N.º Referencia: AGL2007-61948/AGR

MINISTERIO DE CIENCIA E INNOVACIÓN

DIRECCIÓN GENERAL DE INVESTIGACIÓN
Y GESTIÓN DEL PLAN NACIONAL DE I+D+i

PROYECTOS I+D+i, ACCIONES ESTRATÉGICAS Y ERANET

INFORME FINAL

Investigador Principal	**Javier Abadía Bayona**
Título de la actuación	**Estudios sobre la homeostasis de metales en plantas**
Organismo	**Consejo Superior de Investigaciones Científicas**
Centro	**Estación Experimental de Aula Dei**
Departamento	**Nutrición Vegetal**
Fecha de inicio	**1 octubre 2007**
Fecha de finalización	**31 diciembre 2010**

Sr. Subdirector General de Proyectos de Investigación
Este documento no debe remitirse en papel/ por correo postal a la Subdirección

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A. MEMORIA. Resumen de las actividades realizadas y de los resultados del proyecto en relación con los objetivos propuestos (máximo 2.000 palabras).

Destaque su relevancia científica y/o su interés tecnológico.
En el caso de haber obtenido resultados no previstos inicialmente, indique su relevancia para el proyecto.
En caso de resultados fallidos, indíquense las causas.

Las actividades se han realizado como se indica a continuación:

Objective 1. To study metal homeostasis-related changes in plant metabolites

Task 1. En cuanto al "targeted analysis" de metabolitos, se han publicado las bases para la determinación por HPLC/ESI/TOFMS de los complejos metálicos del aminoácido no proteinogénico nicotianamina (2). Se han identificado dos complejos de citrato:Fe en soluciones estándar, uno de los cuales -el complejo Cit_3Fe_3- se ha demostrado que existe en el xilema de tomate mediante HPLC/ESI/TOFMS y HPLC/ICP/MS (12). También se han utilizado técnicas de HPLC/ESI/TOFMS para determinar el contenido en diversas plantas de glutatión oxidado y reducido (7) y ácidos orgánicos (19), así como de quelatos de Fe en hojas, xilema y raíces de plantas (8, 10, 11, 20, 21, 22). Se ha progresado en la caracterización de los efectos, en diversas especies vegetales, de la deficiencia de Fe (3, 6, 9) y de la toxicidad de Cd (4**)**, Zn (5, 15, 18) y Hg (16).

Task 2. Se ha realizado un primer estudio metabolómico/proteómico sobre deficiencia de Fe en puntas de raíz (13). Se están realizando estudios en los cambios en los perfiles metabólicos de diversos materiales, incluyendo materiales de campo, especialmente melocotonero (incluidos en la Tesis de R. Rellán-Álvarez).

Objective 2. To study metal homeostasis-related changes in plant proteins

Task 3. Se ha progresado en la obtención de diversos materiales vegetales, incluyendo protoplastos, vacuolas, envoltura cloroplástica, tonoplasto y membrana plasmática de *Beta vulgaris* (trabajos en preparación). Los investigadores húngaros asociados al proyecto, así como dos de sus becarios (B. Basa y A. Solti), han realizado estancias en nuestro laboratorio para trabajar en aislamiento de membranas cloroplásticas. Todos estos materiales se utilizarán en el proyecto continuación.

Task 4. Se han iniciado los trabajos de separación de proteínas de xilema y floema, y ya se obtiene una buena resolución en geles 2-D y se están comenzando a identificar spots. También se han aislado tilacoides y otras fracciones de plantas deficientes en hierro, controles y con toxicidad de Cd, y se están realizando trabajos de electroforesis 2-D.

Task 5. Se han puesto a punto las técnicas de recogida de spots de los geles 2-D, y el becario asociado al proyecto, G. Lattanzio, ha puesto a punto las técnicas de MS-MS para la secuenciación de proteínas. Se han publicado trabajos sobre cambios en el proteoma de las raíces de remolacha y *Medicago truncatula* por deficiencia de Fe (13, 17) y de las raíces de tomate por el estrés de Cd (14).

Objective 3. To study metal homeostasis-related gradients in plant leaves and roots

Task 6. Se ha publicado información sobre la estructura transversal de hojas de peral y melocotonero (1) y remolacha (23). Se han puesto a punto protocolos experimentales para la obtención de secciones adaxiales finas a partir de discos congelados de hojas con un criotomo. Así mismo, se han abordado técnicas de criofijación en la Univ. de Barcelona, y de microanálisis en el CCMA-CSIC de Madrid (15). Se han obtenido secciones adaxiales de hojas de remolacha en diferentes condiciones nutricionales (15). Uno de los miembros del equipo realizó en 2009 estancias en laboratorios de Japón y Eslovenia adquiriendo diversas técnicas de imagen y localización elemental.

Task 7. Se están realizando análisis de metabolitos en secciones adaxiales de hojas y raíces de remolacha.

Task 8. Se han recogido secciones adaxiales de hoja para la separación de proteínas.

Task 9 (Dissemination)

Se han presentado 41 comunicaciones en Congresos (ver más abajo: 4 Keynotes, 6 Orales, 31 Paneles), y se han publicado ya **24 trabajos SCI** en los que se menciona al proyecto como fuente de financiación (entre ellas 4 reviews: 21, 22, 23 y 24). Las listas de publicaciones y presentaciones están actualizadas en la dirección web del Grupo: http://www.stressphysiology.com/projects.html

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B. RESULTADOS MÁS RELEVANTES ALCANZADOS EN EL PROYECTO (máximo 60 palabras). Indicar únicamente los relacionados con el proyecto.

Dentro de los logros del proyecto señalados en el apartado anterior, reseñe los más relevantes hasta un máximo de tres.

1) Se ha identificado por primera vez un complejo citrato-Fe en el xilema de plantas mediante HPLC/ESI/TOFMS y HPLC/ICP/MS (12).
2) Se ha realizado un primer estudio metabolómico/proteómico sobre deficiencia de Fe en puntas de raíz (13).
3) Se han publicado trabajos sobre cambios en el proteoma de las raíces de remolacha y *Medicago truncatula* por deficiencia de Fe (13, 17) y raíces de tomate por el estrés de Cd (14).

B. RESUMEN DE LOS RESULTADOS DEL PROYECTO

C. Indicar únicamente los relacionados con el proyecto.

Nota: Adjuntar en formato digital la primera página y aquella en la que se mencione a las entidades financiadoras del proyecto, de una selección de las 5-10 publicaciones que se consideren más relevantes.

C1. Formación del personal N.º

Personal formado (4) Irene Orera*, Rubén Rellán-Álvarez, Jorge Rodríguez-Celma, Giuseppe Lattanzio
*(adscrita a otro proyecto AGL)

Personal formado o en formación que se ha transferido al sector industrial:
Doctores (0) Titulados Superiores (0) Técnicos (0)

C2. Tesis doctorales	(2)			
C3. Artículos científicos en revistas	(0)	nacionales	(19)	internacionales
C4. Artículos de divulgación en revistas	(1)	nacionales	(0)	internacionales
C5. Artículos de revisión en revistas	(0)	nacionales	(4)	internacionales
C6. Libros, capítulos de libros y monografías	(0)	nacionales	(2)	internacionales
C7. Conferencias en congresos (por invitación)	(1)	nacionales	(3)	internacionales
C8. Patentes y otros títulos de propiedad	(0)	registrados	(0)	en explotación
industrial	(0)	España	(0)	extranjero

C1. FORMACIÓN DE PERSONAL EN EL PROYECTO, (becarios, técnicos de apoyo, doctorandos…) describir brevemente.

-Becarios pre: 4 (Irene Orera*, Rubén Rellán-Álvarez, Jorge Rodríguez-Celma, Giuseppe Lattanzio)
-Contratados post: 1 (Saúl Vázquez*)
-Técnicos: 1 (Adelina Calviño)
(*adscritos a otro proyecto AGL)

C2. TESIS DOCTORALES REALIZADAS TOTAL O PARCIALMENTE EN EL PROYECTO

Indicar: título, nombre del doctorado, Universidad, Facultad o Escuela, fecha de comienzo, fecha de lectura, calificación y director.

TITULO:	Desarrollo y aplicación de nuevas metodologías analíticas para el estudio de fertilizantes férricos		
DOCTORANDO:	Irene Orera		
UNIVERSIDAD:	Universidad de Zaragoza	FACULTAD:	Ciencias
AÑO:	2010 (July 11)		
CALIFICACION:	Sobresaliente *cum laude* por unanimidad		

TITULO:	Long distance transport and metabolism changes in iron deficient plants		
DOCTORANDO:	Rubén Rellán Álvarez		
UNIVERSIDAD:	Universidad Autónoma de Madrid	FACULTAD:	Ciencias
AÑO:	2011 (February 11)		
CALIFICACION:	Sobresaliente *cum laude*		

C3. ARTÍCULOS CIENTÍFICOS EN REVISTAS (19)

Indicar: autor(es), título, referencia de la publicación.

1. **Fernández V**, Eichert T, **Del Río V**, López-Casado G, Heredia-Guerrero JA, **Abadia A**, Heredia A, **Abadia J** (2008) Leaf structural changes associated with iron deficiency chlorosis in field-grown pear and peach: physiological implications. **Plant Soil** 331, 161-172
2. **Rellán-Álvarez R**, **Abadía J**, **Álvarez-Fernández A** (2008) Formation of metal-nicotianamine complexes as affected by pH, ligand exchange with citrate and metal exchange. A study by electrospray-time of flight mass spectrometry. **Rapid Commun Mass Sp** 22, 1553-1562
3. **López-Millán A-F**, **Morales F**, Gogorcena Y, **Abadía A**, **Abadía J** (2009) Metabolic responses in iron deficient tomato plants. **J Plant Physiol** 166, 375-384
4. **López-Millán A-F**, **Sagardoy R**, **Solanas M**, **Abadía A**, **Abadía J** (2009) Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. **Environ Exp Bot** 65, 376-385
5. **Sagardoy R**, **Morales F**, **López-Millán A-F**, **Abadía A**, **Abadía J** (2009) Effects of zinc toxicity in sugar beet (*Beta vulgaris* L.) plants grown in hydroponics. **Plant Biology** 11, 339-350
6. **Andaluz S, Rodríguez-Celma J, Abadía A, Abadía J, López-Millán A-F** (2009) Time course induction of several key enzymes in *Medicago truncatula* roots in response to Fe deficiency. **Plant Physiol Biochem** 47, 1082-1088
7. Martí MC, Camejo D, Fernández-García N, **Rellán-Álvarez R**, Marques S, Sevilla F, Jiménez A (2009) Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants. **J Hazard Mater** 171, 879-885
8. **Orera I, Abadía A, Abadía J, Álvarez-Fernández A** (2009) Determination of *o*,*o*EDDHA -a xenobiotic chelating agent used in Fe-fertilizers- in plant tissues by liquid chromatography-electrospray mass spectrometry: overcoming matrix effects. **Rapid Commun Mass Sp** 23, 1694-1702
9. **Larbi A**, **Morales F**, **Abadía A**, **Abadía J** (2010) Changes in organic acid and iron concentrations in xylem sap and apoplastic fluid of *Beta vulgaris* in response to iron deficiency and resupply. **J Plant Physiol** 167, 255-260 (doi:10.1016/j.jplph.2009.09.007)
10. **Orera I**, Orduna J, **Abadía J**, **Álvarez-Fernández A** (2010) Electrospray-collision-induced dissociation mass spectrometry: a tool to characterize synthetic polyaminocarboxylate ferric chelates used as fertilizers. **Rapid Commun Mass Sp** 24, 109-119 (doi: 10.1002/rcm.4361)
11. **Orera I**, Rodríguez-Castrillón JA, Moldovan M, García-Alonso JI, **Abadía A**, **Abadía J**, **Álvarez-Fernández A** (2010) Using a dual-stable isotope tracer method to study the uptake, xylem transport and distribution of Fe and its chelating agent from stereoisomers of a xenobiotic Fe(III)-chelate used as fertilizer in Fe-deficient Strategy I plants. **Metallomics** 2, 646-657 (doi:10.1039/C0MT00018C)
12. **Rellán-Álvarez R**, Giner-Martínez-Sierra J, Orduna J, **Orera I**, Rodríguez-Castrillón JA, García-Alonso JI, **Abadía J**, **Álvarez-Fernández A** (2010) Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. **Plant Cell Physiol** 51, 91-102 (doi:10.1093/pcp/pcp170)
13. **Rellán-Álvarez R**, **Andaluz S**, **Rodríguez-Celma J**, Wohlgemut G, Zocchi G, **López-Millán A-F**, Fiehn O, **Álvarez-Fernández A**, **Abadía J** (2010) Changes in the proteomic and metabolic profiles of *Beta vulgaris* root tips in response to iron deficiency and resupply. **BMC Plant Biol** 10:120 (http://www.biomedcentral.com/1471-2229/10/120)
14. **Rodríguez-Celma J**, **Abadía A**, **Abadía J**, **López-Millán A-F** (2010) Changes induced by Cd toxicity in the proteomic profile of tomato roots. **J Proteom** 73, 1694-1706 (doi:10.1016/j.jprot.2010.05.001)
15. **Sagardoy R**, **Vázquez S**, Florez-Sarasa ID, Ribas-Carbó M, Flexas J, **Abadía J**, **Morales F** (2010) Stomatal and mesophyll conductance to CO_2 are the main limitations to photosynthesis in sugar beet plants treated with Zn excess. **New Phytol** 187, 145-158 (doi:10.1111/j.1469-8137.2010.03241.x)
16. Carrasco-Gil S, **Álvarez-Fernández A,** Sobrino-Plata J, Millán R, Carpena-Ruiz RO, LeDuc DL, Andrews JC, **Abadía J**, Hernández LE (2011) Complexation of Hg with phytochelatins is important for plant Hg tolerance. **Plant Cell Environ**, in press (doi: 10.1111/j.1365-3040.2011.02281.x)
17. **Rodríguez-Celma J**, **Lattanzio G**, Grusak MA, **Abadía A**, **Abadía J**, **López-Millán AF** (2011) Changes in the protein profile of *Medicago truncatula* roots in direct and carbonate-induced Fe deficiency conditions: increases in riboflavin synthesis and alterations in C/N metabolism. **J Proteom Res**, in press (doi:10.1021/pr2000623)
18. **Sagardoy R**, **Morales F**, **López-Millán AF**, **Abadía A**, **Abadía J** (2011) Carboxylate metabolism in sugar beet plants grown with Zn excess. **J Plant Physiol**, in press (doi:10.1016/j.jplph.2010.10.012)

19. **Rellán-Álvarez R**, **López-Gomollón S**, **Javier Abadía J**, **Álvarez-Fernández A** (2011) Low molecular mass organic acid determination in plant tissue extracts by liquid chromatography-electrospray Time-Of-Flight mass spectrometry, submitted for publication

C4. ARTÍCULOS DE DIVULGACIÓN EN REVISTAS (1)

Indicar: autor(es), título, referencia de la publicación.

20. **Orera I**, **Abadía J**, **Abadía A**, **Álvarez-Fernández A** (2009) Nuevas metodologías aplicadas a la investigación de quelatos de hierro sintéticos. **Vida Rural** 294, 60-64.

C5. ARTÍCULOS DE REVISIÓN (4)

Indicar: autor(es), título, referencia de la publicación.

21. **Orera I**, **Abadía J**, **Abadía A**, **Álvarez-Fernández A** (2009) Analytical technologies to tackle the biological and environmental implications of iron fertilization with synthetic ferric chelates: the Fe(III)-EDDHA case. **J Hortic Sci Biotechnol** 84, 7-12
22. **Fernández V**, **Orera I**, **Abadía J**, **Abadía A** (2009) Foliar iron fertilisation of fruit trees: present and future perspectives. **J Hortic Sci Biotech** 84, 1-6
23. **Abadía J**, **Vázquez S**, **Rellán-Álvarez R**, **El-Jendoubi H**, **Abadía A**, **Álvarez-Fernández A**, **Ana Flor López-Millán AF** (2011) Towards a knowledge-based correction of iron chlorosis. **Plant Physiol Biochem**, in press (doi:10.1016/j.plaphy.2011.01.026)
24. **El-Jendoubi H**, **Melgar JC**, **Álvarez-Fernández A**, **Sanz M**, **Abadía A**, **Abadía J** (2011) Setting good practices to assess the efficiency of iron fertilizers. **Plant Physiol Biochem**, in press (doi:10.1016/j.plaphy.2011.02.013)

C6. LIBROS, CAPÍTULOS DE LIBROS Y MONOGRAFÍAS (2)

Indicar: autor(es), título, referencia de la publicación.

Sagardoy R, Flexas J, Ribas-Carbó M, **Morales F**, **Abadía J** (2009) Stomatal conductance is the main limitation to photosynthesis in sugar beet plants treated with Zn excess. En: The Proceedings of the International Plant Nutrition Colloquium XVI. UC Davis (Electronic Journal).

Vázquez S, Pinto F, **Abadía A**, **Abadía J** (2009) Elemental microanalysis in leaf transversal sections of peach by SEM/EDXA: Influence of iron nutritional status. En: The Proceedings of the International Plant Nutrition Colloquium XVI. UC Davis (Electronic Journal).

C7. CONFERENCIAS EN CONGRESOS, SIMPOSIOS Y REUNIONES (POR INVITACIÓN)

Indicar: autor(es), nombre del congreso, lugar de celebración, año.

Congresos

Keynotes (4)

2007

XVII Reunión de la Sociedad Española de Fisiología Vegetal-X Congreso Hispano-Luso de Fisiología Vegetal *(Alcalá de Henares, España, 18-21 September, 2007)*

-Abadía J, Álvarez-Fernández A, López-Millán AF, Orera I, Rellán R, Abadía A (2007) Long-distance metal transport in plants

2008

VI International Symposium on Mineral Nutrition of Fruit Crops *(ISHS, University of Algarve, Faro, Portugal, May 19-23, 2008)*

-Fernández V, Abadía J, Abadía A. Foliar fertilisation: a reliable strategy to control plant nutrient deficiencies?

2009

XVI International Plant Nutrition Colloquium *(Sacramento, CA, US, 26-30 August)*

-Rellán-Álvarez R, Andaluz S, López-Millán AF, Fiehn O, Álvarez-Fernández A, Abadía J. Changes in the proteomic and metabolomic profiles of *Beta vulgaris* root tips in response to iron deficiency and resupply

2010

15th International Symposium on Iron Nutrition and Interactions in Plants (Budapest, Hungary, 26-30 June, 2010)

-Abadía J, Vazquez S, Rellán-Álvarez R, Álvarez-Fernández A, López-Millán AF, Abadía A. Towards a knowledge-based correction of iron chlorosis

Conferencias orales (7)

2008

XIV International Symposium on Iron Nutrition and Interactions in Plants *(XIV ISINIP, Beijing, PR China, October 11-15, 2008)*

-Rodríguez-Celma J, Álvarez-Fernández A, Orduna J, Abadía A, Abadía J, López-Millán AF. Root excretion and accumulation of riboflavin derivatives in iron-deficient *Medicago truncatula*

2009

XVIII Reunión de la Sociedad Española de Fisiología Vegetal-X Congreso Hispano-Luso de Fisiología Vegetal *(Zaragoza, España, 8-11 September)*

-Rellán-Álvarez R, Giner-Martínez-Sierra J, Orduna J, Orera I, Rodríguez-Castrillón JA, García-Alonso JI, Abadía J, Álvarez-Fernández A. Iron is transported as a tri-Fe(III), tri-citrate complex in plant xylem sap

-Sagardoy R, Flexas J, Ribas-Carbó M, Morales F, Abadía J. Effects of Zn excess on stomatal conductance in *Beta vulgaris* plants grown in hydroponics

2010

15th International Symposium on Iron Nutrition and Interactions in Plants (Budapest, Hungary, 26-30 June, 2010)

-El Jendoubi H, Melgar JC, Abadía A, Álvarez-Fernández A, Abadía J. Do's and do not's when assessing the efficacy of iron fertilizers

-Rellán-Álvarez R, El Jendoubi H, Rodríguez-Celma J, Wohlgemuth G, Abadía A, Fiehn O, López-Millán AF, Abadía J, Álvarez-Fernández A. Delving into iron deficiency metabolomics

-Solti A, Basa B, Fodor F, Abadía J, Sárvári É. Effect of Cd(II), Zn(II) and Mn(II) on chloroplast iron uptake

6th International Franco-Spanish Workshop on Bio-inorganic Analytical Chemistry (Pau, France, Sept 2010)

-Rellán-Álvarez R, Abadía J, Álvarez-Fernández A. Iron speciation in plant xylem sap using LC-ESI-TOFMS.

