe must be transported from the root cell to the sites of utilization all over the plant. Since up to 80% of the leaf Fe is located in the chloroplast, to arrive at its final destination most of the Fe must cross several biological membrane systems.

The characteristics of this multi-step internal transport system are largely unknown. However, there is evidence in the literature suggesting that some steps in this internal transport system may be impaired by Fe deficiency itself. For instance, in many cases leaves from Fe-deficient plants may have total Fe concentrations similar to those found in leaves from Fe-sufficient plants (Abadía 1992; Morales *et al.* 1998). These high-Fe, low chlorophyll leaves regreen with Fe applications and also with treatments modifying the pH of the leaf apoplast (Tagliavini *et al.* 1999). This suggests that part of the Fe pool in Fe deficient leaves is immobilized in physiologically inactive forms. Therefore, more information is needed on the fine characteristics of the different steps in the Fe transport systems, and also on the changes induced in those systems by Fe deficiency.

Few data are available so far on how Fe enters the leaf cell. Iron is commonly assumed to be transported in the xylem in the ferric form and complexed with organic acids such as citrate (Tiffin 1970). Experiments made with leaf mesophyll disks of *Vigna unguiculata* have indicated the presence of a leaf FC-R able to use Fe(III)-EDTA and Fe(III)-citrate as

*Abbreviations used: BPDS, bathophenanthroline disulfonate; BSA, bovin serum albumin; Chl, chlorophyll; DTT, dithiothreitol; FC, ferric chelate; FC-R, ferric chelate reductase; FCN, ferricyanide; FCN-R, ferricyanide reductase; MOPS-BTP, morpholino-ethane-sulfonic-bis-tris-propane buffer; PM, plasma membrane; PMSF, phenylmethylsulphonyl fluoride; PPFD, photosynthetic photon flux density; PTA, phosphotungstic acid.

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substrates (Brüggemann *et al.* 1993). The FC-R activities of PM isolated from the leaves of Fe-sufficient *V. unguiculata* were also partially characterized by Brüggemann *et al.* (1993). Later, the presence of a leaf mesophyll FC-R, reducing Fe(III)-EDTA, was confirmed in sunflower leaf disks (de la Guardia and Alcántara 1996).

In this paper we have studied the characteristics of the FC-R activities of purified PM preparations from Fe-sufficient and deficient sugar beet. As substrates we have used Fe(III)-citrate and Fe(III)-malate, two of the most likely organic anion Fe carriers in the xylem, and Fe(III)-EDTA, to make possible a comparison of the enzymatic characteristics of the leaf PM FC-R with those previously reported for the root PM FC-R of the same plant species (Susín *et al.* 1996).

Materials and methods

Plant material

Sugar beets (*Beta vulgaris* L. hybrid Monohil, from Hilleshög, Landskrona, Sweden) were grown in a growth chamber. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for 2 more weeks in a nutrient solution (3/8-Hoagland nutrient solution with 40 μM Fe) and then transplanted to 20-L buckets (four plants per bucket) containing half-Hoagland solution (Terry 1980) with 0 or 45 μM Fe. Iron was added as NaFe(III)-EDTA (Sigma). In the zero Fe treatment the pH of the nutrient solution was raised to 7.7 (with 1 mM NaOH and 1 g L^{-1} of solid $CaCO_3$) to simulate conditions usually found in the field that lead to Fe deficiency, whereas the nutrient solutions of the control plants were adjusted every 2 days to pH 5.5 (Susín *et al.* 1994). Plants were grown with a PPFD of 350–400 μmol m^{-2} s^{-1} at 25°C, 80% relative humidity and a photoperiod of 16 h light/8 h dark.

Chlorophyll (Chl) concentration was estimated non-destructively with an SPAD-502 device (Minolta Corp., Osaka, Japan). For calibration, leaves with different degrees of Fe deficiency were first measured with the SPAD and then extracted with 100% acetone and Chl measured spectrophotometrically (Abadía and Abadía 1993). Control leaves were chosen with Chl concentrations above 25 nmol cm^{-2}, whereas leaves from Fe-deficient plants were chosen with 7–10 nmol Chl cm^{-2}.

Plasma membrane isolation

Leaves were harvested from control and Fe-deficient plants. Vesicles enriched in PM were purified from a leaf microsomal fraction by differential centrifugation in an aqueous-polymer two-phase system (Widell and Larsson 1981; Larsson 1985: Larsson *et al.* 1988; Brüggemann *et al.* 1993). Leaves were cut into small pieces and homogenized using a mortar and pestle in a medium containing 330 mM sucrose, 1 mM dithiothreithol (DTT), 2 mM ascorbate, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 5% polyvinylpyrrolidone, 0.1% bovine serum albumin (BSA) and 20 μM leupeptin in 50 mM Hepes–KOH, pH 7.5. A ratio of 6 mL homogenization medium per g (fresh mass) of leaf tissue was used. The homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged at 10 000 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 50 000 *g* for 60 min to pellet the microsomal fraction.

The microsomal fraction (40–50 mg protein) was resuspended in 7.5 mL of a medium containing 330 mM sucrose, 5 mM K phosphate buffer, pH 7.8, 5 mM KCl and 6 mM DTT. This was layered over an aqueous-polymer two-phase system, to yield a 30-g system with a final concentration of 6.5% Dextran T500, 6.5% polyethylene glycol (PEG 3350), 5 mM KCl, 330 mM sucrose and 5 mM K phosphate, pH 7.8. The partitioning was carried out three times (Larsson 1985). The final upper PEG phase was diluted six-fold with a buffer containing 10 mM morpholino-ethane-sulfonic-bis-tris-propane buffer (MOPS-BTP), pH 7.0, 3 mM EDTA and 250 mM sucrose, and centrifuged at 50 000 *g* for 60 min. The resulting pellet was resuspended in the same medium supplemented with 0.5 mM PMSF and 20 μM leupeptin and stored in aliquots at –80°C.

The purity of the PM preparation obtained by aqueous-polymer two-phase partitioning was estimated from the enzymatic activities characteristic of PM, mitochondria inner membrane and Golgi apparatus. The activity of the PM-associated, vanadate sensitive and Mg^{2+} dependent ATPase was assayed by measuring the release of phosphate from ATP at pH 6.5 as in Widell and Larsson (1990). Inorganic phosphate was determined by the formation of a colored phosphomolybdic complex (Widell and Larsson 1990). The activity of cytochrome-*c* oxidase (Hodges and Leonard 1974) was used as a mitochondrial marker. Latent IDPase, a marker of Golgi apparatus, was measured as in Susín *et al.* (1996). Chlorophyll and carotenoids were estimated spectrophotometrically (Lichtenthaler 1987) and by HPLC (de las Rivas *et al.* 1989). Protein was determined by the method of Markwell *et al.* (1981).

Electron microscopy and morphometry

On-section staining with phosphotungstic acid (PTA) was used as a specific marker for PM (Roland 1978; Hall 1983; Morré 1990; Widell and Larsson 1990). Samples of microsomal fractions and purified PM were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde solution, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Thin sections were obtained, placed on nickel grids, incubated for 30 min in 1% periodic acid, and then stained with 1% PTA in 10% chromic acid. From this material at least 30 prints were taken for each treatment and the purity of the PM was estimated by planimetry as described elsewhere (Serrano *et al.* 1994).

Fe-reducing capacities

The ferric chelate reductase (FC-R) activity was monitored by measuring the formation of the Fe(II)-bathophenanthroline disulfonate (BPDS) complex (Bienfait *et al.* 1983). The FC-R activity of PM purified by two-phase partitioning was measured with 5–7 μg protein mL^{-1} in 15 mM MOPS-BTP, pH 6.8, 250 mM sucrose, 5 mM $MgCl_2$, 0.02% Triton X–100, 600 μM BPDS, 500 μM NADH and 500 μM Fe(III)-EDTA (Brüggemann and Moog 1989). The reaction was performed in the dark over a period of 10–15 min. Absorbance was measured at 535 nm and an extinction coefficient of 22.14 mM^{-1} cm^{-1} was used for the estimation of reduced Fe. With FCN absorbance was measured at 420 nm and an extinction coefficient of 1.02 mM^{-1} cm^{-1} was used. Measurements were also made in the absence of PM vesicles to correct for any non-specific Fe reduction. All measurements were performed at room temperature with a Shimadzu 2101 PC computer-controlled spectrophotometer using a 1-nm slit. In other experiments, pH was varied in the range 5.0–8.0 using 15 mM phosphate buffer (pH 5.0), 15 mM Mes–KOH (pH 5.5–6.5) or 15 mM MOPS-BTP (pH 7.0–8.0). To carry out kinetic analyses, different concentrations of Fe-chelate and NADH were used. Anaerobic assays were carried out in the same medium as the aerobic assays, with 42 U mL^{-1} glucose oxidase, 10 mM glucose and 50 μg mL^{-1} catalase (Brüggemann and Moog 1989).

The degree of reductase latency was determined by comparison of the enzyme activities in the presence and absence of detergent. The degree of latency was calculated by the equation: [(rate with detergent) – (rate without detergent)] × 100/(rate with detergent).

Estimation of the free ion activities in solution

The global speciation, including the free activities of the different ionic species in the solutions used for the assays, was estimated with the MINTEQ2A software (Lindsay 1979). The stability constants used for the Fe(III)-citrate complexes were 13.43, 20.18, 21.83, 31.63 and 47.94 for the species $(FeCit)^0$, $(FeCitH)^+$, $(FeCitOH)^-$, $(FeCit_2)^{-3}$ and $(Fe_2Cit_2OH_2)^{-2}$, respectively. The stability constants used for the Fe(II)-citrate complexes

were 9.90 for $(FeCitH)^0$ and 5.68 for $(FeCit)^-$. The dissociation constants used for citric acid were 6.39, 11.15 and 14.28 for $(CitH)^{2-}$, $(CitH_2)^-$ and $(CitH_3)^0$, respectively. Values were corrected for an ionic strength value of zero using the gamma coefficients obtained with the Davies equation (Allison *et al.* 1991). For malate, protonation and Fe(III) complexation was studied using the method proposed by Martell and Motekaitis (1992). A series of pH titrations were performed with malic acid (Merck, 10, 1 and 0.1 mM) or with different malate:Fe ($Fe(NO_3)^3$, Carlo Erba) ratios (1:1, 1:5, 1:10 and 1:25) using a 721 Net Titrino (Metrohn) automatic titrator with TiNET 2.1 software. Total Fe was also assessed by atomic absorption spectrometry. Stability constants were calculated using the programs PKAS and BEST (Martell and Motekaitis 1992) and extrapolated to zero ionic strength using the Davies equation. The possibility of Fe precipitation was not considered in the calculations because, in the actual experiments, precipitation was not observed. The calculations were performed for different constant pe+pH values and considering aerobic conditions.

Fe(III)-chelate preparation

Fe was added as NaFe(III)-EDTA (Sigma), Fe(III)-citrate and Fe(III)-malate. Fe(III)-citrate and Fe(III)-malate were prepared by dissolving $FeCl_3$ in different concentrations of the acid–KOH, at pH 6.0 and 6.5 for citric and malic acid, respectively, to give a final stock of 25 mM Fe at the acid : Fe ratio desired.

Results

Isolation and characterization of PM-enriched preparations

The purity of the PM preparations obtained with the two-phase partitioning method was tested with marker enzymes and electron microscopy. The leaf PM fraction contained 67–76% of the total specific vanadate-sensitive ATPase activity found in the two-phase system. The specific vanadate-sensitive ATPase activity was 2.5- and 2.6-fold higher in PM than in the dextran fraction in isolations from Fe-deficient and control plants, respectively (Table 1). The recoveries of this marker in the PM preparations were 37–45% of the total activities found in the microsomal fractions. The latency of the specific vanadate-sensitive ATPase activity was approximately 79 and 73% in leaf PM from Fe-deficient and control plants, respectively.

Chlorophyll was used as a thylakoid specific marker. Microsomal fractions had 42.6 (–Fe) and 48 (+Fe) μg Chl mg^{-1} total protein. This indicates, assuming thylakoid Chl:protein ratios (m/m) of approximately 0.08 and 0.12 in Fe-deficient and control leaves (Quílez 1994), that thylakoids accounted for 51 and 39% of the total microsomal fraction protein in Fe-deficient and control plants, respectively. Plasma membrane preparations had 0.055 (–Fe) and 0.017 (+Fe) μg Chl mg^{-1} total protein, which indicates that thylakoids accounted only for approximately 0.07 and 0.01% of the total PM protein in Fe-deficient and control plants, respectively.

Mitochondria are often as abundant as PM in the microsomal fractions (Widell and Larsson 1990). The specific activities of Cytochrome-*c* (Cyt-*c*) oxidase were 223 (+Fe) and 106 (–Fe) nmol min^{-1} mg^{-1} protein in microsomal fractions and 9.3 (+Fe) and 6.6 (–Fe) nmol min^{-1} mg^{-1} in plasma membrane preparations. Therefore, the specific activities of Cyt-*c* oxidase remaining in the PM fractions were only 4 and 6% of those found in the microsomal fractions from control and Fe-deficient plants, respectively. The recoveries of the Cyt-*c* oxidase activities in the PM were 0.6 and 1% of those found in the microsomal fractions. These data indicate that contamination by mitochondrial membranes in the PM was lower than 2–3%. The presence of Golgi apparatus in the PM was also significantly reduced in comparison with the microsomal fraction. IDPase activities were 81 (+Fe) and 45 (–Fe) nmol min^{-1} mg^{-1} protein in microsomal fractions and 31 (+Fe) and 21 (–Fe) nmol min^{-1} mg^{-1} in plasma membrane preparations. Therefore, the specific activities of IDPase remaining in the PM fractions were less than half of those found in the microsomal fractions. The recoveries of IDPase activities in the PM were 5.5 and 8.3% of those found in the microsomal fractions.

Furthermore, electron microscopy of the isolated fractions stained with PTA (Fig. 1) indicated that PM vesicles accounted for 95.2±1.9 and 94.3±1.8% of the total membranes in PM preparations from leaves of control and Fe-

Table 1. Protein recoveries and PM-associated activity during PM isolation

Data are the mean (± SE where indicated) of 12 experiments. ATPase activities are in nmol Pi min^{-1} mg^{-1} protein. The ratios of vanadate-sensitive ATPase activity in specific fractions to the ATPase activity of the micro- somal fractions are given in parentheses

	+Fe			–Fe		
	Protein		ATPase	Protein		ATPase
Fraction	μg $μL^{-1}$	mg	activity	μg $μL^{-1}$	mg	activity
Homogenate	7.4	2130	—	6.6	1880	—
First centrifugation pellet	17.1	246	—	7.6	109	—
Supernatant	6.0	1570	—	5.8	1520	—
Microsomal fraction	11.4	49	109 ± 17	12.2	48	134 ± 23
PM vesicles	7.5	7.0	286 ± 27 (2.6)	8.7	8.5	337 ± 49 (2.5)
First lower phase	12.4	25	56 ± 12 (0.5)	12.4	26	37 ± 18 (0.3)
Second lower phase	4.4	1.8	83 ± 23 (0.8)	4.6	2.1	65 ± 17 (0.5)