Paneles (31)

2007

COST859 Meeting *(Israel, September 22-25 2007)*

-Solanas M, López-Millán AF, Abadía J, Abadía A (2007) Effects of Cd in the xylem sap proteome of tomato plants

2008

II "Plant Proteomics in Europe" Meeting *(COST FR 0603, Working Group 2, University of Córdoba, Spain, February 6-8, 2008)*

-López-Millán AF, Rodríguez-Celma J, Abadía A, Abadía J. Effects of Cd in the xylem sap of tomato (*Lycopersicon esculentum*) plants: a proteomic approach

I Jornadas Bienales de Proteómica SEPRO *(Sociedad Española de Proteómica, Barcelona, Spain, February 21-22, 2008)*

-Rodríguez-Celma J, López-Millán AF, Abadía A, Abadía J. Effects of Cd in the root proteome of tomato (*Lycopersicon esculentum*) plants

XIV International Symposium on Iron Nutrition and Interactions in Plants *(XIV ISINIP, Beijing, PR China, October 11-15, 2008)*

-López-Millán AF, Moussaoui S, Fernández V, Abadía J, Abadía A. Deactivation of sugar beet root responses to iron deficiency upon foliar iron application

-Orera I, Rodríguez-Castrillón JA, García Alonso JI, Moldovan M, Abadía J, Abadía A, Álvarez-Fernández A. Iron uptake and distribution in sugar beet plants treated with *racemic* and *meso* Fe(III)-*o,o*EDDHA isomers

-Rellán-Álvarez R, Abadía A, Fiehn O, Abadía J, Álvarez-Fernández A. Changes in the xylem sap metabolome of tomato and lupin with Fe deficiency

-Rellán-Álvarez R, Andaluz S, López-Millán AF, Álvarez-Fernández A, Fiehn O, Abadía J. Proteomic and metabolic profiles of *Beta vulgaris* root tips: changes induced in response to iron deficiency and resupply

XXII Simposio Ibérico de Nutrición Mineral de las Plantas *(Granada, España, October 22-24, 2008)*

-Sagardoy R, Morales F, López-Millán AF, Abadía A, Abadía J. Effects of Zinc toxicity in sugar beet plants grown in hydroponics

-El Jendoubi H, Igartua E, del Río V, Abadía J, Abadía A. Prognosis de la clorosis férrica en frutales a partir de concentraciones de Fe en distintos materiales

Isafruit Meeting *(Gerona, Spain, November 25-27, 2008)*

-Orera I, Abadía J, Abadía A, Álvarez-Fernández A. Determination of the xenobiotic fertilizer *o,o*EDDHA in plant tissues by Liquid Chromatography-Electrospray/Mass Spectrometry

2009

COST FA0603 WG1 MEETING: Technical aspects inherent to Plant Proteomics "Classical and novel approaches in Plant Proteomics" *(Viterbo, Italy, 5-6 May 2009)*

-Rodríguez-Celma J, López-Millán AF, Abadía A, Abadía J. Changes in the *Lycopersicum esculentum* root proteome with Cd stress

XVI International Plant Nutrition Colloquium *(Sacramento, CA, US, 26-30 August)*

-Sagardoy R, Flexas J, Ribas-Carbó M, Morales F, Abadía J. Stomatal conductance is the main limitation to photosynthesis in sugar beet plants treated with Zn excess

-Vázquez S, Pinto F, Abadía A, Abadía J. Elemental microanalysis in leaf transversal sections of peach by SEM/EDXA: Influence of iron nutritional status

XVIII Reunión de la Sociedad Española de Fisiología Vegetal-X Congreso Hispano-Luso de Fisiología Vegetal *(Zaragoza, España, 8-11 September)*

-Orera I, Orduna J, Abadía J, Álvarez-Fernández A. Identification of Fe-containing impurities in commercial fertilizers by collision induced tandem masss spectrometry

-Rodríguez-Celma J, Calviño A, Álvarez-Fernández A, Orduna J, Abadía A, Abadía J, López-Millán AF. Root excretion and accumulation of riboflavin derivatives in iron-deficient *Medicago truncatula*

-Vázquez S, Abadía A, Abadía J. Image techniques: New approaches in metal homeostasis

-Rellán-Álvarez R, Rodriguez-Celma J, López-Millán A-F, Fiehn O, Álvarez-Fernández A, Abadía A, Abadía J. Plant iron deficiency metabolomics

-Sagardoy R, Álvarez-Fernández A, Abadía J. Effects of Zn and Cd toxicity on metal concentrations in the xylem sap of *Beta vulgaris* and *Lycopersicon esculentum*

-Orera I, Abadía A, Abadía J, Álvarez-Fernández A. Study of the plant iron fertilization with synthetic ferric chelates by mass spectrometry

Isafruit Meeting *(Angers, France, November 27-28)*

-Vázquez S, Abadía A, Abadía J. Image techniques: New approaches in metal homeostasis

-Rellán-Álvarez R, Rodriguez-Celma J, López-Millán A-F, Fiehn O, Álvarez-Fernández A, Abadía A, Abadía J. Plant iron deficiency metabolomics

-Orera I, Abadía A, Abadía J, Álvarez-Fernández A. Study of the plant iron fertilization with synthetic ferric chelates by mass spectrometry

2010

15th International Symposium on Iron Nutrition and Interactions in Plants (Budapest, Hungary, 26-30 June, 2010)

-Álvarez-Fernández A, Melgar JC, Abadía J, Abadía A. Fruit quality and yield changes in field-grown pear and peach trees as affected by iron deficiency induced chlorosis

-López-Millán A-F, Rodríguez-Celma J, Abadía A, Grusak MA, Abadía J. Effects of Fe deficiency on the riboflavin synthesis pathway in *Medicago truncatula* plants

-Rodríguez-Celma J, Abadía A, Abadía J, López-Millán AF. Changes in the *Medicago truncatula* root proteome under Fe deficiency

-Sagardoy R, Morales F, López-Millán AF, Abadía A and Abadía J. Zinc toxicity and iron deficiency in sugar beet

-Vázquez S, Abadía A, Abadía J. Micro-localization of iron in iron-deficient and iron-sufficient sugar beet leaves

XVII Congress of the Federation of European Societies of Plant Biology. FESPB 2010 (Valencia, July 4-9)

-Rellán-Álvarez R, Abadía J, Álvarez-Fernández, A. Xylem metabolomics and iron deficiency

28th Horticultural Congress (Lisboa, Portugal, August 22-27)

-El Jendoubi, H., Lastra, M., Melgar, J.C., Abadía, M. and Abadía, J. Evaluation of a soil-applied compound applied to control iron chlorosis in peach trees: regreening and fruit quality

-Álvarez-Fernández, A., Melgar, J.C., Abadía, J. and Abadía, A. Effects of iron deficiency chlorosis on fruit quality and yield in *Pyrus communis* L. and *Prunus persica* L. Batsch

XIII Simposio Ibérico de Nutrición Mineral de las Plantas (San Sebastián, España, Septiembre 5-7)

-Rellán-Álvarez R, El Jendoubi H, Wohlgemuth G, Fiehn O, Abadía A, Abadía J, Álvarez-Fernández, A. Delving into iron deficiency metabolomics

C8. PATENTES Y OTROS TÍTULOS DE PROPIEDAD INDUSTRIAL

Indicar: autor(es), título, registro, entidad titular de la patente, año, países, clase.
Indicar cuáles están en explotación.

Ninguna

C9. OTROS RESULTADOS EXTRAORDINARIOS NO INCLUIDOS EN LOS APARTADOS ANTERIORES

Indicar: naturaleza y autor (es). Descríbalo brevemente en un máximo de 50 palabras.

Ninguno

D. CARÁCTER DE LOS RESULTADOS DEL PROYECTO **(señalar hasta dos opciones)**

(X) Teóricos	(X) Teórico-prácticos
( ) Prácticos	( ) De inmediata aplicación industrial

E. COLABORACIONES

E1. SI EL PROYECTO HA DADO LUGAR A COLABORACIONES CON OTROS GRUPOS DE INVESTIGACIÓN, coméntelas brevemente.

Se han realizado colaboraciones con los investigadores O. Fiehn (Metabolomics Lab, UC Davis, USA) y J.F. Ma (Okayama University, Japón), ambos participantes en el proyecto, así como con F. Fodor y E. Sarvari (Eötvos Lörand Univ., Budapest, Hungría, incorporados al proyecto tras su inicio).

También se han realizado colaboraciones con los grupos de A. Heredia (Univ. de Málaga) en técnicas de análisis de superficie de hojas, con el grupo del Dr. J. I. García Alonso (Universidad de Oviedo) en la utilización de isótopos estables en metrología química, con el Dr. J. Orduna (ICMA-CSIC/Univ. de Zaragoza) para estudios de ESI-MS/MS(Q-TOF), con el Dr. J. M. Andrés, (ICB-CSIC, Zaragoza) en estudios de SEM-EDX en materiales vegetales, y con los grupos de los Drs. L. E. Hernández (UAM Madrid) y F. Sevilla (CEBAS-CSIC, Murcia) en diversas aplicaciones de las técnicas HPLC/TOFMS. Se ha iniciado una colaboración en proteómica con la Dra. Julia Kehr (INIA-UPM, Madrid).

Para análisis relacionados con Biología Molecular se ha contado con la colaboración de Michael Grusak (USDA-ARS-CNRC Houston, US), y para colaboraciones en el área de la proteómica con el grupo del Dr. Zocchi (Univ. De Milán en Italia).

E2. SI HA PARTICIPADO EN PROYECTOS DEL PROGRAMA MARCO DE I+D DE LA UE Y/O EN OTROS PROGRAMAS INTERNACIONALES EN TEMÁTICAS RELACIONADAS CON LAS DE ESTE PROYECTO, indique programa, tipo de participación y beneficios para el proyecto.

Mencione las solicitudes presentadas al Programa Marco de la UE durante la ejecución del proyecto, aunque no hayan sido aprobadas.

Se ha trabajado como participante en el proyecto integrado europeo (FPVI) Isafruit (enero de 2006- Septiembre 2010), en el tema de dinámica de micronutrientes, incluidos metales, en árboles frutales.

Se está trabajando en un proyecto trilateral KBBE (Hot Iron; Programa Nacional de Internacionalización de la I+D+I del PN; Subprograma EUROINVESTIGACIÓN 2008 EUI2008-03618) con los grupos de K. Philippar (Univ. Munich, Alemania), N. von Wirén (Univ. Hohenheim, Alemania), JF Briat/F. Gaymard (INRA Montpellier, Francia), y la empresa Timac-Agro (Pamplona, España), sobre adquisición de hierro por el cloroplasto.

F. PROYECTOS COORDINADOS [1]

Describa las actuaciones de coordinación entre subproyectos, y los resultados de dicha coordinación con relación a los objetivos globales del proyecto.

El proyecto no era coordinado.

G. RELACIONES O COLABORACIONES CON DIVERSOS SECTORES

G1. SI EN EL PROYECTO HA HABIDO COLABORACIÓN CON ENTES PROMOTORES OBSERVADORES (EPO) PARTICIPANTES:

[1] A rellenar solo por el coordinador del proyecto.

1. Describa en detalle la relación mantenida con los EPO's, y la participación concreta de estos en el proyecto, especificando, si procede, su aportación al mismo en todos sus aspectos. (Si se ha modificado la relación y/o el apoyo del EPO, en relación con lo previsto a la aprobación del proyecto, descríbalo brevemente).

La empresa Bruker Biosciences Española S.A. ha constado como apoyo a la ejecución en este proyecto. En todo momento han estado informados del desarrollo de los trabajos, por los que siempre han mostrado interés.

2. Describa, si procede, las transferencias realizadas al (los) EPO (s) de los resultados obtenidos, indicando el carácter de la transferencia y el alcance de su aplicación.

3. Indique si esta colaboración ha dado lugar a la presentación de nuevos proyectos o si se tiene intención de continuarla en el futuro. En caso afirmativo, describa brevemente cómo va a concretarse.

La empresa Bruker Biosciences Española S.A. también consta como apoyo a la ejecución en el proyecto AGL-2010 continuación del presente.

G2. SI EL PROYECTO HA DADO LUGAR A OTRAS COLABORACIONES CON EL ENTORNO SOCIOECONÓMICO (INDUSTRIAL, ADMINISTRATIVO, DE SERVICIOS, ETC.), NO PREVISTAS INICIALMENTE EN EL PROYECTO, descríbalas brevemente.

Ninguna.

H. GASTOS REALIZADOS

H1. GASTOS REALIZADOS EN LA ÚLTIMA ANUALIDAD

Nota: ***Debe cumplimentarse este apartado independientemente de la justificación económica enviada por el organismo.***

1.- Indique el total de gasto realizado en el proyecto:

Concepto	**Total gasto de la anualidad** (€)
Personal	12.466,51
Costes de ejecución	74.787,24
TOTAL GASTO REALIZADO	87.253,75

2.- Comente brevemente si ha habido algún tipo de incidencia en este apartado que desee reseñar.

H2. GASTOS REALIZADOS DURANTE TODO EL PROYECTO

Nota: ***Debe cumplimentarse este apartado independientemente de la justificación económica enviada por el organismo.***

Euros

1. Gastos de personal (indicar número de personas, situación laboral y función desempeñada)

Total 61.828,01 €

2. Material inventariable (describir brevemente el material adquirido)

Total 7.579,49 €

3. Material fungible (describir brevemente el tipo de material)

Total 85.677,37 €

4. Viajes y dietas (describir brevemente)

Total 12.540,50 €

5. Otros gastos (describir brevemente)

Total 28.374,63 €

6. Costes indirectos

Total 41.160,00 €

7. Dotación adicional o complementos salariales, si procede

Total €

TOTAL GASTOS EJECUTADOS DEL PROYECTO	**237.160,00 €**

I. INFORMACIÓN CORRESPONDIENTE A LA ÚLTIMA JUSTIFICACIÓN DE GASTO.

I1. PERSONAL ACTIVO EN EL PROYECTO DURANTE EL ÚLTIMO PERÍODO DE JUSTIFICACIÓN.

En el cuadro siguiente debe recogerse la situación de todo el personal del o de los Organismos participantes que haya prestado servicio en el proyecto en la anualidad que se justifica, o que no haya sido declarado anteriormente, y cuyos costes (salariales, dietas, desplazamientos, etc.), se imputen al mismo.
Si la persona estaba incluida en la solicitud original, marque "S" en la casilla correspondiente y no rellene el resto de las casillas a la derecha.
Indique en la casilla "Categoría Profesional" el puesto de trabajo ocupado, el tipo de contratación: indefinida, temporal, becarios (con indicación del tipo de beca: FPI, FPU, etc.), etc.
En el campo "Función en el proyecto" indique el tipo de función/actividad realizada en el proyecto, (p. e., investigador, técnico de apoyo,…).

Recuerde que:
- En este capítulo solo debe incluir al personal vinculado a los Organismos participantes en el proyecto. Los gastos de personal externo (colaboradores científicos, autónomos…) que haya realizado tareas para el proyecto debe ser incluido en el capítulo de "Varios".
- Las "Altas" y "Bajas" deben tramitarse de acuerdo con las "Instrucciones para el desarrollo de los proyectos de I+D" expuestas en la página web del MICINN.

						Si no incluido en solicitud original:		
Apellido 1	**Apellido 2**	**Nombre**	**NIF/NIE**	**Catgª Profesional**	**Incluido en solicitud original**	**Función en el proyecto**	**Fecha de Alta**	**Observaciones**
Abadía	Bayona	Javier	17850563X	Prof. Inv. CSIC	S			
López	Millán	Ana Flor	29101691K	Cient. Tit. CSIC	S			
Morales	Iribas	Fermín	18204666M	Inv. Cient. CSIC	S			
Álvarez	Fernández	Ana	9392534R	Cient. Tit. CSIC	S			
Rellán	Álvarez	Rubén	71644347Z	Becario predoctoral FPI	S			
Sagardoy	Calderón	Ruth	25186549P	Becario predoctoral FPI	S			
Fiehn		Oliver	249858032	Prof. Tit. Univ. USA	S			
Ma		Jian Feng	TG7146756	Cat. Univ. Japón	S			
Fodor		Ferenc	BA 0771196	Prof. Tit. Univ. Hungría		Trabajos cloroplasto	08/11/2007	Eötvös Lorand University Budapest, Proy. paralelo OTKA-NN74045
Sarvari		Eva	PB189897	Prof. Tit. Univ. Hungría		Trabajos cloroplasto	08/11/2007	Eötvös Lorand University Budapest, Proy. paralelo OTKA-NN74045
Rodríguez	Celma	Jorge		Becario predoctoral JAE		Trabajos laboratorio	28/02/2008	Becario predoctoral JAE-CSIC
Lattanzio		Giuseppe		Becario predoctoral FPI		Trabajos laboratorio	02/11/2008	Becario FPI-MEC adscrito al proyecto
Calviño	Loira	Adelina	52494402F	Contrato Técnico laboratorio temporal		Trabajos Técnico laboratorio	Inicio de cont. 17/12/2007	Técnico de Investigación y Laboratorio (previsto en la propuesta)

I2. GASTOS DE EJECUCIÓN: MODIFICACIONES DE CONCEPTOS DE GASTO CON RESPECTO A LA SOLICITUD ORIGINAL PARA EL ÚLTIMO PERÍODO DE JUSTIFICACIÓN.

Recuerde que los trasvases entre gastos de personal y gastos de ejecución deben tramitarse de acuerdo con las "Instrucciones para el desarrollo de los proyectos de I+D+i" expuestas en la página web del MICINN.

a) Equipamiento:

En el cuadro adjunto, rellene una línea por **cada equipo adquirido** incluido en la justificación de gastos y **no previsto en la solicitud inicial** que dio lugar a la concesión de la ayuda para el proyecto, y justifique brevemente su adquisición. Si se ha adquirido un equipo en sustitución de otro que figuraba en la solicitud de ayuda inicial (por mejorar sus prestaciones, por obsolescencia del anterior…), indíquelo también en la casilla correspondiente.

Identificación del equipo	Importe	Justificación adquisición	Sustituye a ...(en su caso).

b) Viajes/Dietas:

En el cuadro adjunto se justificará la imputación de gasto en viajes y dietas solo en el caso de que este tipo de gasto **no estuviera previsto en la solicitud inicial**

-Se realizó un gasto de 76,2 euros correspondiente a una visita del la Dra. Julia Kehr (INIA-UPM), especialista en Proteómica, para diseño de experimentos.

c) Material fungible:

Se describirá y razonará en el siguiente cuadro la adquisición del material fungible incluido en la justificación de gastos, sólo cuando este tipo de gasto **no estuviera previsto en la solicitud original.**

d) Varios:

Se describirán en el siguiente cuadro los gastos varios más relevantes incluidos en la justificación de gastos y **no previstos en la solicitud original,** justificando brevemente su inclusión. En este apartado se incluirá, entre otros, al personal externo y, en el caso de que el gasto justificado se refiera a colaboraciones científicas, se identificará al colaborador.

-Se enviaron muestras a Taiwan (1028 euros) y a Dinamarca (19,51 euros), para el envío de muestras a laboratorios especializados de análisis.

FIN DEL INFORME FINAL

Plant Soil (2008) 311:161–172
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REGULAR ARTICLE

Leaf structural changes associated with iron deficiency chlorosis in field-grown pear and peach: physiological implications

Victoria Fernández · Thomas Eichert · Víctor Del Río · Gloria López-Casado · José A. Heredia-Guerrero · Anunciación Abadía · Antonio Heredia · Javier Abadía

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Abstract Plants grown in calcareous, high pH soils develop Fe deficiency chlorosis. While the physiological parameters of Fe-deficient leaves have been often investigated, there is a lack of information regarding structural leaf changes associated with such abiotic stress. Iron-sufficient and Fe-deficient pear and peach leaves have been studied, and differences concerning leaf epidermal and internal structure were found. Iron deficiency caused differences in the aspect of the leaf surface, which appeared less smooth in Fe-deficient than in Fe-sufficient leaves. Iron deficiency reduced the amount of soluble cuticular lipids in peach leaves, whereas it reduced the weight of the abaxial cuticle in pear leaves. In both plant species, epidermal cells were enlarged as compared to healthy leaves, whereas the size of guard cells was reduced. In chlorotic leaves, bundle sheaths were enlarged and appeared disorganized, while the mesophyll was more compacted and less porous than in green leaves. In contrast to healthy leaves, chlorotic leaves of both species showed a significant transient opening of stomata after leaf abscission (Iwanoff effect), which can be ascribed to changes found in epidermal and guard cells. Results indicate that Fe-deficiency may alter the barrier properties of the leaf surface, which can significantly affect leaf water relations, solute permeability and pest and disease resistance.

Keywords Cuticle · Epidermis · Iron deficiency chlorosis · Leaf structure · Transpiration

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Introduction

Iron (Fe) deficiency chlorosis is a common abiotic stress affecting plants in many areas of the world. This physiological disorder is mainly found in crops grown in calcareous and/or alkaline soils and occurs as a result of several causes acting simultaneously (Rombolà and Tagliavini 2006). Although Fe is very abundant in the earth's crust, its availability to plants is often restricted by the very low solubility of Fe(III)-oxides

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Formation of metal-nicotianamine complexes as affected by pH, ligand exchange with citrate and metal exchange. A study by electrospray ionization time-of-flight mass spectrometry

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Nicotianamine (NA) is considered as a key element in plant metal homeostasis. This non-proteinogenic amino acid has an optimal structure for chelation of metal ions, with six functional groups that allow octahedral coordination. The ability to chelate metals by NA is largely dependent on the pK of the resulting complex and the pH of the solution, with most metals being chelated at neutral or basic pH values. *In silico* calculations using pKa and pK values have predicted the occurrence of metal-NA complexes in plant fluids, but the use of soft ionization techniques (e.g. electrospray), together with high-resolution mass spectrometers (e.g. time-of-flight mass detector), can offer direct and metal-specific information on the speciation of NA in solution. We have used direct infusion electrospray ionization mass spectrometry (time-of-flight) ESI-MS(TOF) to study the complexation of Mn, Fe(II), Fe(III), Ni, Cu by NA. The pH dependence of the metal-NA complexes in ESI-MS was compared to that predicted *in silico*. Possible exchange reactions that may occur between Fe-NA and other metal micronutrients as Zn and Cu, as well as between Fe-NA and citrate, another possible Fe ligand candidate in plants, were studied at pH 5.5 and 7.5, values typical of the plant xylem and phloem saps. Metal-NA complexes were generally observed in the ESI-MS experiments at a pH value approximately 1–2 units lower than that predicted *in silico*, and this difference could be only partially explained by the estimated error, approximately 0.3 pH units, associated with measuring pH in organic solvent-containing solutions. Iron-NA complexes are less likely to participate in ligand- and metal-exchange reactions at pH 7.5 than at pH 5.5. Results support that NA may be the ligand chelating Fe at pH values usually found in phloem sap, whereas in the xylem sap NA is not likely to be involved in Fe transport, conversely to what occurs with other metals such as Cu and Ni. Some considerations that need to be addressed when studying metal complexes in plant compartments by ESI-MS are also discussed.

Metals such as Mn, Fe, Ni, Cu or Zn are essential for plants, since they participate in numerous metabolic processes in different plant tissues and cell compartments. When these metals are in short supply, plants show deficiency symptoms such as growth reduction and reduced photosynthesis. However, when metals are in excess oxidative stress and other cellular disturbances occur, and plants develop toxicity symptoms.[1] For these reasons, the processes involved in metal acquisition by roots and transport to the different plant organs must be tightly regulated, so that metals can be available where they are needed and in an appropriate chemical form. The tendency toward a relatively stable equilibrium between these interdependent mechanisms, maintained by physiological processes, is usually called metal homeostasis.