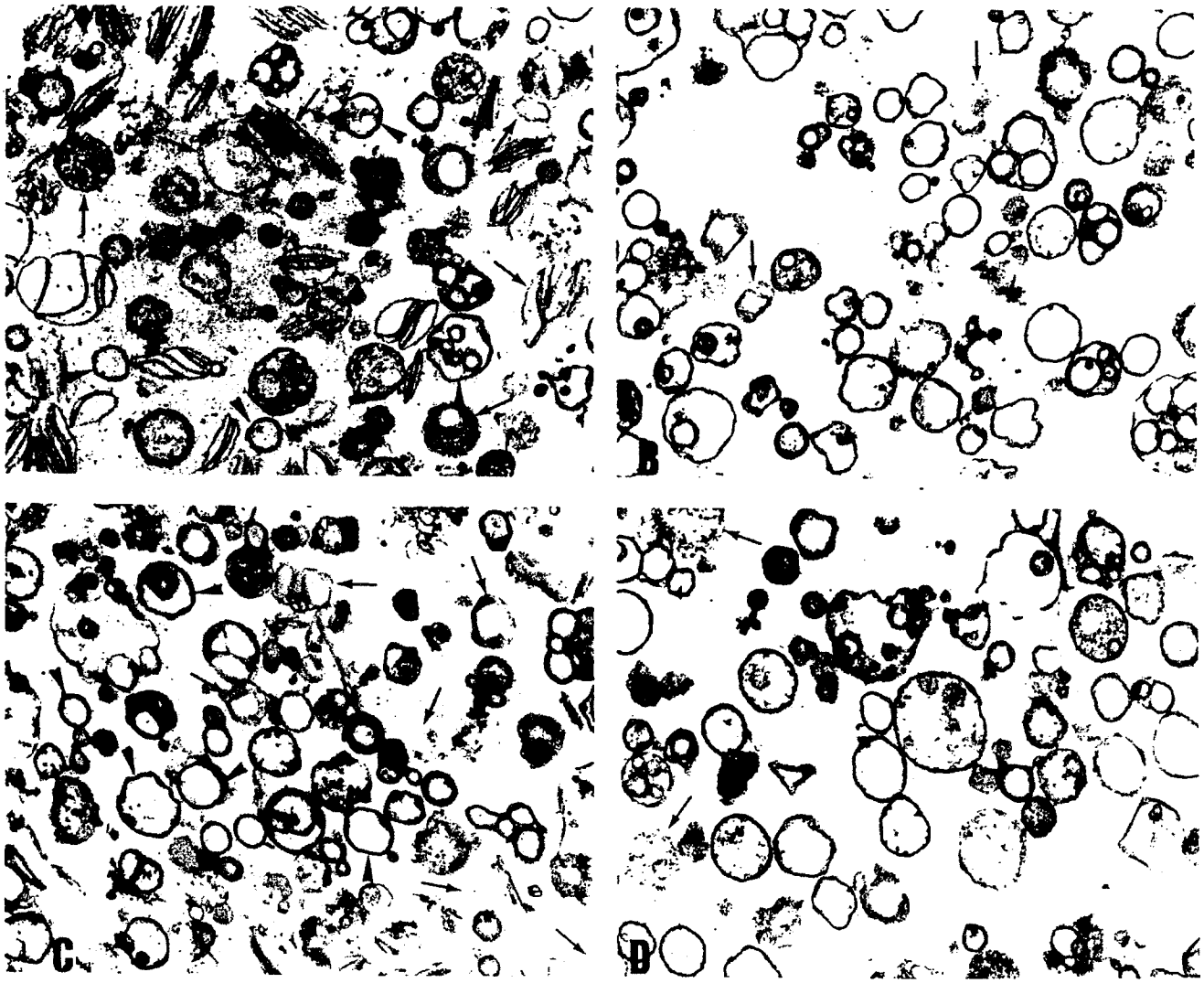

Fig 1. On-section staining of PTA of control (*A* and *B*) and Fe-deficient (*C* and *D*) plants. Microsomal fractions from control (*A*) and Fe-deficient plants (*C*) contain stained PM vesicles (arrowheads) and other non-stained membranes (arrows). Most vesicles in the purified PM fractions from control (*B*) and Fe-deficient plants (*D*) were stained with PTA, indicating their PM origin; only a minor portion of the vesicles in these preparations were unstained (arrows). All sections, × 35 000.

deficient plants, respectively. We conclude that the obtained PM preparations were of sufficient high purity to affirm that the characteristics of the FC-R activities presented in the paper are associated to PM and not to other membrane contaminants.

Enzymatic characteristics of the Fe(III)-EDTA reductase activity in PM

The Fe(III)-EDTA reductase activity was investigated in leaf PM preparations from Fe-sufficient and Fe-deficient plants. The optimal pH for this FC-R activity under aerobic conditions was approximately 6.8 in preparations from control and Fe-deficient leaves (Fig. 2*a*). When the assay was carried out under anaerobic conditions, FC-R activities were higher, confirming a previous report (González-Vallejo *et al.* 1998). The optimal pH for FC-R activity under anaerobic conditions was approximately 6.8 and 7.0 in PM from control and Fe-deficient leaves, respectively (Fig. 2*b*).

Saturation for Fe(III)-EDTA occurred at approximately 500 and 1000 μM in PM from Fe-deficient and control leaves (Fig. 3). The K_m values for Fe(III)-EDTA were not significantly different (103–122 μM) in PM from control and Fe-deficient leaves (Table 2). Iron deficiency induced a V_{max} increase of approximately 1.5-fold (Table 2). The kinetic analysis of the Eadie-Hofstee plots with varying concentrations of NADH (not shown) gave similar K_m values (157–161

μM) with PM from control and Fe-deficient leaves. Detection of optimal FC-R activity required the presence of 0.02% (w/v) Triton X–100; FC-R latencies were 90 and 83% in PM from control and Fe-deficient leaves, respectively, when NADH was used as the electron donor.

Enzymatic characteristics of the FCN-R activity in PM

The FCN reduction by Fe-sufficient plasma membranes with NADH as electron donor showed similar kinetics for the two types of PM preparations (not shown). The K_m values were 119 and 135 μM in PM from control and Fe-deficient leaves, respectively, and the V_{max} increased 1.1-fold with Fe deficiency (Table 2).

Table 2. Enzymatic characteristics of the ferric chelate reductase activities measured in PM vesicles

K_m values are in μM, and V_{max} values are in nmol Fe reduced min^{-1} mg^{-1} protein. The ratio –Fe/+Fe is given in parentheses. Data are mean ± SE of six replications

	K_m		V_{max}	
	+ Fe	– Fe	+ Fe	–Fe
Fe(III)-EDTA (pH 6.8)	103 ± 19	122 ± 19 (1.2)	114 ± 15	175 ± 19 (1.5)
KFeCN (pH 6.8)	119 ± 47	135 ± 1 (1.1)	1086 ± 286	1357 ± 7 (1.3)
Citrate: Fe, 1:5 (pH 6.8)	170 ± 14	157 ± 11 (0.9)	101 ± 2	142 ± 12 (1.4)
Malate: Fe, 1:25 (pH 6.8)	66 ± 3	73 ± 2 (1.1)	86 ± 11	144 ± 11 (1.7)

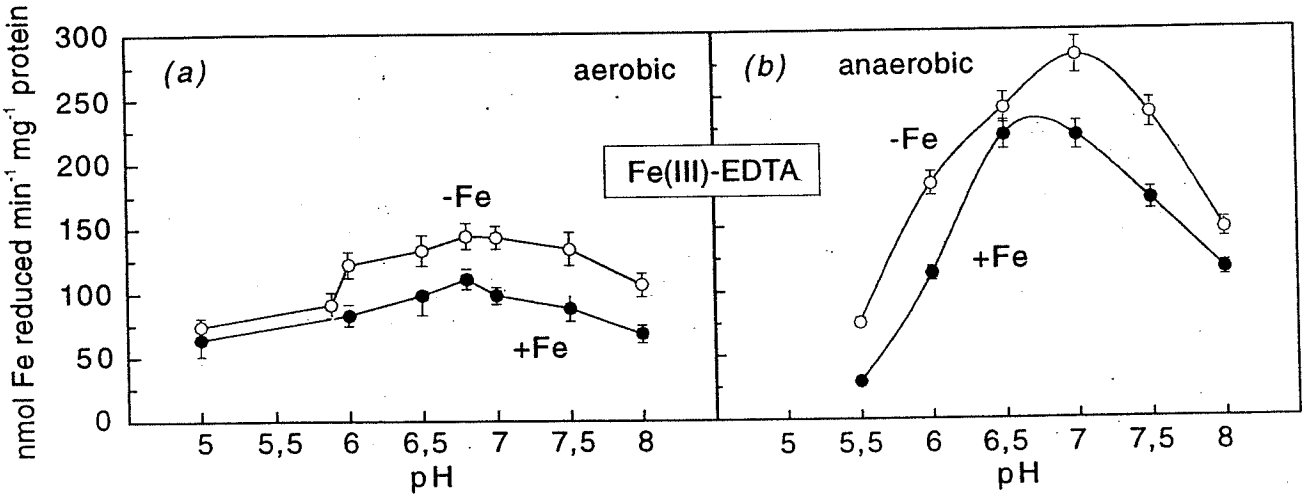

Fig. 2. pH-dependence of the FC-R activity with Fe(III)-EDTA in leaf PM from Fe-deficient and control plants (open and solid symbols, respectively). (*a*) aerobic conditions, (*b*), anaerobic conditions during the assay. Data are the mean ± SE of six replications.

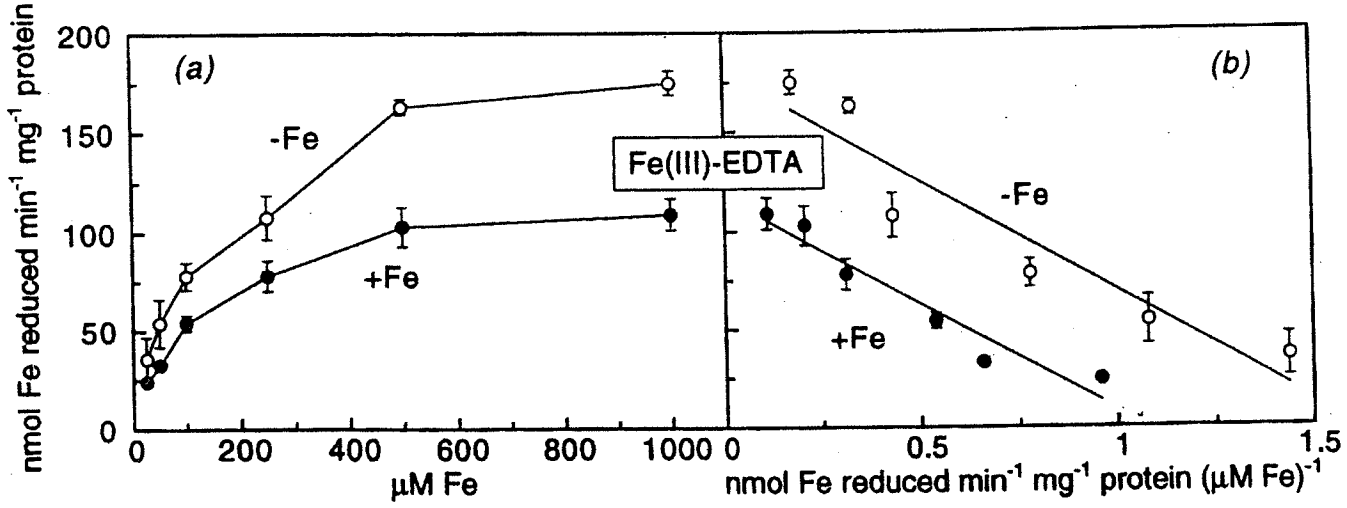

Fig. 3. Changes in leaf PM FC-R activity with the concentration of Fe(III)-EDTA at pH 6.8 (*a*) and corresponding Eadie–Hofstee plot (*b*). Data are the mean ± SE of six replications. Symbols as in Fig. 2.

Enzymatic characteristics of the Fe(III)-citrate reductase activity in PM

The optimal pH for the Fe(III)-citrate reductase activity measured under aerobic conditions was approximately 6.8 in PM preparations from control and Fe-deficient leaves (Fig. 4). The effect of the citrate:Fe ratio on the Fe(III)-citrate reductase activity was investigated at the optimum pH value of 6.8. The activity was markedly dependent on the citrate:Fe ratio used, being low with a 1:1 ratio, reaching a maximum at a 5:1 ratio and decreasing progressively at higher citrate:Fe ratios (Fig. 5*a*). The trend was similar in PM from control and Fe-deficient leaves. When the assay was carried out under anaerobic conditions, the activities showed slight increases (5–20%, depending on the citrate:Fe ratio) in both PM types, confirming a previous report (González-Vallejo *et al.* 1998). However, the dependence of the FC-R activity on the citrate:Fe ratio was similar to that found in aerobic conditions (Fig. 5*b*). The study was extended to a higher citrate:Fe ratio of 500, by using 100 μM Fe instead of the standard 500 μM Fe (at 500 μM Fe it was impossible to reach a 500:1 citrate:Fe ratio). The results (Fig. 5*a*, dashed lines) indicate that the activities continued to decrease when the citrate:Fe ratio increased from 150:1 to 500:1.

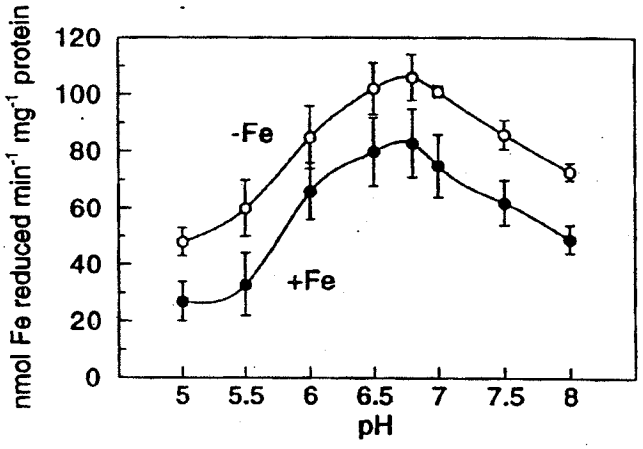

Fig. 4. pH-dependence of the FC-R activity with Fe(III)-citrate (citrate:Fe ratio of 5:1) in leaf PM. Data are the mean ± SE of six replications. Symbols as in Fig. 2.

For Fe(III)-citrate (at a 5:1 citrate:Fe ratio) as substrate, saturation occurred at approximately 500 and 1000 μM in PM from control and deficient leaves, respectively (Fig. 6). The K_m values for Fe(III)-citrate were similar in PM from control and Fe-deficient leaves (Table 3). Iron deficiency induced a V_{max} increase of approximately 1.4-fold.

Enzymatic characteristics of the Fe(III)-malate reductase activity in PM

The Fe(III)-malate reductase activity was investigated in PM preparations from Fe-sufficient and Fe-deficient leaves. The optimal pH for this FC-R activity measured under aerobic conditions was approximately 7.0 and 6.5 in preparations from control and Fe-deficient leaves (Fig. 7). A pH of 6.8 was used to investigate the effect of the malate:Fe ratio on the Fe(III)-malate reductase activity. This activity was lowest with a 2:1 malate:Fe ratio, and increased with higher ratios to reach a maximum at ratios of approximately 25:1 (Fig. 8). The trend was similar in PM from control and Fe-deficient leaves. When the assay was carried out under anaerobic conditions, the activities increased almost 2-fold in both PM types (González-Vallejo *et al.* 1998).