A key element in plant metal homeostasis is the non-proteinogenic amino acid nicotianamine (NA), first discovered by Noma *et al*.[2] Nicotianamine has an optimal structure for chelation of metal ions, with six functional groups that allow octahedral coordination, the distances between functional groups being optimal for the formation of chelate rings. Nicotianamine is known to chelate many metals, including Fe(II) and Fe(III),[3,4] Mn(II), Co(II), Ni(II), Cu(II) and Zn(II).[5,6] The NA stability constants (log K) of the metal-NA complexes with Fe(III), Cu(II), Ni(II), Zn(II), Fe(II) and Mn(II) are 20.6, 18.6, 16.1, 15.4, 12.8 and 8.8, respectively.[3,4,6]

Nicotianamine is thought to be important in the speciation of soluble Fe in different plant compartments,[7] because it is

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Contract/grant sponsor: Spanish Ministry of Education and Science (MEC; co-financed with FEDER); contract/grant number: AGL2006-1416 and AGL2007-61948.

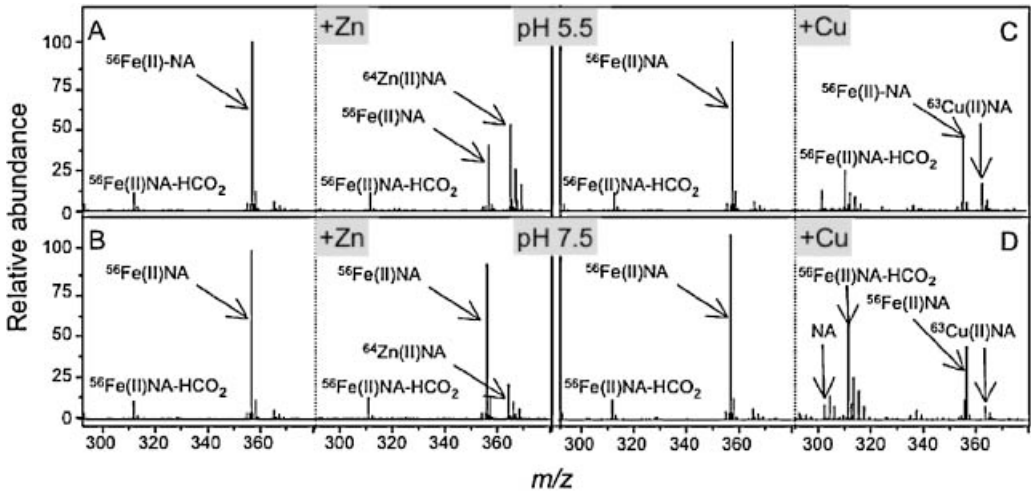

Figure 5. Metal-exchange reactions between Fe(II)-NA and Zn(II) at pH 5.5 (A) and pH 7.5 (B), and between Fe(II)-NA and Cu(II) at pH 5.5 (C) and 7.5 (D). Metal and Fe(II)-NA complex concentrations of the initial solutions were 50 μM.

should be chelating Zn(II) at both pH values. Possibly, the discrepancies between the observed speciation and the *in silico* predictions could be due to the kinetics of the exchange reaction. At pH 5.5, after the addition of Cu(II) to the Fe(II)-NA solution there was a large decrease of the Fe(II)-NA signal, although the signal of Cu(II)-NA was not very large (Fig. 5(C)). This supports the existence of metal exchange, as it could be expected from the values of the stability constants. The low intensity of the Cu(II)-NA complex can be explained by the voltage value used in the experiment (120 V), since the optimal voltage value found for the Cu(II) complex was 90V (Fig. I in the Supplementary Material). Also, signals for free NA and the $[M–H–CO_2]^{-1}$ ion of Fe(II)-NA were observed. At pH 7.5 a similar behaviour was observed, although the peak at m/z 311 corresponding to the $[Fe(II)NA–H–CO_2]^{-1}$ ion was larger than at pH 5.5 (Fig. 5(D)). *In silico* predictions indicate that most of the NA should be chelating Cu(II) at both pH values.

CONCLUSIONS

Results indicate that relatively small changes in pH and changes in the concentrations of citrate and metals can have significant effects in NA speciation in plant fluids such as xylem and phloem sap. In the xylem sap, NA is not likely to complex Fe due to exchange reactions with citrate and other metals, whereas it could chelate other metals such as Cu and Ni. In the phloem sap, NA could still be a good candidate to chelate Fe, specially in the Fe(II) form. Some metal-NA complexes, including Fe(II)-NA and Fe(III)-NA, were found by ESI-MS at lower pH values than those estimated *in silico*, and this effect could be only partially explained by the estimated size of the errors associated to measuring pH in organic-solvent-containing solutions. Our work and recent examples of other researchers have shown the feasibility of ESI-MS to study metal-NA complexes within plant fluids, but some drawbacks inherent to the technique need to be addressed: namely, the need to maintain as much as possible the pH of the plant compartment under study through the whole extraction, separation and analysis process, the possible changes in metal-ligand complex chemistry and the difficulty to assess the true pH value in solutions with a considerable amount of organic solvent, and the possibility that metal redox reactions may occur in the ESI process. Our work has also shown that *in silico* predictions may fail to accurately speciate NA in non-equilibrium solutions such as plant fluids. It should also be mentioned that in real plant samples other metals such as Ca and ligands such as glutathione may affect the interpretations proposed here. However, it would be unrealistic to analyze plant fluids by direct infusion ESI-MS due to matrix effects. To avoid matrix interferences a previous separation technique such as liquid chromatography is mandatory.[36] Direct determination of metal-NA complexes in plant fluids may change the current knowledge on the role of NA in micronutrient plant nutrition.

SUPPLEMENTARY MATERIAL

The supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/0951-4198/suppmat/.

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Metabolic responses in iron deficient tomato plants

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Summary

The effects of Fe deficiency on different metabolic processes were characterized in roots, xylem sap and leaves of tomato. The total organic acid pool increased significantly with Fe deficiency in xylem sap and leaves of tomato plants, whereas it did not change in roots. However, the composition of the pool changed with Fe deficiency, with major increases in citrate concentrations in roots (20-fold), leaves (2-fold) and xylem sap (17-fold). The activity of phospho*enol*pyruvate carboxylase, an enzyme leading to anaplerotic C fixation, increased 10-fold in root tip extracts with Fe deficiency, whereas no change was observed in leaf extracts. The activities of the organic acid synthesis-related enzymes malate dehydrogenase, citrate synthase, isocitrate dehydrogenase, fumarase and aconitase, as well as those of the enzymes lactate dehydrogenase and pyruvate carboxylase, increased with Fe deficiency in root extracts, whereas only citrate synthase increased significantly with Fe deficiency in leaf extracts. These results suggest that the enhanced C fixation capacity in Fe-deficient tomato roots may result in producing citrate that could be used for Fe xylem transport. Total pyridine nucleotide pools did not change significantly with Fe deficiency in roots or leaves, although NAD(P)H/NAD(P) ratios were lower in Fe-deficient roots than in controls. Rates of O_2 consumption were similar in Fe-deficient and Fe-sufficient roots, but the capacity of the alternative oxidase pathway was decreased by Fe deficiency. Also, increases in Fe reductase activity with Fe deficiency were only 2-fold higher when measured in tomato root tips. These values are significantly lower than those found in other plant species, where Fe deficiency leads to larger increases in organic acid synthesis-related enzyme activities and flavin accumulation. These data support the hypothesis that

Abbreviations: AOX, alternative oxidase; CS, citrate synthase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDC, pyruvate decarboxylase; PEPC, phospho*enol*pyruvate carboxylase; SHAM, hydroxy-salicylic acid.

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contrast with other species in which increases were more marked and included other organic anions. Malate and citrate increases in leaves from Fe-deficient tomato can be explained by the contribution of two factors: first, the increases measured in the activities of MDH and CS in the same leaves (1.4- and 3.4-fold, respectively, significant at $p<0.10$ and $p<0.05$), and second, an influx of these acids from the root via xylem sap, as proposed to occur in sugar beet and pear Fe-deficient leaves (López-Millán et al., 2000a, 2001).

In conclusion, this work adds further support to the hypothesis that the extent of activation of several metabolic pathways, including carbon fixation via PEPC, organic acid synthesis-related enzymes and O_2 consumption is different among species, and could determine Fe efficiency. Citrate seems to be a central point in the responses of plants to Fe deficiency, since large increases of citrate concentrations in all plant tissues appear to be conserved among species, whereas the degree of elicitation of other responses could differ considerably.

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Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics

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ABSTRACT

The effects of Cd have been investigated in tomato (*Lycopersicon esculentum*) plants grown in a controlled environment in hydroponics, using Cd concentrations of 10 and 100 μM. Cadmium treatment led to major effects in shoots and roots of tomato. Plant growth was reduced in both Cd treatments, leaves showed chlorosis symptoms when grown at 10 μM Cd and necrotic spots when grown at 100 μM Cd, and root browning was observed in both treatments. An increase in the activity of phospho*enol*pyruvate carboxylase, involved in anaplerotic fixation of CO_2 into organic acids, was measured in root extracts of Cd-exposed plants. Also, significant increases in the activities of several enzymes from the Krebs cycle were measured in root extracts of tomato plants grown with Cd. In leaf extracts, significant increases in citrate synthase, isocitrate dehydrogenase and malate dehydrogenase activities were also found at 100 μM Cd, whereas fumarase activity decreased. These data suggest that at low Cd supply (10 μM) tomato plants accumulate Cd in roots and this mechanism may be associated to an increased activity in the PEPC–MDH–CS metabolic pathway involved in citric acid synthesis in roots. Also, at low Cd supply some symptoms associated with a moderate Fe deficiency could be observed, whereas at high Cd supply (100 μM) effects on growth overrule any nutrient interaction caused by excess Cd. Cadmium excess also caused alterations on photosynthetic rates, photosynthetic pigment concentrations and chlorophyll fluorescence, as well as in nutrient homeostasis.

1. Introduction

Cadmium toxicity in crops has become in a serious problem, especially in developed countries. Cadmium accumulation in soils may come from different sources, including air pollutants and soil applications of commercial fertilizers, sewage sludge, manure and lime (McGrath et al., 1994; McLaughlin et al., 1996; Adams et al., 2004; Kidd et al., 2007). Also, industrial effluents may contain a wide variety of pollutants depending on the industries involved, and in many cases high concentrations of heavy metals have been reported (Iribar et al., 2000). In polluted soils, Cd is generally present as free ions or different soluble forms, and its mobility depends on pH (Bingham, 1979) and on the presence of chelating substances and other cations (Hardiman and Jacoby, 1984). Plants can accumulate Cd during plant growth, and the accumulation often occurs in edible parts, thus endangering crop yield and quality and becoming a potential hazard for human and animal health. Cadmium is suggested to cause damage even at very low concentrations, and healthy plants may contain Cd levels that are toxic for mammals (Chen et al., 2007). Moreover, it is widely recognized that Cd taken up by plants is the main source of Cd accumulation in food (Mahaffey et al., 1975; Pinot et al., 2000).

Most of the information available about Cd physiology in plants comes from studies with the Cd-hyperaccumulator *Thlapsi caerulescens* (Lombi et al., 2002) and Cd-tolerant plants such as *Arabidopsis halleri* (Weber et al., 2006; Zhao et al., 2006), whereas less information is available in commercial crops such as tomato. It is commonly assumed that Cd, as well as other heavy metals, are taken up by transporters of essential elements, because of the lack of specificity of these proteins. There is evidence that metal transporters from different families such as ZIP and Nramp are able to transport several divalent cations, including Cd (Korshunova et al., 1999; Pence et al., 2000; Thomine et al., 2000). Also, it has been described that a Ca transport pathway could be involved

Abbreviations: BPDS, bathophenanthroline disulphonate; Chl, chlorophyll; C_i, sub-stomatal CO_2 concentration; CS, citrate synthase; DW, dry weight; *E*, transpiration rate; Φ_{PSII} and Φ_{exc}, actual and intrinsic photosystem II efficiencies, respectively; F_o and F'_o, minimal Chl fluorescence yield in the dark or after light adaptation, respectively; F_m and F'_m, maximal Chl fluorescence yield in the dark or after light adaptation, respectively; FR, far-red; F_S, Chl fluorescence at steady-state photosynthesis; F_v and F'_v, $F_m - F_o$ and $F'_m - F'_o$, respectively; F_v/F_m, maximum potential PSII efficiency; FW, fresh weight; g_S, stomatal conductance; ICDH, isocitrate deshydrogenase; MDH, malate deshydrogenase; NPQ, non-photochemical quenching; PAR, photosynthetic active radiation; PEPC, phospho*enol*pyruvate carboxylase; P_N, net CO_2 uptake rate per unit leaf area; PPFD, photosynthetic photon flux density; PSII, photosystem II; qP, photochemical quenching; V + A + Z, violaxanthin + antheraxanthin + zeaxanthin.

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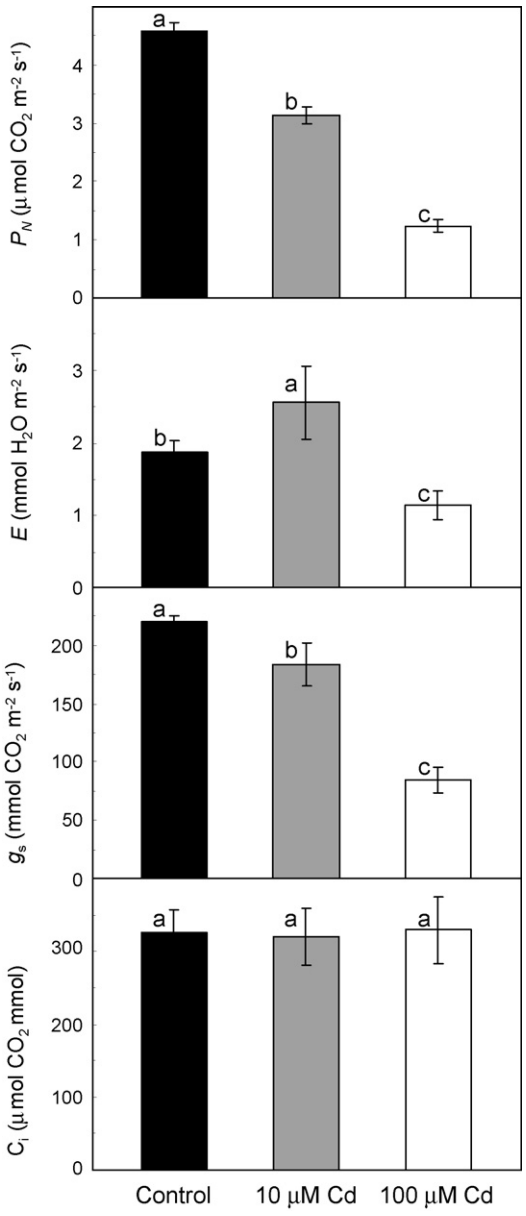

Fig. 5. Gas exchange parameters in leaves of tomato plants grown in absence (control) and in presence of Cd (10 and 100 μM Cd). The incident PPFD was between 130 and 170 μmol m^{-2} s^{-1}. Data are means ± SD of 21 replications (3 batches of plants with 7 replicates per treatment). Columns marked with the same letter are not significantly different (Student's test) at the $p < 0.05$ level.

requirement for Fe was much lower and Fe-deficiency symptoms were no longer evident (Larbi et al., 2002). No de-epoxidation of xanthophyll cycle pigments occurred even when mild leaf chlorosis occurred at 10 μM Cd. The same was observed in sugar beet grown at 50 μM Cd, but not at 10 μM Cd, since at low Cd zeaxanthin and antheraxantin were formed (Larbi et al., 2002). The lack of major effects of Cd on leaf electron transport rates, NPQ and xanthophyll cycle pigments de-epoxidation may support that Cd-treated tomato plants were not affected by photo-inhibitory processes. However, the possibility that the xanthophyll cycle pigments photoprotection mechanism could be inactivated by Cd metal toxicity in tomato (and sugar beet) leaves cannot be ruled out at this stage.

Overall, results suggest that in tomato grown with 10 μM Cd, Cd-induced Fe-deficiency is not the main effect, and that this Cd concentration is enough to lead to direct effects in photosynthesis. However, some signs of a moderate Fe deficiency were present in the 10 μM Cd treatment, including the moderately low leaf Fe concentrations (50 μg g^{-1} DW) and the mild chlorosis symptoms. Root Fe reductase activity, a known sign of Fe-stress, decreased in tomato plants grown with Cd. Heavy metals have been described previously, on one hand, to increase root ferric chelate reductase activity because of the limitations imposed to Fe trafficking (Larbi et al., 2002), and on the other hand, to decrease this activity due to a direct effect on the reductase enzyme itself (Alcántara et al., 1994; Chang et al., 2003). In the case of tomato, the direct effect of Cd apparently overrules any promoting effect associated to the mild Fe deficiency caused by Cd.

Other alterations in micronutrient concentrations were observed in tomato plants grown with Cd. Major changes included increases in root Fe, Zn and Cu concentrations when plants were grown with 100 μM Cd and a progressive decrease in Mn concentration with increased Cd supply. Synergistic effects between high Cd concentrations and Fe, Cu and Zn root accumulation have been described before for other species (Larbi et al., 2002; Liu et al., 2003) and in agreement with our data no such changes were observed in tomato roots with low Cd supply (Dong et al., 2006). It has been hypothesized that increases in root concentrations of divalent metals could be partially explained by Cd interference in nutrient uptake by affecting the permeability of plasma membranes (Dong et al., 2006). Also, the lack of specificity of members of several families of divalent metal transporters such as ZIP and Nramp could contribute to this fact. For instance, a member of the ZIP family, ZIP9, is induced in *Arabidopsis thaliana* roots in presence of Cd and is constitutively highly expressed in the roots of the accumulator *A. halleri* (Weber et al., 2006). Antagonistic effects between high Cd concentrations and Mn uptake and transport have been reported in many studies (Hernández et al., 1998; Larbi et al., 2002; Dong et al., 2006; Wu et al., 2007), and a reduction in Cd uptake in presence of Mn has been observed (Cataldo et al., 1981). Reports are contradictory regarding the influence of Cd in shoot micronutrient concentrations; for instance $CdCl_2$ decreased Zn and Cu shoot concentrations in sugar beet (Larbi et al., 2002) whereas in tomato no differences were found (Dong et al., 2006, and this work). Differences among species may arise from the different micronutrient homeostasis and Cd detoxification mechanisms; for instance, at low Cd sugar beet is able to mobilize more Cd to shoots than tomato (up to 50 and 34% of total Cd, respectively).

In conclusion, at low Cd levels (10 μM), which are possible to occur in intensive horticultural systems, tomato could transport amounts of Cd to the shoot that might become a health hazard, and although decreases in growth and photosynthesis and a mild chlorosis could be noticed quite easily, the detection of Cd excess can only be done through plant tissue Cd analysis. At low Cd supply, tomato plants accumulated Cd in roots and this mechanism may be associated to an increased activity in the PEPC–MDH–CS metabolic pathway via citric acid synthesis by roots. Also, alterations in photosynthesis and photosynthetic pigments may suggest a direct toxic effect of Cd on photosynthesis in addition to a mild Cd-induced Fe deficiency. At high Cd supply (100 μM) effects on growth overrule any nutrient interaction caused by excess Cd.

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RESEARCH PAPER

Effects of zinc toxicity on sugar beet (*Beta vulgaris* L.) plants grown in hydroponics

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ABSTRACT

The effects of high Zn concentration were investigated in sugar beet (*Beta vulgaris* L.) plants grown in a controlled environment in hydroponics. High concentrations of Zn sulphate in the nutrient solution (50, 100 and 300 μm) decreased root and shoot fresh and dry mass, and increased root/shoot ratios, when compared to control conditions (1.2 μm Zn). Plants grown with excess Zn had inward-rolled leaf edges and a damaged and brownish root system, with short lateral roots. High Zn decreased N, Mg, K and Mn concentrations in all plant parts, whereas P and Ca concentrations increased, but only in shoots. Leaves of plants treated with 50 and 100 μm Zn developed symptoms of Fe deficiency, including decreases in Fe, chlorophyll and carotenoid concentrations, increases in carotenoid/chlorophyll and chlorophyll *a*/*b* ratios and de-epoxidation of violaxanthin cycle pigments. Plants grown with 300 μm Zn had decreased photosystem II efficiency and further growth decreases but did not have leaf Fe deficiency symptoms. Leaf Zn concentrations of plants grown with excess Zn were high but fairly constant (230–260 $\mu g \cdot g^{-1}$ dry weight), whereas total Zn uptake per plant decreased markedly with high Zn supply. These data indicate that sugar beet could be a good model to investigate Zn homeostasis mechanisms in plants, but is not an efficient species for Zn phytoremediation.

INTRODUCTION

Zinc is essential for cell physiological processes, and in most living organisms it is the second most abundant transition metal after Fe. Zinc has no redox activity but plays structural and/or catalytic roles in many processes, and is the only metal present in all enzyme classes (Vallee & Auld 1990; Barak & Helmke 1993). Zinc is also essential for plants, and Zn deficiency is a common problem in plants grown in high pH, calcareous soils (as it also found with Fe) (Casona *et al.* 1991; Cakmak *et al.* 1996), whereas in low pH soils Zn availability is generally high (Foy *et al.* 1978; Chaney 1993). When present at high concentrations, Zn can be toxic, and plants affected may show symptoms similar to those found in other heavy metal toxicities, such as those of Cd or Pb (Foy *et al.* 1978). In most cases, excess Zn generates reactive oxygen species and/or displaces other metals from active sites in proteins. Zinc toxicity also induces chlorosis in young leaves, and this has been suggested to result from a Zn-induced Fe or Mg deficiency, based on the fact that the three metals have similar ion *radii* (Marschner 1995). Other common Zn toxicity effects include decreases in tissue water content and changes in the P and Mg concentrations in plant tissues (Marschner 1995).