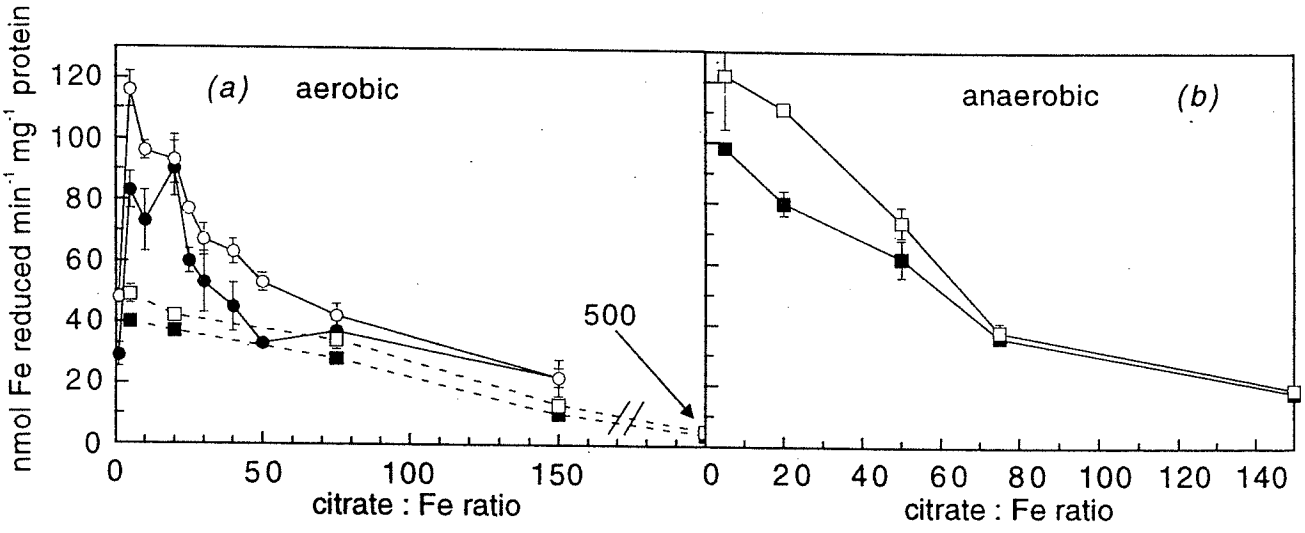

Fig. 5. Changes in leaf PM FC-R activity at different citrate:Fe ratios. (*a*) Aerobic conditions and (*b*) anaerobic conditions during the assay. Dashed lines in (*a*) are data obtained with 100 μM Fe(III)-citrate. Solid lines are data obtained with 500 μM Fe(III)-citrate. Data are the mean ± SE of six replications. Symbols as in Fig. 2.

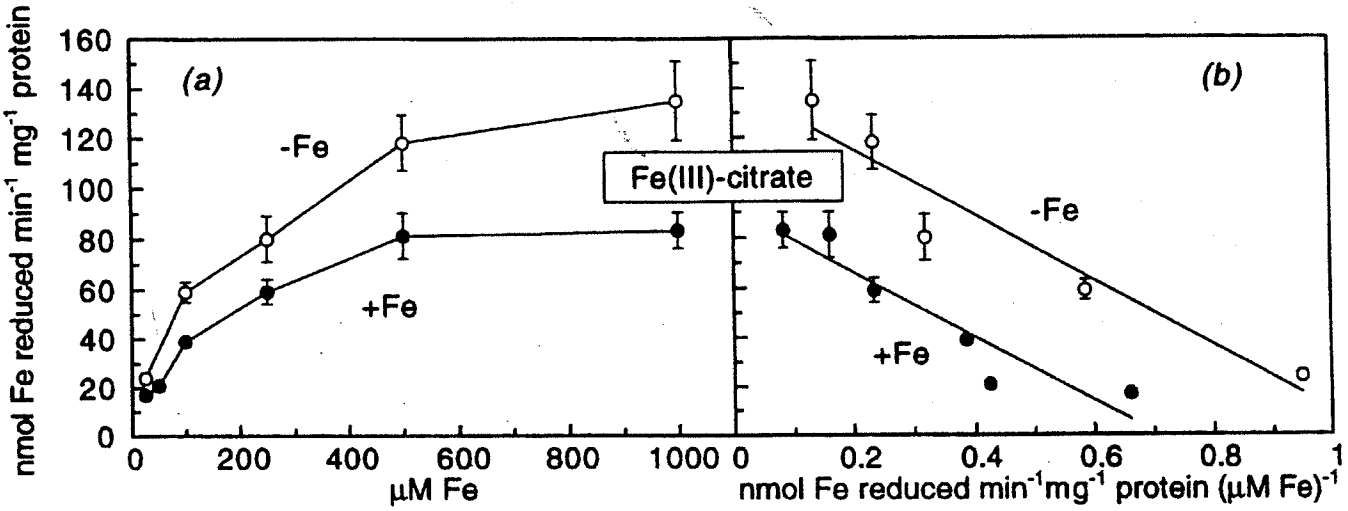

Fig. 6. Changes in leaf PM FC-R activity with the concentration of Fe(III)-citrate (citrate : Fe ratio of 5 : 1) at pH 6.8 (a) and corresponding Eadie–Hofstee plot (*b*). Data are the mean ± SE of six replications. Symbols as in Fig. 2.

For Fe(III)-malate (at a 25 : 1 malate : Fe ratio) as substrate, saturation occurred at approximately 1000 µM (Fig. 9). The K_m values for Fe(III)-malate were similar (66–73 µM) in PM from control and Fe-deficient leaves (Table 3). The increase in V_{max} induced by Fe deficiency was approximately 1.7-fold.

Chemical speciation of chelates

We have attempted to obtain information on the chemical species that may act as substrates for the FC-R by estimating the equilibrium activities of the different species with the MINTEQA2 software, in experiments at different pH values and different chelate : Fe ratios (Table 3). When using Fe(III)-EDTA the major chemical species was always $(FeEDTA)^-$, with some $(Fe(OH)EDTA)^{2-}$ being formed at high pH values (Table 3).

For citrate, the three main possible species were $(FeCit_2)^{3-}$, $(Fe_2OH2Cit_2)^{2-}$ and $(FeOHCit)^-$. At the pH values where the FC-R activity was minimal, the major species was $(FeCit_2)^{3-}$, although the activities of $(Fe_2OH_2Cit_2)^{2-}$ and $(FeOHCit)^-$ were also significant. At higher pH values the activity of the dimer $(Fe_2OH_2Cit_2)^{2-}$ was the largest, followed by those of $(FeOHCit)^-$ and $(FeCit_2)^{3-}$ (Table 3). When the citrate : Fe ratio increased, the estimated activities of $(Fe_2OH_2Cit_2)^{2-}$ and $(FeOHCit)^-$ decreased, whereas that of $(FeCit_2)^{3-}$ increased

Table 3. Equilibrium concentrations of the different chemical species in the conditions used for the FC-R assay

Concentrations were predicted using MINTEQA2 (pe + pH = 14), where pe = Eh/59.2. Eh is the redox potential in mV. Iron concentration was 500 µM. Data are given in µM

Chelate	Chemical species			
Fe(III)-EDTA	$(FeEDTA)^-$	$(Fe(OH)EDTA)^{2-}$	$(Fe(OH)_2EDTA)^{3-}$	All species
pH 5.5	498	1	0	499
pH 7.0	461	36	0.1	497
Fe(III)-citrate	$(Fe_2(OH)_2Cit_2)^{3-}$	$(FeOHCit)^-$	$(FeCit_2)^{3-}$	All species
citrate : Fe 5 : 1				
pH 5.5	88	54	271	413
pH 6.8	183	78	56	316
citrate : Fe 25 : 1				
pH 6.8	75	47	303	425
Fe(III)-malate	$(FeMal)^+$	$(FeOHMal)^0$		All species
malate : Fe 5 : 1				
pH 6.8	0.3	139		139
malate : Fe 25 : 1				
pH 6.8	0.8	308		309

(Table 3). From these data, the species whose activity follows more closely the changes in FC-R activity is $(Fe_2OH_2Cit_2)^{2-}$, whereas the estimated activity of $(FeCit_2)^{3-}$ was not correlated with the extent of reduction. However, it cannot be excluded that $(FeOHCit)^-$ could be the substrate for the FC-R. The very low FC-R activity found for a chelate: Fe ratio of 1:1 does not appear to be due to a low amount of $(Fe_2OH_2Cit_2)^{2-}$ (data not shown). This may be due to the formation of hydroxides when citrate is in short supply.

In the case of malate, the most abundant chemical species was $(FeOHMal)^0$, whereas the activity of $(FeMal)^+$ was always quite low. With high pH the estimated concentration of both species decreased (data not shown). The activities of $(FeMal)^+$ and $(FeOHMal)^0$ increased when the malate: Fe ratio increased (Table 3). The comparison of these data with the FC-R activities indicate that the only possible Fe(III)-malate chemical species that could act as a substrate for the FC-R in the conditions used in this study is $(FeOHMal)^0$.