The mechanisms controlling Zn homeostasis in plants are still not fully known (Hacisalihoglu *et al.* 2004; Broadley *et al.* 2007; Kramer *et al.* 2007). Plant roots acquire Zn predominantly as the divalent ion, and the metal is then distributed throughout the whole plant in a complex series of processes. Several families of plant metal transporters have been identified in recent years (Kramer *et al.* 2007), with at least three being involved in Zn transport through membranes: ZIP (IRT-like proteins) (Grotz *et al.* 1998; Wintz *et al.* 2003), CDF (Cation Diffusion Facilitator proteins) (Blaudez *et al.* 2003; Kim *et al.* 2004; Kobae *et al.* 2004; Kramer 2005) and P_{1B}-type ATPases (HMAs, metal transporting ATPases) (Hussain *et al.* 2004; Papoyan & Kochian 2004; Verret *et al.* 2004; Mills *et al.* 2005). The roles these transporters play in Zn

236 to 259 $\mu g \cdot g^{-1}$ DW, and Zn allocation to the shoot was little changed. Therefore, sugar beet can be used as a good model plant to study Zn homeostasis in non-hyperaccumulator plant species. Treatment with two doses of sewage sludge containing Zn and other metals led to sugar beet leaf Zn concentrations of approximately 75 $\mu g \cdot g^{-1}$ DW (Singh & Agrawal 2007). In four *Datura* species grown in vermiculite with high concentrations of Zn in the nutrient solution, leaf Zn concentrations were higher than 300–500 $\mu g \cdot g^{-1}$ DW (Vaillant *et al.* 2005). Different mechanisms have been implicated in the regulation of Zn homeostasis, including downregulation of Zn root uptake systems, Zn chelation by low-molecular weight compounds, and/or subcellular compartmentalisation of excess Zn in the apoplast or vacuoles (Hall 2002). Most of the knowledge to date comes from the study of Zn hyperaccumulator plants, such as *Arabidopsis halleri* and *Thlapsi caerulescens*, in which the major strategy for Zn detoxification consists of metal sequestration in vacuoles from mesophyll cells (Lasat *et al.* 1998; Kobae *et al.* 2004; Kupper *et al.* 2004). However, in non-hyperaccumulators other mechanisms might make a higher contribution to cope with excess Zn. Further studies should be directed to analyse, in sugar beet plants treated with excess Zn, the underlying mechanisms that contribute in this species to control Zn homeostasis, with special emphasis on chemical speciation in xylem sap, subcellular Zn distribution and the storage forms in vacuoles and/or apoplastic compartments.

Data presented here indicate that sugar beet is not a Zn accumulator and is unlikely to have potential for Zn bioremediation. This contrasts with data obtained for Cd and Pb with the same plant species (Larbi *et al.* 2002). This results from the fact that high Zn causes a very strong growth decrease, whereas the concentration of Zn in tissues does not increase greatly (*i.e.*, 260 μg Zn g^{-1} DW in plants grown with 300 μM Zn in the nutrient solution). In consequence, the amount of Zn removed per plant was larger with 50 than with 100 or 300 μM Zn in the nutrient solution. This is in contrast to Cd and Pb, since sugar beet shoots may contain up to 500 μg Cd or Pb g^{-1} DW in plants grown with 50 μM Cd or 2 mM Pb in the nutrient solution (Larbi *et al.* 2002). Sugar beet plants took up approximately 4, 2 and 1% of the total nutrient solution Zn in the treatments containing 50, 100 and 300 μM Zn, respectively. Considering a possible shoot dry mass production of 1.5 $ton \cdot ha^{-1}$ (usual values in a sugar beet commercial crop are approximately 3 $ton \cdot ha^{-1}$), and a possible leaf Zn concentration of 200 $\mu g \cdot g^{-1}$ DW, metal removal would be approximately 300–600 g Zn ha^{-1}, an amount clearly insufficient for Zn phytoremediation.

In summary, Zn toxicity in sugar beet caused a range of effects, depending on the Zn concentration in the nutrient solution. These included growth decreases, changes in the concentrations of different elements and signs of increased photosynthetic energy dissipation through the violaxanthin cycle pigments. At the highest Zn concentrations tested, plants exhibited a different adaptation strategy, closing stomata and further reducing growth.

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Research article

Time course induction of several key enzymes in Medicago truncatula roots in response to Fe deficiency

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ABSTRACT

Medicago truncatula constitutes a good model for Strategy I plants, since when this plant is challenged with Fe shortage the most important root physiological responses induced by Fe deficiency are developed, including the yellowing of root tips. A better understanding of the mechanisms involved in root adaptation to Fe deficiency in *M. truncatula* may strengthen our ability to enhance Fe efficiency responses in other plant species, especially in different agronomically relevant legumes. Riboflavin concentration, phospho*enol*pyruvate carboxylase (EC 4.1.1.31) and Fe reductase activities, and acidification capacity have been determined in *M. truncatula* roots at different time points after imposing Fe deficiency. Root riboflavin concentrations increased with Fe deficiency and concomitantly *MtDMRL* was upregulated at the transcriptional level, supporting a role for flavins in the Fe deficiency response. Root Fe reductase and phospho*enol*pyruvate carboxylase activities as well as acidification capacity were higher in roots of Fe-deficient than in control plants, and the corresponding genes, *MtFRO1*, *MtPEPC1* and *MtHA1* were also upregulated by Fe deficiency. Expression of these genes and their corresponding physiological activities followed different patterns over time, suggesting the existence of both transcriptional and post-transcriptional regulation.

1. Introduction

Iron is an essential micronutrient for all living organisms, including plants, since it takes part in fundamental biological redox processes, such as respiration and photosynthesis [21]. Although Fe is the fourth most abundant element in the earth's crust, plant Fe deficiency is a worldwide problem, especially in high pH, calcareous soils. In these soils, Fe is present in oxidized forms with low solubility that are not readily available to plants [19]. Plants can be broadly classified into two groups based on the root mechanism for Fe uptake: Strategy I plants, which include dicotyledonous and non-grass monocotyledonous species, and Strategy II plants, which include Poaceae species (see [15] for a review).

When grown under Fe deficiency, Strategy I plant species develop a series of morphological and biochemical changes that increase their capacity for Fe uptake. Morphological changes include the swelling of root tips and formation of lateral roots, root hairs and transfer cells [16,28]. These changes lead to an increase of the root surface that allows plants to maximize Fe uptake. Biochemical changes usually include i) an enhanced excretion of protons to the rhizosphere (mediated by a plasma membrane-bound H^+-ATPase), which lowers soil pH, in turn increasing Fe(III) solubility [9,26,29]; ii) the induction of a plasma membrane ferric reductase enzyme that reduces Fe(III) to Fe(II) in root epidermal cells [22,28]; iii) the activation of a plasma membrane transport system for Fe(II) uptake [6,11]; and iv) the excretion to the growth medium of compounds such as phenolics, organic acids, and flavins, which may facilitate reduction and solubility of external Fe [32,38].

On the other hand, in Strategy I plants several changes occur at the metabolic level, in order to sustain the increased Fe uptake capacity when Fe is scarce (see [40] for a review). These changes include accumulation of organic acids (reviewed in [1]), shifts in the redox state of the cytoplasm [40] and increases in the activity of phospho*enol*pyruvate carboxylase (PEPC) and different enzymes of the Krebs cycle and the glycolitic pathway [1,40]. All hypotheses proposed so far to explain such metabolic changes grant a key role to PEPC [1,40].

Also, some Strategy I plants, when challenged with Fe deficiency, are able to accumulate and excrete to the growth medium

Abbreviations: BPDS, bathophenanthroline disulphonate; DMRL, 6,7-dimethyl-8-ribityllumazine synthase; PAR, photosynthetic active radiation; PEPC, Phospho*enol*pyruvate carboxylase; PPFD, photosynthetic photon flux density.

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Table 1
Forward and reverse specific primers utilized for the semi-quantitative PCR amplification of the different genes. Annealing temperature (T^m, °C), number of PCR cycles and volume of cDNA (μl) used for amplification and the size of the fragments amplified (bp) are also shown.

	Forward primer	Reverse primer	T^m (°C)	No of cycles	Volume cDNA (μl)	Fragment size (bp)
Actin	AAGAGYTAYGARYTNCCWGATGG	TTRATCTTCATGCTRCTRCTWGGAGC	55	25	1	290
MtFRO	GGTGACACGTGGATCATCTG	TTGCAATCCACAGGAACAAA	55	28	2	239
MtPEPC	ATGGCTACTCGTAACATTGAA	CATTATATGGGACACGTTCGT	65	35	3	825
MtHA1	GCACAAGTATGAGATTGTGAAGA	ATCACCAGTCATGCCACAAA	55	30	2	68
MtDMRL	ATGGCTTTATCTGTTTCCACC	CTAATTCAGATGATGCTCGAA	55	30	2	666

Total RNA from roots was isolated using the RNeasy Plant Mini kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentrations of RNAs were assessed using a UV-2101PC spectrometer (Shimadzu, Kyoto, Japan). The structural integrity of the RNAs was checked with non-denaturing agarose gel and ethidium bromide staining. Two plants per treatment were pooled to extract RNA, and three batches of plants were analyzed. A sample aliquot containing 3 μg total RNA was subjected to reverse transcription with 25 μg mL^{-1} oligo (dT) primer, 0.05 mMdNTP mix, and 15 units Cloned AMV Reverse Transcriptase (InVitrogen, Carlsbad, USA) in a volume of 20 μL, according to the manufacturer's instructions. PCR reactions were carried out with the volumes of resulting cDNA solution indicated in Table 1 using gene specific primers (Table 1). Additional reaction components were 75 mM Tris HCL (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 200 μM dNTPs, 2 mM Cl_2Mg, 1 μM gene specific primers and 0.5 units Taq polymerase (Biotools, Madrid, Spain). PCR cycling conditions and fragment size are listed in Table 1. Amplified products from 15 μl of PCR reaction were visualized on a 1% TBE agarose gel containing ethidium bromide. Bands were photographed and band area measured using the Quantity One 4.5.1 Chemidoc EQ software (Bio-Rad, CA, USA), using actin as housekeeper. To calculate normalized signal intensities, each band area was divided by the corresponding area of the actin band. The experiment was repeated 3 times and 2 PCR reactions were carried out for each batch of plants.

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Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants

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ABSTRACT

The refining process in the petrochemical industry generates oil refinery sludges, a potentially contaminating waste product, with a high content of hydrocarbons and heavy metals. Faster degradation of hydrocarbons has been reported in vegetated soils than in non-vegetated soils, but the impact of these contaminants on the plants physiology and on their antioxidant system is not well known. In this study, the effect of the addition of petroleum sludge to soil on the physiological parameters, nutrient contents, and oxidative and antioxidant status in alfalfa was investigated. An inhibition of alfalfa growth and an induction of oxidative stress, as indicated by an increase in protein oxidation, were found. Also, the superoxide dismutase isoenzymes, peroxidase, and those enzymes involved in the ascorbate–glutathione cycle showed significant activity increases, parallel to an enhancement of total homoglutathione, allowing plants being tolerant to this situation. This information is necessary to establish successful and sustainable plant-based remediation strategies.

1. Introduction

The regulations to prevent further undue release of hazardous chemicals into the environment have become stricter over the years, and many countries have started to adopt a tighter line on environmental issues. The currently available decontamination methods are expensive, partially due to the cost of excavating and transporting large quantities of contaminated materials for ex situ treatment, such as chemical inactivation or thermal degradation. Because of this and their low-cost, low maintenance, and environmental friendliness, there is an increasing interest in alternative technologies for in situ applications, in particular those based on biological remediation capability of plants and microorganisms [1]. Phytoremediation may be defined as the use of plants to remove, destroy or sequester hazardous substances from soil environment, and it can be applied to both organic and inorganic pollutants, present in soil substrates, liquid substrates, and air [2]. Its main application has been to remove toxic heavy metals form soil [3]. However, there is a growing interest in broadening this technology to remove/degrade organic pollutants in the environment [4].

The petrochemical industry generates a series of liquid effluents during the refining process which must be treated through depuration processes. The results of these processes are oil refinery sludges, potentially a waste product, that have a high content of petroleum-derived hydrocarbons [5]. Organic contaminants can be stabilized within a soil matrix, taken up by plants and transformed or stored in a non-phytotoxic form. Plants can also stimulate the rhizosphere microbial community that is capable of degrading organic contaminants [6]. Thus, faster degradation of petroleum polycyclic aromatic hydrocarbons (PAHs) has been reported in vegetated soils than in non-vegetated soils, and this has been mediated by root-associated microorganisms [7]. However, it is not completely understood how specific plants increase the remediation of contaminated soils. Kirk et al. [8] showed that alfalfa seems to specifically increase the number of microorganisms capable of degrading complex petroleum hydrocarbons, and in several studies, alfalfa and other plants bearing an abundant root system have been shown to phytoremediate or tolerate several aromatic compounds like phenol, benzene and PAHs [9].

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content was analyzed, we found that the total concentration was higher in plant leaves grown in presence of sludge (Table 6) showing that the synthesis of homoglutathione was induced in the presence of the contaminant. Also, there was a non-significant change in the hGSH/hGSSG ratio, thus the plants grown in contaminated soils showed a lower ratio than plants grown in control soils (Table 6), which indicates an hGSSG accumulation probably because the induced GR activity is insufficient to maintain the homoglutathione pool in its reduced form under these conditions. Thus, it is clear that in spite of the activation of hGSH synthesis, the accumulation of hGSSG reflects its higher sensitivity to be oxidized under these conditions. The preferential oxidation of GSH, in comparison to ASC is in line with the GSH redox couple having a lower redox potential than the ASC [52]. The highly reduced glutathione pool maintained by GR is necessary for active protein function and avoids unspecific formation of mixed disulphide bonds that cause protein inactivation or aggregation. Although the hGSH level increased in alfalfa plants, a rapid ROS mediated oxidation of hGSH could take place in leaves during sludge growth conditions, so the oxidation observed in leaves proteins under sludge might be favoured by the low hGSH/hGSSG found in these plants. However, an important lipid peroxidation increase was not found, by which we interpreted this response to mean that the ASC–GSH cycle plays an important role in the elimination of H_2O_2 in plants under these growth conditions, which may favour plants grown on sludge not presenting a higher important oxidative stress. Apart from the well documented roles of antioxidative enzymes and antioxidants in removing H_2O_2 and other ROS, the importance of glutathione system in relation to others components of the photoprotective and antioxidative defence system should be taken into account. There is a strong interaction between oxidants and antioxidants at the level of gene expression and translation. This implies that there is considerable overlap in the signal transduction cascades that induce GSH synthesis and those involved in defence functions that use GSH, such as glutathione-*S*-transferases (GST) whose primary biochemical function is conjugation, either of xenobiotics or of intermediates and secondary metabolites [55]. On the other hand, the increased demand for GSH in response to metal-induced oxidative stress can be accounted for by activation of pathways involved in sulphur assimilation and cysteine biosynthesis [56]. Plants also contain heavy-metal-binding peptides termed phytochelatins (PCs) (or homo-phytochelatins (h-PC) in leguminous) whose chemical structure suggests that they are not formed as direct results of expression of a metal tolerance gene, but rather as a products of a biosynthetic pathway, with GSH (or hGSH) being the most likely precursor [17,57]. Pb^{2+} and Zn^{2+} whose concentrations increased in sludge, have been reported to be two of the most active metals ions in provoking PC synthesis in plants when exposing plant cells at non-toxic concentrations [58].

4. Conclusions

The presence of sludge in soil induces in alfalfa plants an oxidative stress, as indicated by an increase in protein oxidation, with the alfalfa plants being tolerant to this situation. The oxidative stress could be provoked by the direct toxicity of the petroleum sludge in the soil and/or a result of the contaminant properties that alter the physical and chemical properties of the soil, potentially affecting oxygen transfer, available water uptake, and nutrient mobility. Our data support the idea that contaminants from the petroleum sludge can be used to establish a vegetated cover, and the increased tolerance in plants to the presence of this hazardous material can be achieved through increased tolerance of oxidative stress, enhancing antioxidant enzyme activity and avoiding significant sludge-induced oxidation of their ascorbate and (homo)glutathione pools.

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RCM

Determination of *o,o*EDDHA – a xenobiotic chelating agent used in Fe fertilizers – in plant tissues by liquid chromatography/electrospray mass spectrometry: overcoming matrix effects

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The Fe(III)-chelate of ethylenediamine-*N,N′*-bis(*o*-hydroxyphenylacetic) acid (*o,o*EDDHA) is generally considered as the most efficient and widespread Fe fertilizer for fruit crops and intensive horticulture. The determination of the xenobiotic chelating agent *o,o*EDDHA inside the plant is a key issue in the study of this fertilizer. Both the low concentrations of *o,o*EDDHA expected and the complexity of plant matrices have been important drawbacks in the development of analytical methods for the determination of *o,o*EDDHA in plant tissues. The determination of *o,o*EDDHA in plant materials has been tackled in this study by liquid chromatography coupled to mass spectrometry using several plant species and tissues. Two types of internal standards have been tested: Iron stable isotope labeled compounds and a structural analogue compound, the Fe(III) chelate of ethylenediamine-*N,N′*-bis(2-hydroxy-4-methylphenylacetic) acid (*o,o*EDDHMA). Iron stable isotope labeled internal standards did not appear to be suitable because of the occurrence of isobaric endogenous compounds and/or isotope exchange reactions between plant native Fe pools and the Fe stable isotope of the internal standard. However, the structural analogue Fe(III)-*o,o*EDDHMA is an adequate internal standard for the determination of both isomers of *o,o*EDDHA (*racemic* and *meso*) in plant tissues. The method was highly sensitive, with limits of detection and quantification in the range of 3–49 and 11–162 pmol g^{-1} fresh weight, respectively, and analyte recoveries were in the range of 74–116%. Using this methodology, both *o,o*EDDHA isomers were found in all tissues of sugar beet and tomato plants treated with 90 μM Fe(III)-*o,o*EDDHA for 24 h, including leaves, roots and xylem sap. This methodology constitutes a useful tool for studies on *o,o*EDDHA plant uptake, transport and allocation. Copyright © 2009 John Wiley & Sons, Ltd.

Fertilizers containing Fe(III)-chelate derivatives from synthetic aminopolycarboxilate strong binding chelating agents have been used to alleviate Fe-deficiency problems in fruit crops and intensive horticultural systems since the 1950s.[1] Specifically, the synthetic Fe(III)-chelate of ethylenediamine-*N,N′*-bis(*o*-hydroxyphenylacetic) acid, commonly known as Fe(III)-*o,o*EDDHA, is considered by many authors as the most efficient Fe(III)-chelate to control Fe chlorosis in crops grown in calcareous soils.[2] The presence of *o,o*EDDHA in plants was first proposed from ^{14}C measurements in plant tissues treated with ^{14}C-labeled Fe(III)-*o,o*EDDHA, including leaves, roots, stems and xylem sap exudate from soybean,[3,4] bean,[4] pea, peanut, sunflower, millet, wheat and corn.[5] In spite of the wide use and high efficiency of these Fe(III)-chelates, the mechanisms of plant uptake, transport and allocation are not yet completely elucidated.[6] This is in part due to the lack of analytical methodologies capable of determining, in a specific way, the very low concentrations of Fe(III)-chelate occurring in complex matrices such as plant extracts.

Few attempts have been made until now to determine directly *o,o*EDDHA in plant tissue extracts. Extraction and determination of Fe(III)-*o,o*EDDHA has been reported only three times, carrying out quantification always by spectrophotometric detection in the visible (VIS) spectral range. First, Fe(III)-*o,o*EDDHA was extracted from leaves and stems of tomato with a mixture of water and amyl alcohol, and quantified directly in the plant extract by spectrophotometric detection at 480 nm.[7] Using tobacco leaves, the same extraction procedure for Fe(III)-*o,o*EDDHA was modified by the addition of lead acetate to reduce interfering components from plant tissues.[8] More recently, *o,o*EDDHA

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used by Bienfait *et al.* apparently seem to facilitate a preferential occurrence of the *meso* isomer.[9]

Results show for the first time that Fe(III)-*o,p*EDDHA is also present in leaves, roots and xylem sap of plants treated with commercial Fe(III)-EDDHA products (Fig. 3). However, quantification was not possible because of the lack of commercially available standards. Further research is needed to design and validate an appropriate methodology to determine this compound.

CONCLUSIONS

The method developed permits the determination by HPLC/ESI-MS of the xenobiotic *o,o*EDDHA chelating agent used in Fe fertilizers, with extreme selectivity, high sensitivity and sufficient accuracy and reproducibility, in a wide range of species and plant tissues. Samples tested include sugar beet leaves and roots, tomato leaves and roots and peach leaves and fruits. The results presented in this paper demonstrate the need for a careful evaluation and proper choice of the internal standard (IS) used for quantification in complex matrices such as plant materials, when using HPLC/ESI-MS-based methods. Iron stable isotope labeled Fe-*o,o*EDDHA does not appear to be a suitable IS, mainly because of the occurrence of isotope exchange reactions during extraction and/or sample treatment. An adequate IS would probably be any ^{13}C-, ^{15}N- or ^{17}O-stable isotope labeled chelating agent (*o,o*EDDHA), but they are not commercially available. A structural analogue, one of the Fe(III)-*o,o*EDDHMA isomers, has been confirmed to be an adequate IS for *o,o*EDDHA determination in plant tissues by HPLC/ESI-MS, therefore constituting a useful tool for studies on *o,o*EDDHA plant uptake, transport and allocation. *o,o*EDDHA was found in all plant tissues tested in tomato and sugar beet plants treated with moderate (90 μM) Fe(III)-*o,o*EDDHA doses for only one day.

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Changes in iron and organic acid concentrations in xylem sap and apoplastic fluid of iron-deficient *Beta vulgaris* plants in response to iron resupply

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ABSTRACT

In this study, the effects of Fe resupply on the composition of the xylem sap and apoplastic fluid of Fe-deficient sugar beet plants were investigated. Experiments were carried out in growth chambers with plants grown in hydroponics, and Fe resupply to Fe-deficient plants was carried out by adding 45 μM Fe(III)–EDTA to the nutrient solution. In the short term (within 24 h), Fe resupply caused marked changes in the xylem sap and apoplastic fluid composition and in leaf physiological parameters when *de novo* chlorophyll (Chl) synthesis was still beginning. Major changes included: (i) 10- and 5-fold increases in Fe concentrations in apoplastic fluid and xylem sap, respectively; (ii) marked decreases in the concentrations of organic acids in apoplastic fluid, but not in xylem sap and (iii) large decreases in the citrate/Fe ratios, both in apoplastic fluid and in xylem sap. Two to four days after Fe resupply, xylem sap and apoplastic fluid Fe and organic acid concentrations and pH reached values similar to those obtained in Fe-sufficient leaves. Leaf mesophyll ferric chelate-reductase (FC-R) activities and photosynthetic rates increased gradually during recovery from Fe deficiency.

Introduction

Iron (Fe) is an essential nutrient for plants, and is required for a wide range of biological functions (Marschner, 1995). In Fe-deficient plants, plant growth and development are compromised and leaves have low photosynthesis rates (Abadía, 1992; Larbi et al., 2006). At the root level, Fe deficiency induces a number of physiological and biochemical responses in many species, in what is called "Strategy I" response (Hell and Stephan, 2003; Schmidt, 2006). Iron-deficient plants accumulate organic acids, mainly citrate (Cit) and malate, in roots, xylem sap, leaf apoplastic fluid and whole leaves (Nikolic and Römheld, 1999; López-Millán et al., 2000b, 2001a, 2009). In roots of Fe-deficient plants, CO_2 dark fixation and organic acid synthesis increase, likely due to the major increase in the activities of phospho*enol*pyruvate carboxylase (PEPC) and other enzymes (Rabotti et al., 1995; De Nisi and Zocchi, 2000; López-Millán et al., 2000a; Rombolà et al., 2002). The changes induced by Fe deficiency on mitochondrial structure and function also indicate increased communication between the cytosolic and mitochondrial pools of organic acids (Vigani et al., 2009).

Organic acid accumulation in Fe-deficient plants can improve long-distance Fe transport (López-Millán et al., 2000a, 2001b), and since the production of organic acids is protogenic, it could also promote control of cytosolic pH and feed the increased activity of the plasma membrane (PM) H^+-ATPase (Zocchi, 2006). Another possible function of the organic acid export from roots to leaves is the use of C compounds for basic leaf maintenance processes, such as respiration, when photosynthesis is impaired (Abadía et al., 2002). Also, the excretion of organic acids from roots to the rhizosphere can improve Fe availability (Tyler and Ström, 1995; Jones, 1998).

Iron resupply to Fe-deficient plants leads to increases in chlorophyll (Chl) concentrations and photosynthetic activity within a few days in annual species (Nishio et al., 1985; Larbi et al., 2004) and within weeks in trees (Larbi et al., 2003). Iron resupply to Fe-deficient plants also decreases organic acid concentrations, mainly Cit and malate, in roots, xylem sap and whole leaves (López-Millán et al., 2001a, 2001c). In the short term, the effects of Fe resupply have been less studied, although there is a lag-phase of 1–2 d in which leaf Fe concentrations increase rapidly (Thoiron et al., 1997; López-Millán et al., 2001a), whereas Chl concentrations increase much more slowly (Nishio and Terry, 1983; Larbi et al., 2004). Even with the minor Chl increases found after 24 of Fe resupply, a marked shift in the de-epoxidation state of the xanthophyll cycle pigments occurs (Larbi et al., 2004), suggesting that major changes in leaf metabolism occur a short time after Fe resupply.

Abbreviations: Chl, chlorophyll; FC-R, ferric chelate-reductase; PEPC, phospho*enol*pyruvate carboxylase; PM, plasma membrane

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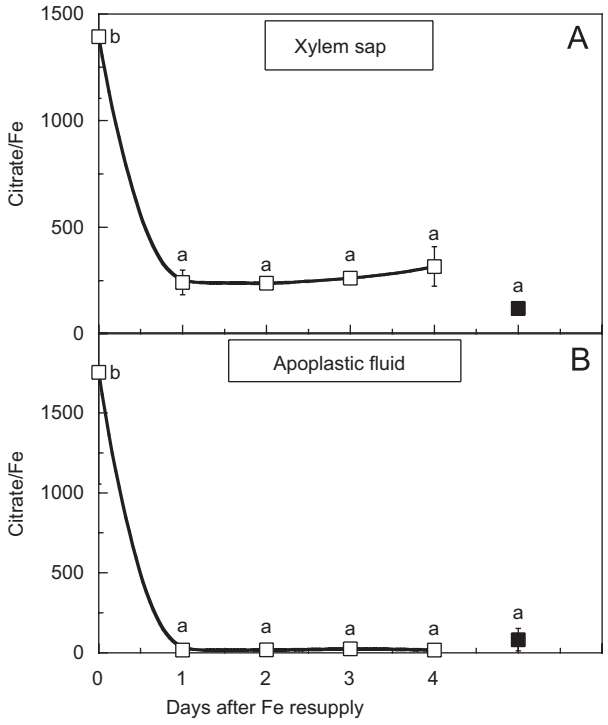

Fig. 6. Citrate:Fe ratios in xylem sap (A) and leaf apoplastic fluid (B) after adding Fe to the nutrient solution. Citrate:Fe xylem sap and apoplastic fluid in Fe-sufficient plants (solid square on the right side), Fe-deficient plants (open square at day 0) and Fe-deficient plants resupplied with Fe (open squares). Data are means ± SE of 3 replicates. Values with the same letter were not significantly (Duncan's test) different at the $p \leq 0.05$ probability level.

known to decrease with Fe deficiency (De la Guardia and Alcántara, 1996; Gónzalez-Vallejo et al., 2000; Rombolà et al., 2000; Larbi et al., 2001). The changes observed in the composition of apoplastic fluid after 24 h of Fe resupply in this study could enhance FC-R activities because: (i) higher FC-R activities will be triggered by the increases in the concentrations of the unknown Fe-containing substrate(s); (ii) the marked decreases in apoplastic fluid Cit/Fe ratios (from approximately 1700 to 50; Fig. 6B) are likely to improve Fe uptake by mesophyll cells, since leaf PM FC-R activity increases 10-fold when the Cit/Fe molar ratio decreased from 500 to 50 (González-Vallejo et al., 1999) and (iii) the optimal mesophyll FC-R activity (measured in excised leaf disks with 500 μM Fe-EDTA at pH 6.5) also increased by 10–20% after 24 h of Fe resupply (although this increase was only significant at $p < 0.10$; Fig. 4), possibly due to increases in reducing power availability associated with the increase in photosynthetic rates (Fig. 5).

One day after Fe resupply, organic acid concentrations had decreased in apoplastic fluid (this study) and whole leaves (López-Millán et al., 2001a), but did not change markedly in xylem sap (this study) and whole roots (López-Millán et al., 2001c). This suggests that, at this short Fe resupply time, leaf organic acids are actively consumed (depleting apoplastic and symplastic pools), while the transport of organic acids from the roots to the shoots *via* xylem (*i.e.*, anaplerotic, non-autotrophic C export) is still similar to that occurring in Fe-deficient plants. The reason for the leaf organic acid consumption upon Fe resupply is not known, although the rapid change that occurs in the thylakoid xanthophyll pigment de-epoxidation status upon Fe resupply (Larbi et al., 2004) suggests drastic pH changes in the lumen and possibly other leaf compartments. A decrease in the leaf activities of PEPC, MDH and G-6-P-DH and an increase in the activity of fumarase have also been observed after 24 h of Fe resupply in sugar beet (López-Millán et al., 2001a).

After 2–4 d of Fe resupply, the characteristics of xylem sap and apoplastic fluid approached those of the Fe-sufficient plants. Xylem sap and apoplastic Fe and organic acid concentrations had decreased towards values found in the controls, whereas pH values had also increased to values only slightly lower than those obtained in Fe-sufficient controls. Citrate/Fe ratios remained stable at 2–4 d after Fe resupply both in xylem sap and apoplastic fluid, with values similar to those of Fe-sufficient plants (Fig. 6). The decrease in xylem C transport at these longer Fe resupply times (2–4 d) is likely to be associated with the progressive decreases in root enzymatic activities involved in organic acid metabolism triggered by Fe resupply (López-Millán et al., 2001c). The gradual improvement of leaf photosynthetic rates with re-greening after Fe resupply would make non-autotrophic C export from roots to leaves unnecessary.

In summary, Fe resupply to Fe-deficient sugar beet plants, *via* increases in Fe concentrations in the nutrient solution, caused marked changes within 24 h, when *de novo* Chl synthesis was still beginning. Changes found include: (i) large increases in Fe concentrations in apoplastic fluid and xylem sap; (ii) a marked decrease in the concentrations of organic acids in apoplastic fluid, but not in xylem sap; (iii) marked changes in the Cit/Fe ratios, both in apoplastic fluid and in xylem sap and (iv) increases in leaf mesophyll FC-R activities and photosynthetic rates. Later on, 2–4 d after Fe resupply, Fe and organic acid concentrations and pH in xylem sap and apoplastic fluid shifted towards values similar to those obtained in Fe-sufficient leaves.

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RCM

Electrospray ionization collision-induced dissociation mass spectrometry: a tool to characterize synthetic polyaminocarboxylate ferric chelates used as fertilizers

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Fertilizers based on synthetic polyaminocarboxylate ferric chelates have been known since the 1950s to be successful in supplying Fe to plants. In commercial Fe(III)-chelate fertilizers, a significant part of the water-soluble Fe-fraction consists of still uncharacterized Fe byproducts, whose agronomical value is unknown. Although collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) is a valuable tool for the identification of such compounds, no fragmentation data have been reported for most Fe(III)-chelate fertilizers. The aim of this study was to characterize the CID-MS^2 fragmentation patterns of the major synthetic Fe(III)-chelates used as Fe-fertilizers, and subsequently use this technique for the characterization of commercial fertilizers. Quadrupole-time-of-flight (QTOF) and spherical ion trap mass analyzers equipped with an electrospray ionization (ESI) source were used. ESI-CID-MS^2 spectra obtained were richer when using the QTOF device. Specific differences were found among Fe(III)-chelate fragmentation patterns, even in the case of positional isomers. The analysis of a commercial Fe(III)-chelate fertilizer by high-performance liquid chromatography (HPLC) coupled to ESI-MS(QTOF) revealed two previously unknown, Fe-containing compounds, that were successfully identified by a comprehensive comparison of the ESI-CID-MS^2(QTOF) spectra with those of pure chelates. This shows that HPLC/ESI-CID-MS^2(QTOF), along with the Fe(III)-chelate fragmentation patterns, could be a highly valuable tool to directly characterize the water-soluble Fe fraction in Fe(III)-chelate fertilizers. This could be of great importance in issues related to crop Fe-fertilization, both from an agricultural and an environmental point of view.

Iron is an essential micronutrient for plants, required for important metabolic processes such as respiration, photosynthesis, nitrogen fixation and the synthesis of DNA and hormones.[1] Iron deficiency (also called Fe chlorosis) is a widespread nutritional disorder that limits crop yields in many agricultural areas of the world. Since the incidence of Fe-deficiency in crops has increased markedly in recent years,[2] the use of Fe-fertilizers is now greater than ever. The efficiency of Fe-fertilizers derived from synthetic polyaminocarboxylate Fe(III)-chelates has been known since the 1950s. The application of these Fe(III)-chelates is considered to be the most effective way to control Fe-deficiency and, in spite of the high cost, these fertilizers are now commonly used in soil-less horticulture as well as in high value field-grown crops.[3]

The synthetic polyaminocarboxylate compounds used to produce Fe-fertilizers are strong binding chelating agents from the ethylenediaminecarboxylic acid family, and include ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEEDTA), cyclohexane-1,2-diaminetetraacetic acid (CDTA), ethylenediamine-*N*,*N*′-bis(*o*-hydroxyphenylacetic) acid (*o*,*o*EDDHA), ethylenediamine-*N*,*N*′-bis(2-hydroxy-4-methylphenylacetic) acid (EDDHMA), ethylenediamine- *N*,*N*′-bis(5-carboxy-2-hydroxyphenylacetic) acid (EDDCHA) and ethylenediamine- *N*,*N*′-bis(2-hydroxy-5-sulphophenylacetic) acid (EDDHSA). All these compounds have high denticity (5 to 8 donor groups available for metal chelation), high affinity for Fe(III), and a structure that allows the formation of highly stable Fe(III)-chelate complexes via simultaneous coordination of several donor groups in a given chelating agent molecule to a single Fe(III) atom. Therefore, the most common coordination arrangement described for the chelation of Fe(III) by these chelating agents is a mononuclear Fe(III)-chelate complex with 1:1 stoichiometry, where Fe is generally found in a six-coordinate, roughly octahedral field, with the chelating agent coordinating as a sexadentate one.

In spite of the wide use of these fertilizers, the biological and environmental implications of this agronomical practice are still not fully known, with most of the studies being focused on Fe(III)-EDTA and Fe(III)-DTPA. These

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(persistence, mobility, metal mobilization, etc.) points of view.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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Using a dual-stable isotope tracer method to study the uptake, xylem transport and distribution of Fe and its chelating agent from stereoisomers of an Fe(III)-chelate used as fertilizer in Fe-deficient Strategy I plants

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A dual-stable isotope tracer experiment was carried out with Fe-deficient sugar beet plants grown hydroponically and resupplied with differentially Fe labeled *racemic* and *meso* Fe(III)-chelates of the ethylendiamine di(*o*-hydroxyphenylacetic) acid (*o*,*o*EDDHA). No short-term Fe isotope exchange reactions occurred in the nutrient solution and plants did not discriminate between ^{54}Fe and ^{57}Fe. After 3–6 h, stable Fe isotopes, chelating agents and chelates were analyzed in roots, xylem sap and leaves by ICP-MS and HPLC-ESI/TOFMS. Ferric chelate reductase rates, xylem transport and total uptake were 2-fold higher with the *meso* isomer than with the *racemic* one. Both chelating agent isomers were incorporated and distributed by plants at similar rates, in amounts one order of magnitude lower than those of Fe. After 6 h of Fe resupply, most of the Fe acquired was localized in roots, whereas most of the chelating agent was in leaves. In a separate experiment, Fe-deficient sugar beet and tomato plants were treated with different concentrations of Fe(III)-*o*,*o*EDDHA (with a *meso*/*racemic* ratio of 1). The xylem sap Fe concentration at 24 h was unaffected by the chelate concentration, with xylem Fe(III)-*o*,*o*EDDHA accounting for 1–18% of total Fe and xylem *meso*/*racemic* ratio close to 1. Although most of the Fe coming from Fe(III)-*o*,*o*EDDHA was taken up through a reductive dissociative mechanism, a small part of the Fe may be taken up *via* non-dissociative mechanisms.

Introduction

Ethylendiamine di(*o*-hydroxyphenylacetic) acid (*o*,*o*EDDHA) is a xenobiotic water-soluble compound, known for its high Fe(III)-binding ability.[1] In biomedical studies, Fe(III)-*o*,*o*EDDHA has been used in magnetic resonance imaging[2] and positron emission tomography.[3] In agricultural practice, the deficiency of Fe in crops (also called Fe-chlorosis) is commonly remedied by soil applications of Fe(III)-*o*,*o*EDDHA.[4] Iron chlorosis is characterized by a marked decrease in leaf chlorophyll, that in turn results in losses in crop quality and yield;[5] the most common cause of Fe deficiency is low soil Fe bioavailability, due to the occurrence of this metal in oxy-hydroxide insoluble forms. The efficiency of Fe(III)-*o*,*o*EDDHA as an Fe fertilizer is due to the remarkable stability over a wide range of pH values[1] and the low reactivity in soils.[6] In spite of the high cost of Fe(III)-*o*,*o*EDDHA, fertilization with this compound is one of the most widespread practices to control Fe-chlorosis in high-value crops and soil-less horticulture.[4]

Iron uptake from this and other Fe(III)-chelates is carried out in the so-called Strategy I plant species by a root ferric chelate reductase (FCR) enzyme.[7,8] Radioactivity assays using ^{59}Fe(III)–^{14}C-EDDHA in the nutrient solution confirmed that Fe-deficient Strategy I plants have a splitting Fe uptake mechanism, with final root ^{59}Fe/^{14}C ratios being very different in Fe-deficient and Fe-sufficient plants (approximately 25 and 6, respectively).[9] In the case of Fe-deficient plants most of the EDDHA remains in the soil solution, possibly facilitating further solubilization and transport of native Fe to the rhizosphere, in what has been called 'shuttle effect'.[4,10]

In spite of the existence of the splitting uptake mechanism, both synthetic Fe(III) chelating agents and Fe(III)-chelates have been found in plants after fertilization. Radioactivity assays with ^{14}C labeled Fe(III)-EDDHA found significant amounts of ^{14}C in Strategy I and II plants,[9,11,12] indicating that either the whole chelating agent or one or more breakdown product(s) could enter the plant. Iron(III)-EDDHA was also found, using UV-Vis detection, in xylem exudates of zinnia, sunflower and soybean,[13] as well as in tobacco,[14] pepper, lettuce and tomato tissues.[15] Recently, significant amounts of both Fe(III)-*o*,*o*EDDHA and *o*,*o*EDDHA have been found in sugar beet and tomato tissues using a very selective and sensitive technique, HPLC-ESI/TOFMS.[16]

Commercially available Fe(III)-*o*,*o*EDDHA fertilizers contain a mixture (approximately 1 : 1) of two groups of

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Conclusion

The usefulness of dual-stable Fe isotope tracer experiments in plant Fe nutrition has been proven, since plants did not discriminate between ^{54}Fe and ^{57}Fe and no short-term Fe isotope exchange reactions occurred with the Fe(III)-chelates used. The *meso* isomer appears to be the major contributor to the exceptional efficiency of Fe(III)-*o*,*o*EDDHA to deliver Fe to plants in nutrient solution, with rates of FCR, xylem transport and total uptake (in both cases considering the Fe previously chelated) 2-fold higher than those found for the *racemic* isomer. Both isomers of the chelating agent were incorporated and distributed by plants at similar rates, in amounts one order of magnitude lower than those of Fe. After 6 h of Fe resupply, most of the Fe acquired was still localized in roots, whereas most of the chelating agent was localized in leaves. Although most of the Fe coming form the Fe(III)-*o*,*o*EDDHA was taken up by the plant through a dissociative reduction mechanism, a small part of the Fe delivered by the Fe(III)-*o*,*o*EDDHA may be taken up *via* non-dissociative mechanisms, probably using the transpiration stream as the driving force for entry, and this may be important in the short term after Fe fertilization and also when root FCR activity is down-regulated.

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PLANT & CELL PHYSIOLOGY

Identification of a Tri-Iron(III), Tri-Citrate Complex in the Xylem Sap of Iron-Deficient Tomato Resupplied with Iron: New Insights into Plant Iron Long-Distance Transport

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The identification of Fe transport forms in plant xylem sap is crucial to the understanding of long-distance Fe transport processes in plants. Previous studies have proposed that Fe may be transported as an Fe–citrate complex in plant xylem sap, but such a complex has never been detected. In this study we report the first direct and unequivocal identification of a natural Fe complex in plant xylem sap. A tri-Fe(III), tri-citrate complex (Fe_3Cit_3) was found in the xylem sap of Fe-deficient tomato (*Solanum lycopersicum* Mill. cv. 'Tres Cantos') resupplied with Fe, by using an integrated mass spectrometry approach based on exact molecular mass, isotopic signature and Fe determination and retention time. This complex has been modeled as having an oxo-bridged tri-Fe core. A second complex, a di-Fe(III), di-citrate complex was also detected in Fe–citrate standards along with Fe_3Cit_3, with the allocation of Fe between the two complexes depending on the Fe to citrate ratio. These results provide evidence for Fe–citrate complex xylem transport in plants. The consequences for the role of Fe to citrate ratio in long-distance transport of Fe in xylem are also discussed.

Keywords: Iron deficiency Iron-citrate Mass spectrometry Xylem sap Iron transport.

Abreviations: B3LYP, hybrid density functional method; DFT, density functional theory; ESI-MS, electrospray ionization-mass spectrometry; EXAFS, extended X-ray absorption fine structure; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; IDA, isotope dilution analysis; IPD, isotope pattern deconvolution; LOD, linits of detection; NA, nicotianamine; Q-ICP-MS, quadrupole-inductively coupled plasma-mass spectrometry; TOF, time of flight; XANES, X-ray absorption near edge structure; SXRF, synchroton X-ray fluorescence.

This paper is dedicated to the memory of Dr. Arthur Wallace, a pioneer in the study of plant iron nutrition.

Introduction

The mechanisms of long-distance Fe transport in plants have remained elusive until now. In the case of xylem sap, Fe is assumed to be transported as complexed forms, because free ionic forms [Fe(II) and Fe(III)] can be toxic and are also prone to undergo precipitation at the neutral or slightly acidic pH values typical of xylem sap. Increases in carboxylate concentrations in plant xylem exudates with Fe deficiency were reported in several papers published in the 1960s by Brown and co-workers. Iron was first suggested to be transported bound to malate (Tiffin and Brown 1962), but later citrate (Cit), which also increases markedly in stem exudates of many plant species when Fe-deficient (Brown 1966) and co-migrates with Fe during paper electrophoresis (Tiffin 1966a, Tiffin 1966b, Tiffin 1970, Clark et al. 1973), was considered the most likely candidate for Fe transport.

The identity of Fe–Cit complexes in the xylem sap has only been hypothesized by means of in silico calculations using total concentrations of possible Fe complexing agents (including carboxylates) and Fe, and the known stability constants of Fe-containing complexes, always assuming that chemical equilibrium was achieved. Using this approach, several Fe–Cit species were predicted to be the most abundant Fe complexes in the xylem sap whereas other potential plant metal chelators such as nicotianamine (NA) were ruled out (von Wirén et al. 1999, Rellán-Álvarez et al. 2008) as possible xylem Fe carriers. NA function as an Fe chelator might be restricted to the cytoplasm and in Fe phloem loading (Curie et al. 2008). Citrate recently has been found by using molecular biology techniques to play a role in long-distance Fe transport. Xylem sap loading

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Iron–citrate complex molecular modeling

All theoretical calculations were performed by using the Gaussian 03 program (Frisch et al. 2003). The molecular geometry of $(Fe_3OCit_3)^{2-}$ was optimized assuming C_{3h} symmetry. The chemistry model used consisted in the Becke's three-parameter exchange functional combined with the LYP correlation functional (B3LYP) (Becke 1993) and the LanL2DZ basis set as indicated in the Gaussian 03 program (Frisch et al. 2003). In order to achieve the convergence of the wavefunction, an initial guess was obtained using the same chemistry model on the closed shell $(Fe_3OCit_3)^{2-}$ species.