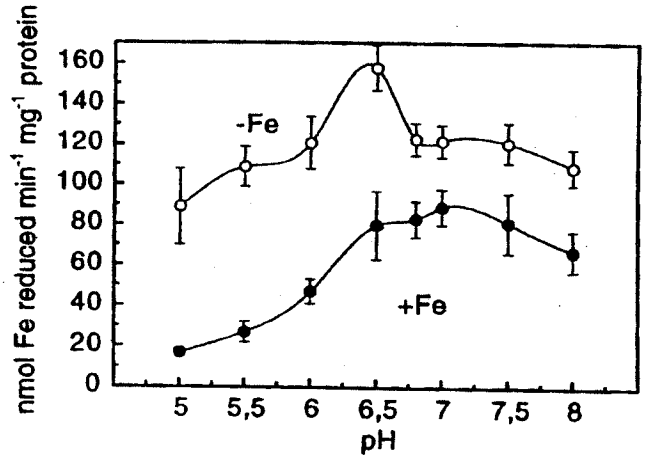

Fig. 7. pH-dependence of the FC-R activity with Fe(III)-malate (malate: Fe ratio of 25:1) in leaf PM. Data are the mean ± SE of six replications. Symbols as in Fig. 2.

Discussion

To our knowledge sugar beet is the first species where the FC-R activities of leaf PM isolated from Fe-deficient plants have been characterized. Studies published so far with other species have described only the FC-R characteristics of PM from Fe-sufficient leaves. These included studies with purified leaf PM from *V. unguiculata* (with Fe(III)-EDTA and Fe(III)-citrate; Brüggemann *et al.* 1993) and PM-enriched microsomes from kiwifruit (with Fe(III)-citrate and Fe(III)-malate; Rombolá *et al.* 1999). Some studies have also been

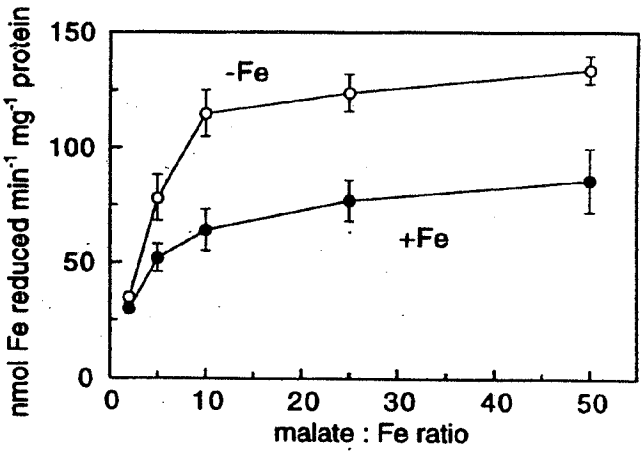

Fig. 8. Changes in leaf PM FC-R activity at different malate: Fe ratios. Data are the mean ± SE of six replications. Symbols as in Fig. 2.

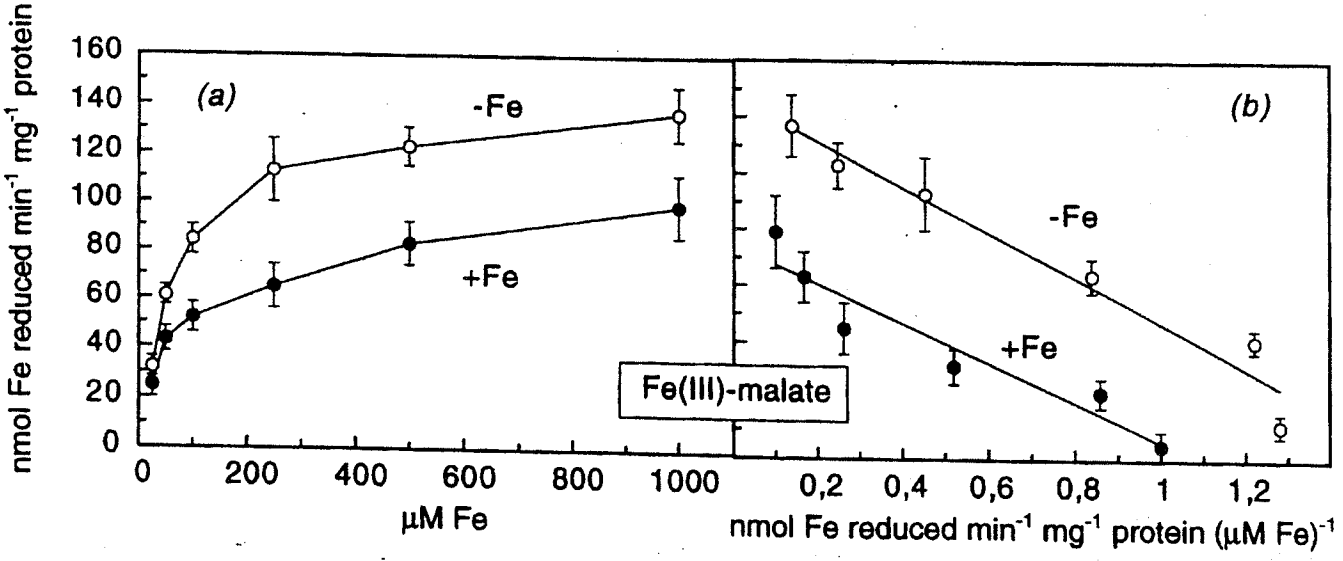

Fig. 9. Changes in leaf PM FC-R activity with the concentration of Fe(III)-malate (malate: Fe ratio of 25:1) at pH 6.8 (*a*) and corresponding Eadie–Hofstee plot (*b*). Data are the mean ± SE of six replications. Symbols as in Fig. 2.

done with Fe-sufficient spinach (Fe(III)-EDTA, Fe(III)-citrate and Fe(III)-malate; Moog, pers. comm.). In sugar beet, Fe-deficiency was associated with 1.5- to 2-fold increases in the V_{max} of the PM FC-R, on a protein basis, when compared to the Fe-sufficient controls. This increase is quite similar to the 2-fold increase found when comparing the FC-R activities of PM fractions purified from the roots of the same plants (Susín *et al.* 1996).

The natural organic anion complexes Fe(III)-citrate and especially Fe(III)-malate, despite their low stability, are good substrates for the FC-R enzyme present in leaf PM preparations. The affinities were in the order Fe(III)-malate > Fe(III)-EDTA > Fe(III)-citrate, with apparent K_m values of 66, 103 and 170 (Fe-sufficient) and 73, 122 and 157 (Fe-deficient), for Fe(III)-malate, Fe(III)-EDTA and Fe(III)-citrate, respectively. The K_m values found here are of the same order of magnitude as those reported for the PM FC-R activities of Fe-sufficient *V. unguiculata* leaves (K_m values of 97–137 and 173 μM for Fe(III)-citrate and Fe(III)-EDTA, respectively; Brüggemann *et al.* 1993), and PM enriched microsomes from Fe-sufficient kiwifruit leaves (K_m values of 96 and 399 μM for Fe(III)-malate and Fe(III)-citrate, respectively; Rombolá *et al.* 1999). The K_m values found are also in the same range than those found for PM obtained from the roots of sugar beet (Susín *et al.* 1996). In Fe-deficient and -sufficient sugar beet leaf PM the FC-R maximal rates followed the order Fe(III)-EDTA > Fe(III)-citrate ≥ Fe(III)-malate, although rates were not too different for the natural chelates and Fe(III)-EDTA. Rates found for Fe(III)-citrate and Fe(III)-malate were within the range 82–89% and 75–82%, respectively, of the rates with Fe(III)-EDTA.

Citrate has been long assumed to be a carrier for Fe(III) in the xylem (Tiffin 1970), although malate was initially thought to be involved in Fe transport (Tiffin and Brown 1962). Other unidentified anionic Fe(III) carrier forms may be also present in the xylem (Cataldo *et al.* 1988). Both citrate and malate are present in the mM range in xylem and apoplast of sugar beet (A. F. López-Millán, unpubl. data), and are therefore likely candidates as Fe(III) substrates for the FC-R.

Data shown in this paper confirm preliminary data indicating that anaerobiosis increases FC-R activity in isolated PM from sugar beet leaves (González-Vallejo *et al.* 1998). Furthermore, they show that the pH dependence of the PM FC-R activity is similar in anaerobic and aerobic conditions. Also, the increase in FC-R activity with anaerobiosis occurred in a similar way in PM from Fe-sufficient and Fe-deficient leaves, indicating that the mechanism by which anaerobiosis increases FC-R activity is not affected by Fe deficiency. Anaerobiosis has been reported to increase FC-R activities in PM from barley, tomato and *Vigna ungiculata* (Brüggemann and Moog 1989; Brüggemann *et al.* 1990; Brüggemann *et al.* 1993), as well as in other plant materials such as protoplasts (Macri *et al.* 1992) and leaf disks (de la Guardia and Alcántara 1996). Oxygen has been proposed as an acceptor for the ferricyanide reductase in PM systems (Döring *et al.* 1990; Moog and Brüggemann 1994) and also for the root FC-R enzyme induced by Fe deficiency (Cakmak *et al.* 1987).

The computer speciation data may provide information on the chemical species acting as substrates for the FC-R. In the case of Fe(III)-malate, a single major chemical species is predicted for the experimental conditions used, $(FeOHMal)^0$. This species was compatible with the FC-R rates in the experiment with different malate : Fe ratios. However, the pH dependence of the Fe(III)-malate reductase was not explained by the concentration of $(FeOHMal)^0$, and is likely to be due to true pH dependence of the enzyme.

In the case of citrate the data are complex and difficult to interprete. The dependence of the FC-R activities on the citrate : Fe ratios found here was quite different to that found by Holden *et al.* (1991) in PM from tomato roots. In the present study, the FC-R rates increased 2- or 3-fold from a citrate : Fe ratio of 1 : 1 to a 5 : 1 ratio, and there was a progressive and significant decrease in activity from the 5 : 1 to a 50 : 1 ratio. From the three Fe(III)-citrate species, $(Fe_2OH_2Cit_2)^{2-}$ and $(FeOHCit)^-$ seem possible substrates for the FC-R enzyme under the conditions used in our experiments. The relative concentration of the species $(FeOHCit)^-$ and that of its dimer $(Fe_2OH_2Cit_2)^{2-}$ depend mainly on the total citrate concentration, the former species being less abundant when total citrate is high. Our data suggest that the significant decrease in FC-R activity found at high citrate : Fe ratios is likely to be due to the increase found for the $(FeCit_2)^{3-}$ species. Two other factors that have been mentioned as possible reasons for low FC-R values at high citrate : Fe ratios are citrate-reductase competition for Fe(III) and/or citrate-BPDS competition for Fe(II), and subsequent reoxidation of Fe(II) to Fe(III) (Holden *et al.* 1991). The competition between citrate and reductase was already taken into account in our speciation calculations by considering a constant pe+pH value, which simulates the existence of a non-restricted electron donor. Also, from the stability constants reported in the literature it is unlikely that citrate can compete with BPDS for Fe(II).

The activity of the leaf PM FC-R enzyme has been suggested to be strictly regulated by pH (Mengel 1995). If this hypothesis holds true, an increase in apoplastic pH caused by Fe deficiency could inactivate the FC-R enzyme, in turn causing the accumulation of Fe in the apoplast of the Fe-deficient leaves (Mengel 1995; Kosegarten *et al.* 1998). The optimal pH values found for the FC-R in sugar beet leaf PM in this work were in the range 6.5–7.0 (6.8–7.0, 6.8–6.8 and 6.5–7.0 for Fe(III)-EDTA, Fe(III)-citrate and Fe(III)-malate, respectively). These pH optimal values are similar to those found previously for PM from Fe-sufficient leaves of *V.*

unguiculata (pH optimum 6.5–6.8 for Fe(III)-citrate) (Brüggemann *et al.* 1993), and somewhat higher than those found for the FC-R in PM enriched microsomes from Fe-sufficient leaves of kiwifruit (6.5 and 6.0–6.2 for Fe(III)-citrate and Fe(III)-malate, respectively) (Rombolá *et al.* 1999). The optimum pH range for the sugar beet leaf PM FC-R is higher than that found in the apoplast of the same plant, which is within the range 5.8–6.2 (A.F. López-Millán, unpubl. data). Our data indicate that changes in pH within the range 5.8–7.5 would cause only moderate (<30%) changes in FC-R activity with Fe(III)-citrate and Fe(III)-malate. Marked decreases in FC-R (>50% of the maximum) were found only at pH values outside the 5.5–8.0 range. This may suggest that strict modulation of the FC-R enzyme by pH is unlikely to occur in sugar beet leaf PM. It has been recently reported that part of the total FC-R activity obtained *in vitro* with root PM membranes treated with Triton X-100 may be due to a *cis*-type electron transport activity (both the donor and acceptor site located in the cytoplasmic side of the membrane) (Schmidt and Bartels 1998). Therefore, the pH dependence of FC-R measured *in vitro* may or may not correlate with the true *in vivo* pH dependence. Further experiments using isolated protoplasts are in progress to investigate the pH dependence of the FC-R enzyme.

Conclusions

In summary, we have characterized the FC-R activities of leaf PM isolated from Fe-deficient and Fe-sufficient sugar beet. Iron deficiency was associated with 1.5- to 2-fold increases in PM FC-R activity when rates were calculated on a protein basis. We found that the natural complexes Fe(III)-citrate and especially Fe(III)-malate are good substrates for the FC-R enzyme present in leaf PM preparations, with apparent K_m values being lowest for Fe(III)-malate. The optimal pH for the activity of the FC-R in sugar beet leaf PM was always within the range 6.5–7.0.

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References

Abadía, J. (1992). Leaf responses to Fe deficiency: a review. *Journal of Plant Nutrition* **15**, 1699–1713.

Abadía, J., and Abadía, A. (1993). Iron and plant pigments. In 'Iron Chelation in Plants and Soil Microorganisms'. (Eds L.L. Barton and B.C. Hemming.) pp. 327–343. (Academic Press: New York.)

Allison, J.D., Brown, D.S., and Novo-Gradak, K.J. (1991). A geochemical assessment model for environmental systems v. 3.0. Washington DC, Environmental Research Laboratory, United States Environmental Protection Agency.

Bienfait, H.F., Bino, R.J., van der Bliek, A.M., Duivenvoorden, J.F., and Fontaine, J.M. (1983). Characterization of ferric reducing activity in roots of Fe-deficient *Phaseolus vulgaris*. *Physiologia Plantarum* **59**, 196–202.

Bienfait, H.F. (1985). Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. *Journal of Bioenergetics and Biomembranes* **17**, 73–83.

Bienfait, H.F. (1988). Mechanisms in Fe-efficiency reactions of higher plants. *Journal of Plant Nutrition* **11**, 605–629.

Brüggemann, W., and Moog, P.R. (1989). NADH-dependent Fe^{3+}EDTA and oxygen reduction by plasma membrane vesicles from barley roots. *Physiologia Plantarum* **75**, 245–254.

Brüggemann, W., Moog P.R., Nakagawa, H., Janiesch, P., and Kuiper, P.J.C. (1990). Plasma membrane-bound NADH: Fe3+-EDTA reductase and iron deficiency in tomato (*Lycopersicum esculentum*). Is there a turbo reductase? *Physiologia Plantarum* **79**, 339–346.

Brüggemann, W., Maas-Kantel K., and Moog P.R. (1993). Iron uptake by leaf mesophyll cells: the role of the plasma membrane-bound ferric-chelate reductase. *Planta* **190**, 151–155.

Cakmak, I., van de Wetering, D.A.M., Marschner, H., and Bienfait, H.F. (1987). Involvement of superoxide radical in extracellular ferric reduction by iron-deficient bean roots. *Plant Physiology* **85**, 310–314.

Cataldo, D.A., McFadden, K.M., Garland, T.R., and Wildung, R.E. (1988). Organic constituents and complexation of Nickel(II), Iron(III), Cadmium(II), and Plutonium(IV) in soybean xylem exudates. *Plant Physiology* **86**, 734–739.