Supplementary data

Supplementary data are available at PCP online.

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RESEARCH ARTICLE Open Access

Changes in the proteomic and metabolic profiles of *Beta vulgaris* root tips in response to iron deficiency and resupply

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Abstract

Background: Plants grown under iron deficiency show different morphological, biochemical and physiological changes. These changes include, among others, the elicitation of different strategies to improve the acquisition of Fe from the rhizosphere, the adjustment of Fe homeostasis processes and a reorganization of carbohydrate metabolism. The application of modern techniques that allow the simultaneous and untargeted analysis of multiple proteins and metabolites can provide insight into multiple processes taking place in plants under Fe deficiency. The objective of this study was to characterize the changes induced in the root tip proteome and metabolome of sugar beet plants in response to Fe deficiency and resupply.

Results: Root tip extract proteome maps were obtained by 2-D isoelectric focusing polyacrylamide gel electrophoresis, and approximately 140 spots were detected. Iron deficiency resulted in changes in the relative amounts of 61 polypeptides, and 22 of them were identified by mass spectrometry (MS). Metabolites in root tip extracts were analyzed by gas chromatography-MS, and more than 300 metabolites were resolved. Out of 77 identified metabolites, 26 changed significantly with Fe deficiency. Iron deficiency induced increases in the relative amounts of proteins and metabolites associated to glycolysis, tri-carboxylic acid cycle and anaerobic respiration, confirming previous studies. Furthermore, a protein not present in Fe-sufficient roots, dimethyl-8-ribityllumazine (DMRL) synthase, was present in high amounts in root tips from Fe-deficient sugar beet plants and gene transcript levels were higher in Fe-deficient root tips. Also, a marked increase in the relative amounts of the raffinose family of oligosaccharides (RFOs) was observed in Fe-deficient plants, and a further increase in these compounds occurred upon short term Fe resupply.

Conclusions: The increases in DMRL synthase and in RFO sugars were the major changes induced by Fe deficiency and resupply in root tips of sugar beet plants. Flavin synthesis could be involved in Fe uptake, whereas RFO sugars could be involved in the alleviation of oxidative stress, C trafficking or cell signalling. Our data also confirm the increase in proteins and metabolites related to carbohydrate metabolism and TCA cycle pathways.

Background

Two different strategies of Fe uptake have been described in plants. The so-called chelation strategy (or Strategy II), which is mainly found in graminaceous plants, is based on the excretion of phytosideropores (PS) to the rhizosphere. Phytosideropores rapidly chelate Fe(III), to form Fe(III)-PS chelates that are subsequently transported into the root cells through a specific transporter. The so-called reduction strategy (or Strategy I) relies on the coordinated action of a membrane bound Fe reductase, that reduces Fe(III) to Fe(II) [1], an Fe(II) uptake transporter [2] and an H^+ -ATPase that lowers the pH of the rhizosphere [3], is mainly used by non graminaceous plants, including *Beta vulgaris*. The reduction strategy includes root morphological, physiological and biochemical changes that lead to an increased capacity for Fe uptake. Morphological changes include root tip swelling, development of transfer cells and an increase in the number of

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Changes induced by two levels of cadmium toxicity in the 2-DE protein profile of tomato roots

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ABSTRACT

Tomato is an important crop from nutritional and economical points of view, and it is grown in greenhouses, where special substrates and the use of recycled water imply an increased risk of Cd accumulation. We investigated tomato root responses to low (10 μM) and high (100 μM) Cd concentrations at the root proteome level. Root extract proteome maps were obtained by 2-DE, and an average of 121, 145 and 93 spots were detected in the 0, 10 and 100 μM Cd treatments, respectively. The low Cd treatment (10 μM) resulted in significant and higher than 2-fold changes in the relative amounts of 36 polypeptides, with 27 of them identified by mass spectrometry, whereas the 100 μM Cd treatment resulted in changes in the relative amounts of 41 polypeptides, with 33 of them being identified. The 2-DE based proteomic approach allowed assessing the main metabolic pathways affected by Cd toxicity. Our results suggests that the 10 μM Cd treatment elicits proteomic responses similar to those observed in Fe deficiency, including activation of the glycolytic pathway, TCA cycle and respiration, whereas the 100 μM Cd treatment responses are more likely due to true Cd toxicity, with a general shutdown of carbon metabolism and increases in stress related and detoxification proteins.

1. Introduction

Cadmium is highly toxic to plants and animals [1]. In particular, Cd toxicity in crops has become a serious problem, especially in developed countries. Cadmium is released into the environment by human activities such as mining, agricultural use of commercial fertilizers, sewage sludge, manure and lime and industrial activities that release air pollutants and effluents with high Cd concentrations [2,3]. Food chain contamination is the main Cd exposure risk for humans, and Cd taken up by plants is accepted to be the main source of Cd accumulation in foods [4]. Cadmium is suggested to cause damage even at very low concentrations, and healthy plants may contain Cd levels that are toxic for mammals [5].

In polluted soils, Cd is generally present as a free ion or in other soluble forms, and its mobility depends on pH and on the presence of chelating substances and other cations [6]. It is accepted that Cd is taken up by roots via Fe/Zn transporters because of the low metal specificity of these proteins. There is evidence that metal transporters from different families such as ZIP and Nramp are able to transport several divalent cations, including Cd [7,8]. Also, it has been described that a Ca

Abbreviations: ACN, Acetonitrile; GADPH, Glyceraldehyde 3-phosphate dehydrogenase; GDH, Glutamate dehydrogenase; GST, Glutathione S-transferase; HSP, Heat shock protein; KOBAS, KEGG orthology-based annotation system; MDAR, Monodehydroascorbate reductase; MDH, Malate dehydrogenase; PCs, Phytochelatins; PDH, Pyruvate dehydrogenase; PTMs, Post translational modifications; TCA, Tricarboxylic acid cycle.

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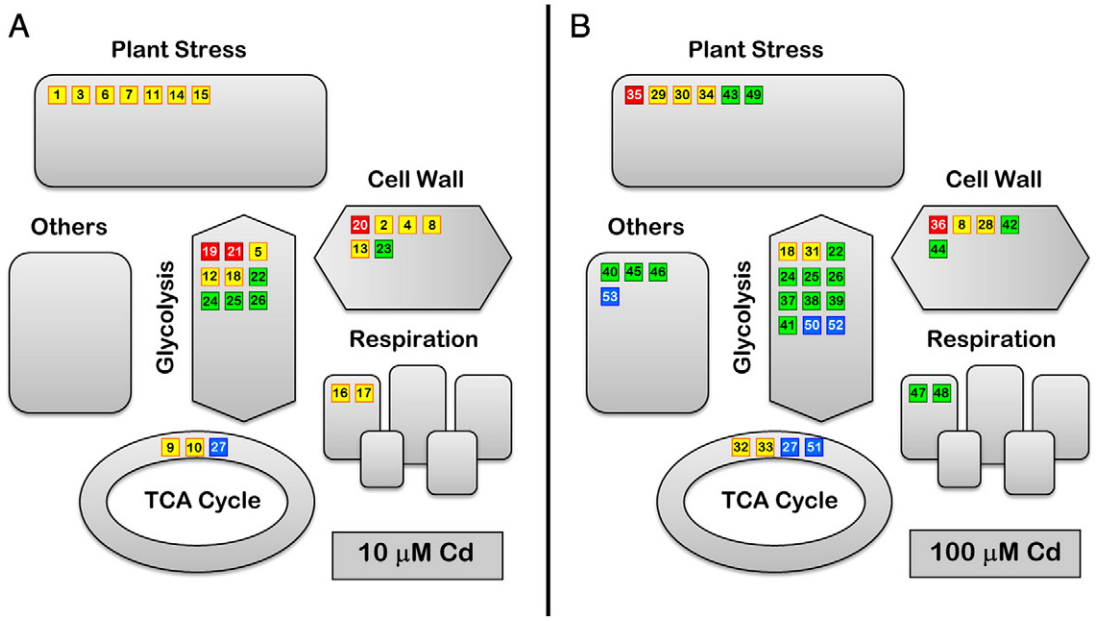

Fig. 4 – Changes in metabolic pathways as affected by Cd. Panels A and B are for 10 and 100 μM Cd treated plants, respectively. Pathways related to the identified proteins were integrated according to the KEGG database. A statistical Student *t*-test was performed to show relevant changes between samples. Red symbols mean newly detected proteins in Cd treated roots and yellow symbols proteins showing increases in intensity compared to control (using a 2-fold threshold change). The same threshold (decreases larger than 50%) was selected for proteins showing decreases in intensity (green symbols). Blue symbols indicate proteins not detected in Cd treated roots. Numbers correspond to those in Table 1.

as tomato, C and energy metabolism increase, whereas in tolerant plants, such as poplar, C metabolism decreased and energy metabolism did not change [30].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2010.05.001.

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Stomatal and mesophyll conductances to CO_2 are the main limitations to photosynthesis in sugar beet (*Beta vulgaris*) plants grown with excess zinc

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Key words: *Beta vulgaris* (sugar beet), CO_2 mesophyll conductance, CO_2 stomatal conductance, photosynthesis limitations, zinc (Zn) excess.

Summary

- The effects of zinc (Zn) toxicity on photosynthesis and respiration were investigated in sugar beet (*Beta vulgaris*) plants grown hydroponically with 1.2, 100 and 300 μM Zn.
- A photosynthesis limitation analysis was used to assess the stomatal, mesophyll, photochemical and biochemical contributions to the reduced photosynthesis observed under Zn toxicity.
- The main limitation to photosynthesis was attributable to stomata, with stomatal conductances decreasing by 76% under Zn excess and stomata being unable to respond to physiological and chemical stimuli. The effects of excess Zn on photochemistry were minor. Scanning electron microscopy showed morphological changes in stomata and mesophyll tissue. Stomatal size and density were smaller, and stomatal slits were sealed in plants grown under high Zn. Moreover, the mesophyll conductance to CO_2 decreased by 48% under Zn excess, despite a marked increase in carbonic anhydrase activity. Respiration, including that through both cytochrome and alternative pathways, was doubled by high Zn.
- It can be concluded that, in sugar beet plants grown in the presence of excess Zn, photosynthesis is impaired due to a depletion of CO_2 at the Rubisco carboxylation site, as a consequence of major decreases in stomatal and mesophyll conductances to CO_2.

Abbreviations: A_N, net photosynthesis; AOX, alternative oxidase; APX, ascorbate peroxidase; BL, DL, MCL, NSL and SL, biochemical conductance, diffusional conductance, mesophyll conductance, and nonstomatal and stomatal limitations, respectively; C_a, C_C and C_i, atmospheric, chloroplastic and substomatal CO_2 concentrations, respectively; CA, carbonic anhydrase; Chl, chlorophyll; ETR, electron transport rate; Φ_{PSII}, actual photosystem II efficiency; FAAS, flame atomic absorption spectroscopy; F_o, minimal Chl fluorescence in the dark; F_v/F_m, maximum potential photosystem II efficiency; g_m and g_s, CO_2 mesophyll and stomatal conductances, respectively; J_{max}, *in vivo* maximum rate of electron transport driving regeneration of RuBP; Mn-SOD, manganese-superoxide dismutase; PPFD, photosynthetic photon flux density; PSII and PSI, photosystems II and I, respectively; $V_{c,max}$, *in vivo* maximum rate of Rubisco carboxylation; WC, water content.

nature of this stomatal seal, and the mechanism for its accumulation under excess Zn.

The causes of the decreased CO_2 mesophyll conductance were also investigated using LT-SEM. High Zn-grown plants had a lower leaf porosity than control plants, with the surface of leaf mesophyll cells being less exposed to intercellular air spaces than those of control leaves. Also, chloroplasts were smaller and the interaction of chloroplasts with cell membranes was hampered as a result of the changes in shape, and both factors would increase the length of the CO_2 diffusion pathway in the cytosol. All these factors have been shown to be determinants of g_m under very different experimental conditions (Sharkey *et al.*, 1991; Flexas *et al.*, 2008; Evans *et al.*, 2009; Li *et al.*, 2009).

The photochemistry-related parameters F_v/F_m, ETR and Φ_{PSII} were not decreased significantly under high Zn, consistent with previous observations in sugar beet grown in relatively low PPFDs (Sagardoy *et al.*, 2009) as well as in other species (Van Assche & Clijsters, 1986a; Schuerger *et al.*, 2003; Dhir *et al.*, 2008). This indicates that decreased photosynthesis was not caused by impaired leaf photochemistry. Zn treatments did not induce the operation of alternative sinks for electrons. On the one hand, this evidence comes from the close to 0 intercept of the relationship between Φ_{PSII} and Φ_{CO2} under nonphotorespiratory conditions (Laisk & Loreto, 1996; Long & Bernacchi, 2003). On the other hand, the ETR : A_N ratios increased under Zn excess, from 6.7 in the controls to 10.8 in the 300 μM Zn treatment (calculated from Table 2); values for ETR : A_N + respiration (values taken from Table 5) ratios were lower, at 6.6 (controls) and 10.1 (300 μM Zn), whereas those for the ETR : A_N + respiration + photorespiration (not shown) ratios were lower still, at 5.2 (controls) and 6.9 (300 μM Zn), indicating photorespiration as the cause of the increased ETR : A_N ratios in excess Zn-grown plants and ruling out the existence of alternative sinks for electrons under Zn excess. The slope of the relationship between Φ_{PSII} and Φ_{CO2} was not affected by the Zn treatments, which suggests that there were no changes either in leaf absorbance or in energy partitioning between PSI and PSII. Photosynthetic biochemistry was also unaffected by Zn, based on the *in vivo* estimates of $V_{c,max}$ and J_{max}. Nevertheless, Zn excess increased the activity of CA, a metalloprotein with Zn in its active center, although only at 300 μM. Zn was previously reported to inhibit CA *in vitro* at high concentrations (Ivanov *et al.*, 2007). Although it has been suggested that CA is involved in the regulation of mesophyll conductance to CO_2 (Gillon & Yakir, 2000), g_m did not increase under excess Zn, but instead decreased by 44% compared with the control. This decrease, moderate when compared with the much greater decrease in stomatal conductance, may be related to the increase in CA activity.

Dark respiration increased markedly in Zn-treated plants, as observed previously in other species (Ismail & Azooz, 2005). The increase in total respiration was associated with significant increases in the activity of both cytochrome (v_{cyt}) and alternative (v_{alt}) pathways. Data obtained in this study are not consistent with previous data suggesting a Zn-induced preferential increase in AOX (Webster & Gadd, 1999), or an inhibitory effect of Zn on AOX (Affourtit & Moore, 2004). The cytochrome pathway is associated with the growth component of respiration and results in high ATP production, whereas AOX is associated with the maintenance component of respiration and results in lower ATP production (Florez-Sarasa *et al.*, 2007). In the case of the sugar beet plants in our study, where growth of high-Zn plants was severely reduced, increased ATP synthesis through increased cytochrome respiration would probably be used to increase ion uptake, exchange and compartmentalization (Lambers *et al.*, 2005), to minimize the impact of Zn toxicity. The direct effect of increased respiration on the decrease in net photosynthesis would have been very small (approx. 3%); assuming that the measured rates of dark respiration also applied during the light period, the increased respiration would have decreased the total reduction in photosynthesis induced by excess Zn from 42% to 39% and from 48% to 45% under 100 μM and 300 μM Zn, respectively. The actual effect was probably even smaller, as the rates of respiration in the light are often lower than in the dark (Priault *et al.*, 2006; Juszczuk *et al.*, 2007).

In conclusion, 100–300 μM Zn resulted in large reductions in sugar beet biomass (> 50%) and photosynthetic rates (40–50%), whereas leaf respiration rates doubled through increased activity of both the cytochrome and alternative pathways, probably resulting in increases in capacities for ion compartmentalization and Zn exclusion. Under excess Zn, stomatal conductance was reduced by 70%, and stomata became insensitive to environmental variables such as leaf water status, exogenously applied ABA and VPD. In high Zn-treated plants, stomata were round in shape and smaller than in control plants and, in many cases, were covered by a wax-like seal of unknown nature. Excess Zn, therefore, affected primarily stomatal conductance, apparently through alterations of guard cell development (lower stomatal density on the leaf surface) and guard cell function. Leaf photochemistry and photosynthetic biochemistry were not significantly affected by high Zn. Mesophyll conductance to CO_2 also showed 44% decreases, despite concomitant 2-fold increases in CA, possibly as a result of changes in mesophyll ultrastructure and chloroplast size and arrangement with respect to the mesophyll cell plasma membrane.

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Complexation of Hg with phytochelatins is important for plant Hg tolerance

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ABSTRACT

Three-week-old alfalfa (*Medicago sativa*), barley (*Hordeum vulgare*) and maize (*Zea mays*) were exposed for 7 d to 30 μM of mercury ($HgCl_2$) to characterize the Hg speciation in root, with no symptoms of being poisoned. The largest pool (99%) was associated with the particulate fraction, whereas the soluble fraction (SF) accounted for a minor proportion (<1%). Liquid chromatography coupled with electro-spray/time of flight mass spectrometry showed that Hg was bound to an array of phytochelatins (PCs) in root SF, which was particularly varied in alfalfa (eight ligands and five stoichiometries), a species that also accumulated homophytochelatins. Spatial localization of Hg in alfalfa roots by microprobe synchrotron X-ray fluorescence spectroscopy showed that most of the Hg co-localized with sulphur in the vascular cylinder. Extended X-ray Absorption Fine Structure (EXAFS) fingerprint fitting revealed that Hg was bound *in vivo* to organic-S compounds, i.e. biomolecules containing cysteine. Albeit a minor proportion of total Hg, Hg–PCs complexes in the SF might be important for tolerance to Hg, as was found with *Arabidopsis thaliana* mutants *cad2-1* (with low glutathione content) and *cad1-3* (unable to synthesize PCs) in comparison with wild type plants. Interestingly, high-performance liquid chromatography-electrospray ionization-time of flight analysis showed that none of these mutants accumulated Hg–biothiol complexes.

Key-words: biothiols; EXAFS; mass spectrometry; mercury; phytochelatins; soluble fraction; X-ray absorption spectroscopy.

Abbreviations: DEAE, diethylaminoethyl; DTT, dithiothreitol; GSH, glutathione; hGSH, homoglutahione; hPCs, homophytochelatins; MF, microsomal fraction; PCs, phytochelatins; PF, particulate fraction; SF, soluble fraction.

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INTRODUCTION

Mercury (Hg) accumulation is considered a global environmental threat, and its trade is restricted due to its bioaccumulation and biomagnification in diverse ecosystems (Leonard *et al*. 1998). Mercury has no nutritional role, and exposure of biological systems to relatively low Hg concentrations results in serious toxicity (Nriagu 1990). Although little is known about the precise mechanism of toxicity exerted by Hg in plants, cellular integrity and biological activity might be compromised due to its strong affinity for sulfhydryl residues of proteins and other biomolecules (Van Assche & Clijsters 1990; Hall 2002). Mercury has also been found to be a potent inducer of oxidative stress (Cho & Park 2000; Rellán-Álvarez *et al*. 2006a), and an oxidative burst appeared in alfalfa root epidermal cells after a brief exposure to 30 μM Hg (Ortega-Villasante *et al*. 2007), in spite of its limited redox activity (Schützendübel & Polle 2002).

Mercury accumulates preferentially in roots (4- to 10-fold the concentration found in shoots) of several plant species such as *Pisum sativum* (Beauford, Barber & Barringer 1977), *Brassica napus* (Iglesia-Turiño *et al*. 2006), *Zea mays* (Rellán-Álvarez *et al*. 2006a) and *B. chinensis* (Chen, Yang & Wang 2009). Therefore, most of the toxic effects of Hg are observed in roots. A large proportion of Hg was found associated with cell wall materials in *P. sativum* and *Mentha spicata* (Beauford *et al*. 1977), *Nicotiana tabacum* (Suszcynsky & Shann 1995) and *Halimione portulacoides* (Válega *et al*. 2009). Although the mobility of Hg within the plant may be limited by root cell walls, the distribution in root cells or tissues is not presently clear. This objective can be achieved using techniques such as microprobe synchrotron X-ray fluorescence spectrometry (μ-SXRF), which is capable of providing spatially resolved metal data (Punshon, Guerinot & Lanzirotti 2009).

Once heavy metals enter the cell, additional defence mechanisms involve the synthesis of organic ligands that could form metal complexes with reduced biological activity. Among these compounds, phytochelatins (PCs) are known to bind Cd and other toxic elements by means of

observations that neither Hg–GSH nor Hg–hGSH complexes could be found in the SF of barley, maize and alfalfa roots, and with the findings in Hg-treated *B. chinensis*, *O. sativa* and *M. vulgare* (Chen *et al*. 2009; Krupp *et al*. 2009). We tested the sensitivity of our HPLC-ESI-TOFMS method by analysing samples of *A. thaliana* Col-0 spiked with a 25 μM Hg:50 μM GSH mixture, and we observed the characteristic $[Hg(GSH)_2–H]^-$ ion at m/z 813.1. The $Hg(GSH)_2$ complex was not detected *in vivo*, in spite of being the endogenous GSH concentration found in 30 μM Hg-treated Col-0 leaves (257.4 nmol g^{-1} FW) 5-fold higher than the concentration of spiked GSH (Supporting Information Fig. S4). The absence of Hg–GSH complexes could be partially explained by the fact that PCs containing a larger number of sulfhydryl residues than GSH or hGSH will bind Hg more strongly, as Mehra *et al*. (1996) showed *in vitro* by circular dichroism and HPLC-UV/visible spectroscopy. These results imply that despite GSH accumulation under Hg stress, PCs are the biothiols that contribute to Hg detoxification in plants.