Chaney, R.L., Brown, J.C., and Tiffin, L.O. (1972). Obligatory reduction of ferric chelates in iron uptake by soybeans. *Plant Physiology* **50**, 208–213.

Döring O., Lüthje S., Hilgendorf F., and Böttger M. (1990). Membrane depolarization by hexacyanoferrate (III), hexabromo-iridate (IV) and hexacloro-iridate (IV). *Journal of Experimental Botany* **41**, 1055–1061.

González-Vallejo, E.B., Susín, S., Abadía, A., and Abadía, J. (1998). Changes in sugar beet leaf plasma membrane Fe(III)-chelate reductase activities mediated by Fe deficiency, assay buffer composition, anaerobiosis and the presence of flavins. *Protoplasma* **205**, 163–168.

de la Guardia, M.D., and Alcántara, E. (1996). Ferric chelate reduction by sunflower (*Helianthus annuus* L.) leaves: influence of light, oxygen, iron deficiency and leaf age. *Journal of Experimental Botany* **47**, 669–675.

Hall, J.L. (1983). Plasma membranes. In 'Isolation of Membranes and Organelles from Plant Cells'. (Eds J.L. Hall and A.L. Moore.) pp. 55–81. (Academic Press: London, UK.)

Hodges, T.K., and Leonard, R.T. (1974). Purification of a plasma membrane-bound adenosine triphosphate from plant roots. *Methods in Enzymology* **32**, 392–406.

Holden, M.J., Luster, D.G., Chaney, R.L., Buckhout, T.J., and Robinson, C. (1991). Fe^{3+}-chelate reductase activity of plasma membranes isolated from tomato (*Lycopersicon esculentum* Mill.) roots: comparison of enzymes from Fe-deficient and Fe-sufficient roots. *Plant Physiology* **97**, 537–544.

Kosegarten, H., Schwed, U., Wilson, G., and Mengel, K. (1998). Comparative investigation on the susceptibility of faba bean (*Vicia faba* L.) and sunflower (*Helianthus annuus* L.) to iron chlorosis. *Journal of Plant Nutrition* **21**, 1511–1528.

Larsson, C. (1985). Plasma membranes. In 'Modern Methods of Plant Analysis'. (Eds H.F. Linskens and J.F. Jackson.) Vol. 1, pp. 35–46. (Springer-Verlag: Berlin, Germany.)

Larsson, C., Widell, S., and Sommarin, M. (1988). Inside-out plant plasma membrane vesicles of high purity obtained by aqueous two-phase partitioning. *FEBS Letters* **229**, 289–292.

Lichtenthaler, H.K. (1987). Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. *Methods in Enzymology* **148**, 350–382.

Lindsay, W. L. (1979) 'Chemical Equilibria in Soils.' (Wiley-Interscience: New York.)

Macri, F., Braidot, E., Petrussa, E., Zancani, M., and Vianello A. (1992). Ferric ion and oxygen reduction at the surface of the protoplasts and cells of *Acer pseudoplatanus*. *Botanica Acta* **105**, 97–103.

Markwell, M., Haas, S.M., Tolbert, N.E., and Lieber, L.L. (1981). Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods in Enzymology* **72**, 296–303.

Marschner, H., Römheld, V., and Kissel, M. (1986). Different strategies in higher plants in mobilization and uptake of iron. *Journal of Plant Nutrition* **9**, 695–713.

Marschner, H., and Römheld, V. (1994). Strategies of plants for acquisition of iron. *Plant and Soil* **165**, 261–274.

Martell, A.E., and Motekaitis, R.J. (1992). 'Determination and Use of Stability Constants.' (VCH Pubs.: New York.) 200 pp.

Mengel, K. (1994). Iron availability in plant tissues-iron chlorosis on calcareous soils. *Plant and Soil* **165**, 275–283.

Moog, P.R., and Brüggemann, W. (1994). Iron reductase systems on the plant plasma membrane. A review. *Plant and Soil* **165**, 241–260.

Morales, F., Grasa, R., Abadía, A., and Abadía, J. (1998). Iron chlorosis paradox in fruit trees. *Journal of Plant Nutrition* **21**, 815–825.

Morré, D.J. (1990). Plasma membrane cytochemistry. In 'The Plant Plasma Membrane'. (Eds C. Larsson and I.M. Moller.) pp. 76–92. (Springer-Verlag: Berlin, Germany.)

Quílez, R. (1994). Changes Induced by Iron Deficiency and the Mutation LCM in the Organization of the Photosynthetic Apparatus of *Beta vulgaris*. PhD Thesis (in Spanish). University of Zaragoza, Spain.

Rivas, de las J., Abadía, A., and Abadía, J. (1989). A new reversed phase-HPLC method for resolving all major higher plant photosynthetic pigments. *Plant Physiology* **91**, 190–192.

Roland, J.C. (1978). General preparation and staining of thin sections. In 'Electron Microscopy and Cytochemistry of Plant Cells'. (Ed. J.L. Hall.) pp. 1–62. (Elsevier/Noth-Holland Biomedical Press: Amsterdam, The Netherlands.)

Rombolá, A.D., Brüggemann, W., Tagliavini, M., Marangoni, B., and Moog, P.R. (1999). Iron source affects Fe reduction and re-greening of kiwifruit (*Actinidia deliciosa*) leaves. *Journal of Plant Nutrition* (in press).

Rubinstein, B., and Luster, D.G. (1993). Plasma membrane redox activity: components and role in plant processes. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 131–155.

Schmidt, W., and Bartels, M. (1998). Orientation of the NADH-linked ferric chelate (turbo) reductase in plant plasma membranes from roots of *Plantago lanceolata*. *Protoplasma* **203**, 186–193.

Schmidt, W. (1999). Mechanisms and regulation of reduction-based iron uptake in plants. *New Phytologist* **141**, 1–26.

Serrano, A., Córdoba, F., González-Reyes, J.A., Navas, P., and Villalba, J.M. (1994). Purification and characterization of two distinct NAD(P)H dehydrogenases from onion (*Allium cepa* L.) root plasma membrane. *Plant Physiology* **106**, 87–96.

Susín, S., Abián, J., Peleato, M.L., Sánchez-Baeza, F., Abadía, A., Gelpí, E., and Abadía, J. (1994). Flavin excretion from roots of iron-deficient sugar beet (*Beta vulgaris* L.). *Planta* **193**, 514–519.

Susín, S., Abadía, A., González-Reyes, J.A., Lucena, J.J., and Abadía, J. (1996). The pH requeriment for *in vivo* activity of the iron-deficiency-induced *turbo* ferric chelate reductase. *Plant Physiology* **110**, 111–123.

Tagliavini, M., Abadía, J., Rombolá, A.D., Abadía, A., Tsipouridis, C., and Marangoni B. (1999). Agronomic means for the control of iron chlorosis in deciduous fruit plants. *Journal of Plant Nutrition* (in press).

Terry, N. (1980). Limiting factors in photosynthesis I. Use of iron stress to control photochemical capacity *in vivo*. *Plant Physiology* **65**, 114–120.

Tiffin, L.O. (1970). Translocation of iron citrate and phosporous in xylem exudate of soybeans. *Plant Physiology* **41**, 515–518.

Tiffin, L.O., and Brown, J.C. (1962). Iron chelates in soybean exudates. *Science* **135**, 311–313.

Welkie, G.W., and Miller, G.W. (1993). Plant iron uptake physiology by nonsiderophore systems. In 'Iron Chelation in Plants and Soil Microorganisms'. (Eds L.L. Barton and B.C. Hemming.) pp. 345–370. (Academic Press: New York.)

Widell, S., and Larsson, C. (1981). Separation of presumptive plasma membranes from mitochondria by partition in an aqueous polymer two-phase system. *Physiologia Plantarum* **51**, 368–374.

Widell, S., and Larsson, C. (1990). A critical evaluation of markers used in plasma membrane purification. In 'The Plant Plasma Membrane'. (Eds C. Larsson and I.M. Moller.) pp. 16–43. (Springer-Verlag: Berlin, Germany.)

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