Phytochelatins are synthesized by the condensation of a molecule of γ-glutamylcysteine on GSH that could contain the thiol group blocked by the transpeptidase activity of phytochelatin synthase (Vatamaniuk *et al*. 2000). Taking into account the strong affinity of Hg for thiol residues, an alteration of PCs and GSH metabolism catalysed by phytochelatin synthase might explain the restricted variety of PCs or hPCs variants found in plants exposed to Hg in comparison with those treated with Cd or As (Table 3; Cobbett & Goldsbrough 2002; Haydon & Cobbett 2007). In this sense, the absence of $Hg–(GSH)_2$ in all root SF and $Hg–PC_3$ in barley root SF, which should be expected in the canonical series of Hg–biothiol complexes, could also depend on the particular molecular stability (*i.e.* strength of bonds or susceptibility to oxidative modifications) and subcellular compartmentalization (*i.e.* vacuolar sequestration) occurring during the detoxification of Hg. Therefore, future work should be directed to characterize the dynamics of Hg–biothiol complexes formation using isotopic labelling and HPLC-ICPMS to quantify precisely their cellular concentration, information necessary to understand the contribution of each Hg species to detoxification.

In summary, plants accumulated several classes of Hg–PC complexes. Biothiols may constitute a sink of soluble Hg, although the major proportion of the toxic metal was found associated with the particulate fraction. Albeit a minor proportion of plant Hg, Hg–PCs in the SF contribute to the ultimate fate of Hg in plants. It is plausible that Hg is mainly retained in the roots, interacting with cell wall components, and only when this barrier is overridden, soluble Hg binds to biothiols. EXAFS fingerprint fits suggest that the bulk of Hg is associated with thiols or cysteine, corresponding to cysteine-related components (probably proteins), which is in agreement with the data from μ-SXRF. Incidentally, the major structural protein in cell walls is extensin, a highly glycosylated protein which contains several residues of cysteine in a so-called Cys-rich domain (Baumberger *et al*. 2003). Therefore, as most Hg accumulates in the particulate fraction, speciation of Hg in cell wall components could be the major task for future work. The precise contribution of each compartment to the tolerance of plants is still in debate, and more sensitive and accurate techniques are needed to analyse the distribution of Hg at the subcellular level and to quantify the amount of Hg bound to the different ligands that accumulate in the plants.

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ARTICLE

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Root Responses of *Medicago truncatula* Plants Grown in Two Different Iron Deficiency Conditions: Changes in Root Protein Profile and Riboflavin Biosynthesis

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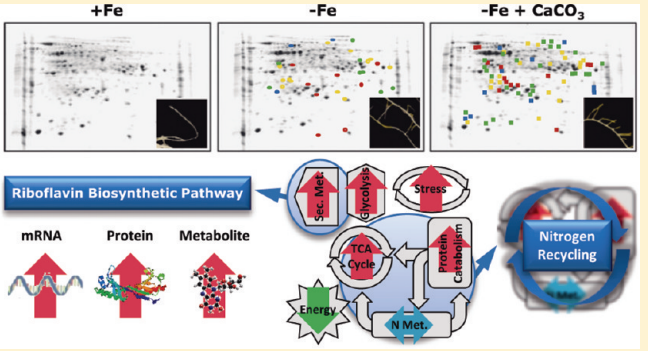
Supporting Information

ABSTRACT: Iron deficiency is a yield-limiting factor with major implications for field crop production in one-third of the world's agricultural areas, especially those with high soil $CaCO_3$. In the present work, a two-dimensional gel electrophoresis proteomic approach was combined with a study on the riboflavin synthesis pathway, including qPCR and riboflavin determination, to investigate Fe-deficiency responses in *Medicago truncatula* plants grown with and without $CaCO_3$. Iron deficiency caused a *de novo* accumulation of DMRLs and GTPcII, proteins involved in riboflavin biosynthesis, as well as marked increases in root riboflavin concentrations and in the expression of four genes from the riboflavin biosynthetic pathway. Two novel changes found were the increased accumulation of proteins related to N recycling and protein catabolism. Other identified changes were consistent with previously found increases in glycolysis, TCA cycle, and stress-related processes. All effects were more marked in the presence of $CaCO_3$. Our results show that the riboflavin biosynthesis pathway was up-regulated at the genomic, proteomic, and metabolomic levels under both Fe-deficiency treatments, especially in the presence of $CaCO_3$. Results also indicate that N recycling occurs in *M. truncatula* upon Fe deficiency, possibly constituting an additional anaplerotic N and C source for the synthesis of secondary metabolites, carboxylates, and others.



KEYWORDS: Calcium carbonate, DMRLs, iron, C/N metabolism, riboflavin, root, two-dimensional gel electrophoresis

INTRODUCTION

Iron is an essential micronutrient for all living organisms including plants, since it takes part in fundamental biological redox processes such as photosynthesis and respiration. Although Fe is the fourth most abundant element in the earth's crust, the bioavailability for plants is low, due to its poor solubility in the rhizosphere at neutral or basic pH. Therefore, Fe deficiency is a yield-limiting factor with major implications for field crop production in many agricultural areas of the world. For instance, it has been estimated that 20−50% of fruit trees in the Mediterranean basin suffer from Fe deficiency.[1] The most prevalent cause of Fe deficiency in this area, where calcareous soils are predominant, is the presence of high $CaCO_3$ concentrations. Plants can be broadly classified into two groups based on their mechanism of Fe acquisition: Strategy I plants use a Fe(III) reduction-based mechanism and include dicotyledonous and non-Graminaceae monocotyledonous species, whereas Strategy II plants use a Fe(III) chelation-based mechanism and include Graminaceae species.[2] The response of Strategy II plants to Fe shortage includes the biosynthesis and secretion of phytosiderophores, which specifically bind Fe(III) with high affinity, and the subsequent uptake of Fe(III)-PS complexes from the rhizosphere by specific cellular transport systems.[3]

When Fe is scarce, Strategy I plants develop morphological and biochemical changes leading to an increase in their Fe uptake capacity. Morphological changes include swelling of root tips and formation of lateral roots, root hairs, and transfer cells that increase the root surface in contact with the external medium, thereby increasing the Fe uptake capability.[4,5] Biochemical changes aimed to increase Fe uptake by roots and Fe solubility in the soil solution include the induction of a plasma-membrane Fe(III)-reductase[6,7] and an Fe(II) transporter,[8,9] and an enhanced proton extrusion capacity, the latter being supported by a plasma-membrane H^+-ATPase, whose activity lowers soil pH favoring the solubilization of inorganic Fe.[10,11] Other metabolic changes occur in order to sustain the elevated energy requirements for Fe uptake; these include accumulation of organic acids, mainly malate and citrate,[12] shifts in the redox

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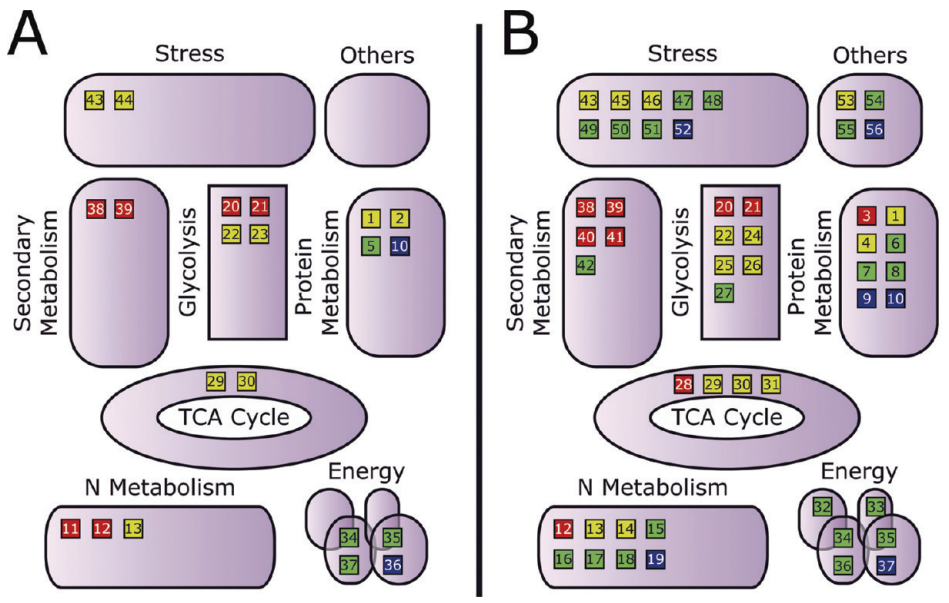

Figure 6. Changes in metabolic pathways as affected by Fe deficiency. Panels A and B are for 0 μM Fe and 0 μM Fe plus $CaCO_3$ treated plants, respectively. Pathways related to the identified proteins were integrated according to the KEGG database and GO annotation. A statistical Student *t* test was performed to show relevant changes between samples. Red symbols mark newly detected proteins in Fe-deficient roots, and yellow symbols proteins with increased intensity compared to controls (using a 2-fold threshold change). The same threshold (decreases larger than 50%) was selected for proteins with decreased intensity (green symbols). Blue symbols indicate proteins not detected in Fe-deficient roots. Numbers correspond to those in Table 1.

Other changes found support previous observations in Fe-deficient plants. For instance, the decrease in the mitochondrial complex I supports the reorganization of the electron transport chain with Fe deficiency, possibly involving increases in the activities of alternative dehydrogenases.[15] Also, stress-related proteins accounted for 10−17% of the changes in the protein profile in the 0 μM Fe and 0 μM Fe plus $CaCO_3$ treatments, respectively. A major change in both treatments was an increase in glutathione transferase, which conjugates glutathione to cytotoxic products. A reorganization of the SODs was also observed, most likely to compensate for FeSOD decreases. The increase in MDAR in the 0 μM Fe plus $CaCO_3$ treatment could compensate for the decrease in APX, an Fe-containing protein. An enhancement of the glycolytic pathway and TCA cycle agree with previous studies.[12,16] As also occurs in Fe-deficient tomato roots,[32] a decrease in fructokinase was found, suggesting that glucose is preferred over fructose as initial substrate in the glycolytic pathway, possibly related to the use of starch as an energy source instead of sucrose. This hypothesis is consistent with the low photosynthetic rates in Fe-deficient plants that would cause a shortage of available sucrose.

CONCLUSIONS

An overview of the results is shown in Figure 6. In general, more dramatic changes occur in plants exposed to $CaCO_3$, where the high external pH may account for some of the observed changes, but the same general response was observed. Fe deficiency caused a *de novo* accumulation of DMRLs and GTPcII, proteins involved in Rbfl biosynthesis, along with increases in the corresponding mRNA levels, suggesting that these enzymes may be control steps for the Fe-regulation of Rbfl synthesis. Overall, our results also suggest the occurrence of N recycling, possibly providing an additional anaplerotic N and C source for the synthesis of secondary metabolites, carboxylates and others. This was associated with increases in proteolysis, the already widely reported increases in glycolysis and TCA cycle, and changes in the electron transport chain in the mitochondria.

ASSOCIATED CONTENT

Supporting Information

Supporting material and methods: Identification of putative *DMRLs*, *GTPcII*, *ribD* and *ribE* genes in *Medicago truncatula*. Supporting results: Identification of putative *DMRLs*, *GTPcII*, *ribD* and *ribE* genes in *Medicago truncatula*; forward and reverse specific primers utilized for qRT-PCR; univariate statistical analysis of 2-DE gels; summary of protein profiling results; multivariate statistical analysis of 2-DE gels. This material is available free of charge via the Internet at http://pubs.acs.org.

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Short communication

Carboxylate metabolism in sugar beet plants grown with excess Zn

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ABSTRACT

The effects of Zn excess on carboxylate metabolism were investigated in sugar beet (*Beta vulgaris* L.) plants grown hydroponically in a growth chamber. Root extracts of plants grown with 50 or 100 μM Zn in the nutrient solution showed increases in several enzymatic activities related to organic acid metabolism, including citrate synthase and phospho*enol*pyruvate carboxylase, when compared to activities in control root extracts. Root citric and malic acid concentrations increased in plants grown with 100 μM Zn, but not in plants grown with 50 μM Zn. In the xylem sap, plants grown with 50 and 100 μM Zn showed increases in the concentrations of citrate and malate compared to the controls. Leaves of plants grown with 50 or 100 μM Zn showed increases in the concentrations of citric and malic acid and in the activities of citrate synthase and fumarase. Leaf isocitrate dehydrogenase increased only in plants grown with 50 μM Zn when compared to the controls. In plants grown with 300 μM Zn, the only enzyme showing activity increases in root extracts was citrate synthase, whereas the activities of other enzymes decreased compared to the controls, and root citrate concentrations increased. In the 300 μM Zn-grown plants, the xylem concentrations of citric and malic acids were higher than those of controls, whereas in leaf extracts the activity of fumarase increased markedly, and the leaf citric acid concentration was higher than in the controls. Based on our data, a metabolic model of the carboxylate metabolism in sugar beet plants grown under Zn excess is proposed.

Introduction

Zinc is an essential element for plant cell physiological processes, but can also be toxic when present in excess (Broadley et al., 2007). Agricultural soils are often contaminated with heavy metals due to anthropogenic sources, and in these soils, some crops may take up large amounts of Zn that can be stored in edible parts (Broadley et al., 2007). High concentrations of Zn in fruits and vegetables pose a threat to food quality and safety, and a risk to animal and human health (White and Broadley, 2005). Plant roots acquire Zn as Zn(II), and then the metal is distributed throughout the whole plant in a complex series of processes not yet fully elucidated, involving several families of metal transporters (Krämer et al., 2007; Pilon et al., 2009; Puig and Peñarrubia, 2009; White and Broadley, 2009). In the xylem, Zn could be transported chelated by different small molecules, including organic acids, histidine and nicotianamine (NA) (Broadley et al., 2007; Trampczynska et al., 2010). With Zn excess, a large part of the Zn in the cell can be chelated by organic acids, amino acids such as histidine and NA, phytate and metallothioneins (Callahan et al., 2006; Broadley et al., 2007), and most likely stored in vacuoles.

In the model plant sugar beet (*Beta vulgaris* L.), which has a great capacity to accumulate heavy metals (Larbi et al., 2002), Zn toxicity symptoms include Fe deficiency-induced chlorosis in young leaves, altered plant mineral composition, and growth decreases (Sagardoy et al., 2009). Zinc excess in sugar beet increases leaf respiration rates and decreases photosynthetic rates due to reductions in stomatal and mesophyll conductance to CO_2 associated with changes in stomatal frequency, morphology and functioning, and also to changes in mesophyll ultrastructure, leading to a CO_2 depletion in the sub-stomatal chamber and at the Rubisco carboxylation site (Sagardoy et al., 2010). Our aim was to investigate the effects of high Zn concentrations in the carboxylate metabolism of *B. vulgaris*, measuring the activities of enzymes involved in these processes in roots and leaves and carboxylate concentrations in roots, xylem sap and leaves.

Materials and methods

Plant material

Sugar beet (*B. vulgaris* L. cv. Orbis) was grown hydroponically in a growth chamber in controlled conditions (Sagardoy et al., 2009). Seeds were germinated, pre-cultured for 2 weeks in control conditions and then treatments were imposed (Sagardoy et al., 2009). A concentration of 1.2 μM $ZnSO_4$ was used as the Zn-sufficient con-

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In leaves, the total carboxylate pool increased in 50 and 100 μM Zn-grown plants (1.6- and 1.3-fold, respectively) compared to controls. This was due to increases in citric (2.4- and 2.6-fold) and malic acid (1.7- and 2.0-fold) concentrations in plants grown with 50 and 100 μM Zn, respectively (Table 2). Oxalic acid concentrations increased 1.5-fold in 50 μM Zn-grown plants, and did not change in 100 μM Zn plants. Succinic acid concentrations did not change with the Zn treatments (Table 2).

Based on our data, a metabolic model of the carboxylate metabolism in sugar beet plants grown under Zn excess is proposed (Fig. 1). Our results indicate that several changes in the root carboxylate metabolism occur upon treatment with 50–100 μM Zn (Fig. 1A): (i) an increase of anaplerotic C fixation associated to increases in the root activities of CS and PEPC; (ii) an alteration in TCA activity, based on the decreases in ICDH (at 100 μM Zn) and fumarase (at 50 μM Zn) activities; and (iii) an increased flow of carboxylates from roots to leaves *via* xylem, supported by the several-fold increase in the total carboxylate pool in the xylem sap and the slight (at 100 μM Zn) or no (at 50 μM Zn) change of the same pool in roots (Table 2). Moreover, calculations of C flow in xylem sap based on transpiration rates (Sagardoy et al., 2009) and carboxylate concentrations indicate that in 50–100 μM Zn-grown plants, C flow was higher (1.5 and 1 μmol C m^{-2} s^{-1}, respectively) than in control plants (0.4 μmol C m^{-2} s^{-1}). In leaves, in contrast to what happens in roots, PEPC activity did not change, TCA activity was enhanced and leaf citric and malic acid concentrations increased 2–3-fold in the 50–100 μM Zn-grown plants (Tables 1 and 2). These results suggest that carboxylates transported from roots are used as respiratory substrates to support metabolism in leaves with low photosynthetic rates. This conclusion is also supported by the increases in leaf respiration observed under Zn excess (Sagardoy et al., 2010). Increases in xylem carboxylate concentrations and root PEPC activity have also been described in other stresses causing photosynthetic damage, such as Cd toxicity, Fe deficiency and P stress (Johnson and Allan, 1994; López-Millán et al., 2000b; Wei et al., 2007). Based on these observations, we hypothesize that anaplerotic C fixation in roots and subsequent transport of carboxylates to shoots may constitute a general mechanism to cope with situations causing reduced photosynthetic activity.

Effects of 300 μM Zn

When using 300 μM Zn, Zn(II) concentrations in the nutrient solution were estimated to be 279 μM, but leaves still had approximately 250 μg Zn g^{-1} DW, values similar to those found in plants grown with 50–100 μM Zn (Sagardoy et al., 2009).

Root extracts from plants grown with 300 μM Zn showed a significant increase in the activity of CS (2-fold) when compared to the activity measured in controls, whereas the activities of ICDH, fumarase and PEPC decreased by 80, 35 and 80%, respectively, and MDH activity was no longer detected (Table 1). In these roots, the total carboxylate pool increased 1.7-fold (Table 2); citric and oxalic acid concentrations increased (4- and 1.8-fold, respectively), whereas succinic acid concentration decreased by 59%, and no significant changes were found for malic acid when compared to controls (Table 2).

In xylem sap from 300 μM Zn-grown plants, the total carboxylate pool increased 1.8-fold when compared to controls. Citric and malic acid concentrations increased (3.8- and 2.7-fold, respectively), whereas succinic acid concentration decreased by 78% and no significant changes were observed for oxalic acid (Table 2). The transpiration rates of the 300 μM Zn-grown plants were markedly diminished (by 75%) when compared to controls (Sagardoy et al., 2009).

In leaves, significant increases were observed in the activities of fumarase and CS (5.7- and 1.6-fold), whereas MDH, PEPC and ICDH activities decreased by 78, 54 and 48%, respectively, when compared to controls (Table 1). The total carboxylate pool in leaves decreased by 32% when compared to controls, due to major decreases (45%) in oxalic and succinic acid concentrations (Table 2).

Therefore, the responses observed at 300 μM Zn concerning the carboxylate metabolism were different from those observed with 50 and 100 μM Zn (Fig. 1B). The large decrease in root PEPC activity suggests that anaplerotic C fixation *via* PEPC did not take place in roots, and the C flow to the shoots decreased to values similar to those of control plants (0.3 μmol C m^{-2} s^{-1}); the carboxylate concentrations increased but major decreases in transpiration occurred. Root and leaf CS activities were higher than those of the controls, probably associated with the high citrate concentrations in roots and the increased leaf TCA activity.

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Analytical technologies to study the biological and environmental implications of iron-fertilisation using synthetic ferric chelates: the case of Fe(III)-EDDHA – a review

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SUMMARY
The most commonly used and efficient compound for iron (Fe)-fertilisation of fruit crops grown in calcareous soils is the synthetic Fe(III)-chelate of ethylenediamine-N,N'-di-(*ortho*-hydroxyphenyl) acetic acid, usually known as Fe(III)-*o*,*o*-EDDHA. However, the mechanism(s) of plant Fe uptake from this compound, and the environmental implications of its use, are still not completely understood. This lack of information is due, in part, to the lack of suitable analytical methods capable of determining the very low concentrations of this Fe(III)-chelate which may occur in complex matrices such as plant tissues and fluids after Fe-fertilisation. In this report, the main issues for studies on the biological and environmental implications of fertilisation with synthetic Fe(III)-chelates are discussed, focussing on new possibilities offered by recently developed analytical technologies.

Despite of the relatively low Fe requirements of plants and the high abundance of Fe in soils, Fe-deficiency is a nutritional disorder that limits crop yields in many agricultural areas of the World. Fruit tree crops such as peach, pear, kiwifruit, apricot, plum, cherry, and avocado are sensitive to shortages of Fe. The cause of Fe-deficiency is generally a combination of limited bio-availability of Fe in the soil, which occurs particularly in calcareous and alkaline soils, and the use of susceptible genotypes that have insufficient activation of one or more Fe-deficiency defence mechanisms. Iron-deficiency has an important economic impact on the fruit sector because it can reduce fruit yield and quality (Álvarez-Fernández *et al*., 2006), and also because Fe-fertilisation is expensive (200 – 400 € ha^{-1} $year^{-1}$; Rombolà and Tagliavini, 2006).

Iron-fertilisation is the best and most commonly used technique to correct for Fe-deficiency in established fruit tree orchards. The active ingredients can be either inorganic or organic Fe-containing compounds. Foliar fertilisation with inorganic Fe compounds (e.g., $FeSO_4$) or some organic Fe complexes, including natural (e.g., citrate) and synthetic ligands such as ethylenediamine tetraacetic acid [Fe(III)-EDTA; Figure 1A], N-(2-hydroxyethyl) ethylenediaminetriacetic acid [Fe(III)-HEEDTA; Figure 1B], and di-ethylenetriamine pentaacetic acid [Fe(III)-DTPA; Figure 1C], could alleviate Fe-deficiency, although this method is still not very common (Abadía *et al*., 2004). Trunk injection with liquid Fe fertilisers, or solid branch implants of Fe compounds are even less frequent, in spite of the long-lasting efficacy that can be obtained with one application per year (Abadía *et al*., 2004). The most widely-used Fe-fertilisation technique for fruit crops grown in calcareous soils is an annual soil application of expensive synthetic Fe(III)-chelates such as ethylenediamine-N,N'-di-(*ortho*-hydroxyphenyl) acetic acid [Fe(III)-*o*,*o*-EDDHA; Figure 1D] and analogues such as ethylenediamine-N-(ortho-hydroxyphenylacetic)-N'-

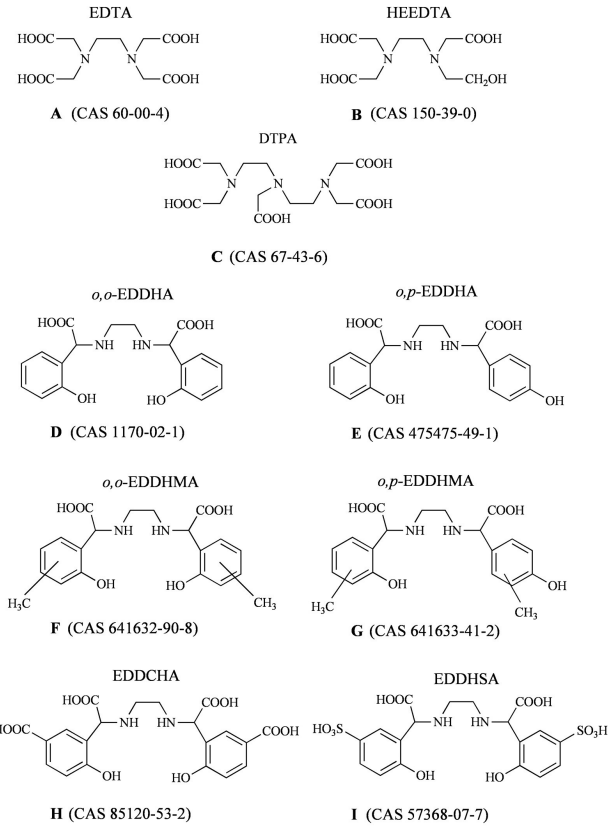

FIG. 1
Chemical structures and abbreviated names of Fe(III)-chelating agents (Panels A–I) allowed by current EU Commission Regulation No. 162/2007 in Fe-fertilisers (Anon, 2007). CAS Numbers of the compounds are indicated below the formulae.

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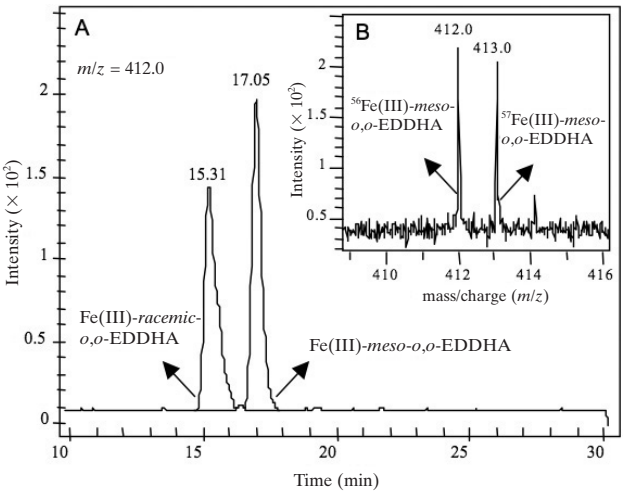

FIG. 3.
Chromatogram (Panel A) and mass spectrum (inset, Panel B) of a xylem sap sample extracted from tomato plants treated with Fe(III)-*o,o*-EDDHA. The inset (Panel B) shows a zoomed mass spectrum (409-416 *m/z*) at a retention time of 17.05 min.

whereas only one recent study deals with the photochemical and redox behaviour of Fe(III)-*o,o*-EDDHA at different pHs (Gómez-Gallego *et al*., 2005). At typical environmental pH values (4 – 8), the low reduction potential of Fe(III)-*o,o*-EDDHA makes it unreactive in photochemically- or chemically-induced electron transfer processes, which invalidates photodegradation as an alternative mechanism for environmental elimination. The persistence of chelates in the soil depends on their polarity and solubility (following the order: Fe(III)-EDDHSA > Fe(III)-EDDCHA >> Fe(III)-*o,o*-EDDHA > Fe(III)-*o,o*-EDDHMA), and this controls the movement of Fe(III)-chelates to the lower soil layers with an excess of water (Lucena, 2003).

Phytotoxicity studies are less common, and have been based on the appearance of symptoms (e.g., necrosis, necrotic spots, leaf malformations, etc.) and a decrease in biomass, as well as changes in leaf mineral composition (e.g., Fe, Mn, Zn, Cu, P, etc.). Although the concentration of Fe in leaves usually increases in plants treated with Fe(III)-chelates, a poor correlation is commonly found between leaf Fe concentration and the severity of plant toxicity symptoms (Broschat and Moore, 2004). Fe(III)-EDDHA toxicity frequently causes a reddish stain in the foliage. In bean plants, the phytotoxic level in the nutrient solution was 4 mM Fe(III)-EDDHA (Wallace and Wallace, 1983); whereas African marigold and zonal geranium plants showed mild toxic effects at 1 mM, with moderate toxic effects at 2 mM and 4 mM Fe(III)-EDDHA (Broschat and Moore, 2004). This study also found that Fe-EDDHA was less toxic than Fe-EDTA or Fe-DTPA, and slightly more toxic than $FeSO_4$.

The toxicological effects of EDDHA have been studied mainly in medical applications, when this chelating agent is used as a Fe chelating drug for patients with hemochromatosis. The median lethal dose of *o,o*-EDDHA (LD_{50}) was 53 mg kg^{-1} for intervienal-treated rats and mice (Rosenkrantz *et al*., 1986), and 0.30 mg cm^{-2} of soil for slugs' (*Deroceras reticulatum*) eggs exposed for 12 d (Iglesias *et al*., 2002).

FUTURE RESEARCH

Application of the most recent analytical techniques offers an excellent tool to increase our knowledge on the biological and environmental implications of fertilisation with synthetic Fe(III)-chelates. A better understanding of their mechanisms of action could rationalise their use, improve efficiency, and minimise their environmental effects. Finally, the presence of these xenobiotic compounds in plants makes it necessary to study their toxicological effects and persistence in edible plant parts.

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Foliar iron-fertilisation of fruit trees: present knowledge and future perspectives – a review

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SUMMARY
Iron (Fe)-deficiency is a common physiological disorder affecting fruit crops in many areas of the World. Foliar Fe-fertilisation is a common agricultural strategy to control Fe-deficiency under field conditions. However, variable responses to Fe sprays have often been described and foliar Fe-fertilisation cannot yet be considered a reliable strategy to control plant Fe-deficiency. The lack of understanding of some factors relating to the penetration, distribution, and bio-activity of leaf-applied, Fe-containing solutions hinders the development of effective Fe formulations for foliar treatment. The current state-of-the-art and future perspectives for foliar Fe-fertilisation, as a strategy to control Fe-deficiency in fruit crops, is discussed.

Iron (Fe)-deficiency chlorosis is a widespread physiological disorder affecting many fruit crops and is a limiting factor for production, especially under high pH, calcareous soil conditions, such as those prevailing in many agricultural areas with a Mediterranean climate. Typical symptoms of Fe-deficiency include the development of interveinal chlorosis, starting from the apical leaves, reduction of shoot growth, defoliation during the growing season and, ultimately, tree death (Rombolà and Tagliavini, 2006). Iron chlorosis has deleterious effects on fruit production, reducing the number of fruits per tree, fruit size, total yield, and affecting fruit quality parameters such as colour, firmness, or acidity (Álvarez-Fernández *et al*., 2003; 2006).

There is scientific evidence that Fe-fertilisation increases fruit quality and yield in many crops (Álvarez-Fernández *et al*., 2006). Iron-fertilisation is a standard agricultural practice in fruit production areas that suffer from plant Fe-deficiency. Strategies to alleviate Fe-chlorosis in fruit crops include: (i) the use of rootstocks tolerant to soil conditions that induce the development of the disorder and with improved Fe-uptake mechanisms; (ii) modifying soil characteristics; and/or (iii) treatment with Fe-substances *via* root, trunk, or canopy application(s) (Abadía *et al*., 2004; Lucena, 2006). Iron-fertilisation of roots is the most reliable and widely-used technique to control Fe-deficiency, and commercial Fe(III)-EDDHA-based products are the most effective fertilisers used to correct Fe-chlorosis under severe soil conditions (Lucena, 2006). However, such chemicals are expensive and may perform differently according to the particular Fe(III)-EDDHA formulation (Cerdán *et al*., 2007).

Foliar Fe-fertilisation could be a cheaper and more targeted strategy to correct plant Fe-chlorosis (Abadía *et al*., 2002; Álvarez-Fernández *et al*., 2004; Fernández *et al*., 2008a), but the response to Fe sprays has been shown to vary according to many plant-related, environmental, and physico-chemical factors (Fernández and Ebert, 2005). Problems of reproducibility and interpretation of results from foliar and cuticular Fe-application studies have been described (Fernández and Ebert, 2005). Our current limited understanding of the factors involved in the penetration, translocation, and bio-availability of leaf-applied Fe fertilisers makes it difficult to develop effective spray formulations for agricultural purposes. At present, foliar nutrition is only considered to be a valuable complement to the application of nutrients *via* the root system (Weinbaum, 1996).

In general, the penetration of Fe-containing solutions will be influenced by plant factors, environmental conditions, the nature of the spray solution, and the method of application (Currier and Dybing, 1959). Similarly, the roles of active and passive processes involved in the penetration and subsequent physiological effects of foliar-applied nutrient solutions remain controversial (Jyung and Wittwer, 1964; Zhang and Brown, 1999).

The effectiveness of leaf-applied, Fe-containing solutions is normally assessed on the basis of their re-greening capacity, tissue Fe-absorption rate, and Fe-translocation from the site of treatment (Fernández, 2004; Fernández *et al*., 2006; 2008a). Therefore, in response to foliar treatment with a Fe-containing solution, at least three distinct key processes can be distinguished, in theory, although they are difficult to separate from one another: (i) the penetration of foliar-applied Fe through the leaf surface; (ii) the distribution of Fe from the site of application; and (iii) the active involvement of exogenous Fe in physiological processes.

An account of the state-of-the-art concerning foliar Fe-fertilisation of fruit trees and the key factors to be considered for the development of more effective Fe-containing formulations is provided in the following sections.

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surfactant was ionised due to the presence of the Fe(III)-EDTA chelate.

Usually, Fe(III)-chelates are prepared by addition of $FeCl_3$ to the corresponding chelating agent, in this case K_2EDTA. Therefore, the hypothesis that Cl^- ions may be responsible for the ionisation of the polymer was subsequently tested. The mass spectrum of $FeCl_3$ plus 0.1% (v/v) Surfactant 1 is represented in Figure 2D. An identical polymer to the one observed in Figure 2C was formed. These results suggest that the Cl^- ions present in the Fe(III)-chelate solution may induce ionisation of the surfactant. The same polymer was observed in the mass spectrum of Fe(III)-EDTA synthesised from Na_2EDTA and $FeCl_3$ with Surfactant 1 (data not shown).

In summary, ions derived from the synthesis of the Fe(III)-chelate could induce ionisation of non-ionic surfactants due to "salting out" effects (Mackay, 1997), as shown above. This could affect the performance of surfactants as adjuvants in foliar sprays. Research is in progress to understand the interactions between Fe-substances and surfactants suitable for foliar application.

CONCLUSIONS AND FUTURE PERSPECTIVES

The performance of Fe-sprays is affected by many plant-related, environmental, and physico-chemical factors, which are currently not fully understood. Research should focus on investigating the potential interactions between formulation components using modern analytical techniques such as those described above. Efforts should be made to understand the relevance of the physico-chemical properties of spray solutions to design optimised Fe-containing formulations, and the significance of changes in the leaf surface in relation to the foliar uptake of agrochemicals. The process of penetration of a leaf-applied, Fe-containing solutions is not fully understood and should be investigated further, since foliar uptake is a prerequisite for leaf-cell Fe utilisation. Research on suitable foliar treatment strategies to ensure optimal plant coverage should also proceed. Similarly, information on plant Fe metabolism will facilitate the selection of bio-active Fe-containing compounds. The role of physiological processes and environmental factors in foliar Fe uptake and distribution should also be investigated further using intact leaves and following a holistic approach.

In summary, more knowledge relating to the role of Fe in plants, and on the effects of environmental, plant physiological, or leaf morphological factors, adopting a multi-disciplinary approach, is required for the development of effective Fe-spray formulations to correct widespread Fe-deficiency in fruit trees.

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Review

Towards a knowledge-based correction of iron chlorosis

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ABSTRACT

Iron (Fe) deficiency-induced chlorosis is a major nutritional disorder in crops growing in calcareous soils. Iron deficiency in fruit tree crops causes chlorosis, decreases in vegetative growth and marked fruit yield and quality losses. Therefore, Fe fertilizers, either applied to the soil or delivered to the foliage, are used every year to control Fe deficiency in these crops. On the other hand, a substantial body of knowledge is available on the fundamentals of Fe uptake, long and short distance Fe transport and subcellular Fe allocation in plants. Most of this basic knowledge, however, applies only to Fe deficiency, with studies involving Fe fertilization (i.e., with Fe-deficient plants resupplied with Fe) being still scarce. This paper reviews recent developments in Fe-fertilizer research and the state-of-the-art of the knowledge on Fe acquisition, transport and utilization in plants. Also, the effects of Fe-fertilization on the plant responses to Fe deficiency are reviewed. Agronomical Fe-fertilization practices should benefit from the basic knowledge on plant Fe homeostasis already available; this should be considered as a long-term goal that can optimize fertilizer inputs, reduce grower's costs and minimize the environmental impact of fertilization.

1. Introduction

Iron (Fe) deficiency chlorosis is a major nutritional disorder in crops growing on calcareous soils. This deficiency is particularly important in fruit tree species, causing decreases in tree vegetative growth, marked fruit yield and quality losses and a decrease in the life span of orchards (for reviews see [1,2]). Therefore, Fe fertilizers, either applied to the soil or delivered to the foliage, are provided to these crops every year to control Fe deficiency, and the use of Fe-fertilization is increasing. The amounts of Fe needed depend on the crop, and in peach trees they are in the range of 1–2 g per tree and per year [3]. In many cases, Fe-fertilization is done at just one or a few specific time points, for instance in the case of chelate soil applications, trunk and branch injections and foliar sprays. In other cases, the application is done on a more frequent basis and with a more diluted Fe fertilizer, such as in the case of fertirrigation.

Improving current Fe chlorosis practical correction methods will need taking into account the state-of-the-art of all related scientific knowledge, integrating physiological, biochemical and agronomical data. With this aim, we review here the recent research on Fe-fertilizers, including the development and application of new advanced analytical techniques that allow for the specific and sensitive detection of low concentrations of these Fe compounds, not only in growth media, but also in plant tissues. We also summarize and discuss the substantial basic physiological and biochemical knowledge obtained in the last years on how plants acquire, transport and utilize Fe. In all cases, Fe-fertilization leads to episodes of high Fe concentration in the rhizosphere and the roots (in cases of soil or growth substrate fertilization) or in plant shoot tissues (in cases of foliar fertilization and fertilizer injections). However, how these high-Fe episodes caused by fertilization may affect plant Fe uptake and transport processes is much less known, and this review also focuses on these poorly explored interactions.

2. Iron fertilizers

Increasing the amount of crop-available Fe has long been carried out by means of Fe-fertilizer application to soils and irrigation water, as well as to plant seeds, roots, shoots and foliage. Iron fertilizers are grouped into three main classes: inorganic Fe-compounds, synthetic Fe-chelates and natural Fe-complexes (for reviews see [2–7]).

Fertilizers based on inorganic Fe-compounds include soluble ones such as Fe salts (e.g., $Fe_2(SO_4)\cdot 7H_2O$) and insoluble

Abbreviations: BPDS, 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid; *o,o*EDDHA, ethylenediamine-*N*-*N*′bis(*o*-hydroxyphenylacetic) acid; EDTA, ethylenediamine tetraacetic acid; FCR, Fe chelate reductase; MA, mugineic acid; NA, nicotianamine; PS, phytosiderophore.

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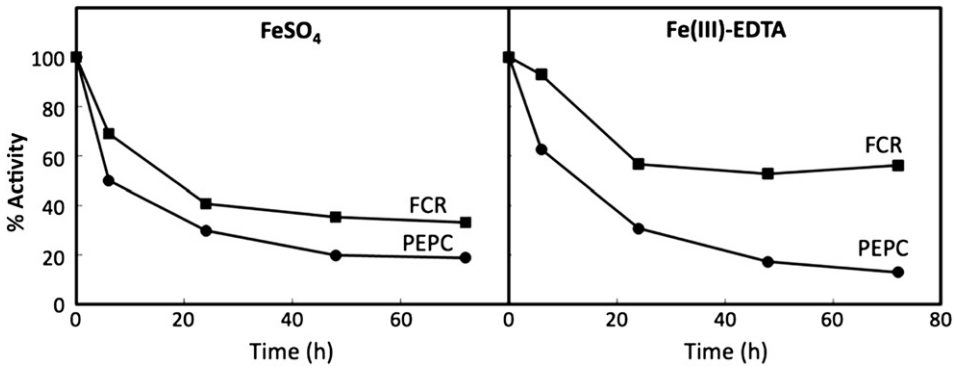

Fig. 8. Deactivation of root Fe-deficiency responses with foliar Fe fertilization. Percentage of initial phospho*enol*piruvate carboxylase (PEPC; circles) and Fe-reductase (FCR; squares) activities in root extracts from sugar beet (*Beta vulgaris* L.) Fe-deficient plants resupplied with Fe. Measurements were done at different time-points after a single Fe foliar spray with 2 mM $FeSO_4$ or Fe(III)-EDTA. Data are means ± SD of 9 plants used as replicates.

may cause Fe immobilization when foliar fertilization is applied, ultimately lowering treatment efficacy. The use of high-resolution image techniques should clarify this hypothesis.

There is also evidence that foliar Fe-fertilization could de-activate plant root Fe uptake mechanisms. In tobacco plants, a foliar spray of 100 μM Fe(III)-EDTA caused, 24 h after treatment, a decrease in root expression of *NtFRO1* and *NtIRT1* gene transcript levels to values similar to those found in control plants, whereas a lower Fe concentration (10 μM) did not cause such effects [87]. In Fe-deficient sugar beet plants, a foliar Fe application of 2 mM Fe (III)-EDTA or $FeSO_4$ (this is a commonly used Fe concentration in foliar sprays that can be very efficient in field conditions) caused decreases in root FCR activities of 10–30 and 40–65% at 6 and 24 h after application, respectively, with the decreases being larger for $FeSO_4$ (Fig. 8). The decrease upon Fe-fertilization was even larger for the activity of root phospho*enol*pyruvate carboxylase, another enzyme elicited by Fe-deficiency (Fig. 8).

All these data indicate that foliar fertilization may de-activate root Fe-reduction strategy responses very rapidly, suggesting that root responses are down-regulated directly by an Fe-dependent signal perhaps Fe itself-, possibly *via* phloem. Therefore, it should be carefully assessed whether foliar fertilization management techniques could have a deleterious effect by de-activating root responses to Fe deficiency.

6. Concluding remarks

For the optimization of the Fe-fertilization strategies it will be crucial to further improve the basic knowledge on the long and short-transport of Fe, xylem loading and unloading, Fe immobilization and the Fe acquisition processes by mesophyll leaf cells and subcellular compartments.

Iron trafficking within the plant involves the passage through many environments with different pH values and chemical composition. This implies that Fe should change from one to another chemical specie(s) in each of the corresponding environment interfaces. Furthermore, in each environment Fe could be in several different forms, and we are only starting to unravel their identity and localization by using advanced analytical technologies.

Also, it is often assumed that Fe contained in Fe-fertilizers would be taken up, transported and utilized following mechanisms and processes present in Fe-deficient plants. However, there is emerging evidence that Fe-resupply caused by Fe-fertilization could change the physiology and biochemistry of these Fe-deficient plants. Upon Fe-fertilization (either to the roots or the shoots), some of the mechanisms elicited by Fe-deficiency will be modulated or de-activated in the short term. Furthermore, some Fe-fertilizers such as Fe(III)-chelates could enter the plant directly, without using known uptake pathways. Therefore, comprehensive studies on the physiology of Fe-resupplied plants, including the effects of the different kinds of Fe-fertilization on the modulation of the reduction-based and chelation-based Fe acquisition strategies, are highly needed.

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Setting good practices to assess the efficiency of iron fertilizers

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ABSTRACT

The most prevalent nutritional disorder in fruit tree crops growing in calcareous soils is Fe deficiency chlorosis. Iron-deficient, chlorotic tree orchards require Fe-fertilization, since chlorosis causes decreases in tree vegetative growth as well as fruit yield and quality losses. When assessing the effectiveness of Fe-fertilizers, it is necessary to use sound practices based in the state-of-the art knowledge on the physiology and biochemistry of Fe deficiency. This review provides an overview on how to carry out the assessment of the efficiency of Fe-fertilizers, discussing common errors found in the literature, outlining adequate procedures and giving real examples of practical studies carried out in our laboratory in the past decade. The review focuses on: i) the design of Fe-fertilization experiments, discussing several issues such as the convenience of using controlled conditions or field experiments, whether fertilizer assessment experiments should mimic usual fertilization practices, as well as aspects regarding product formulations, dosages, control references and number of replicates; ii) the assessment of chlorosis recovery upon Fe-fertilization by monitoring leaf chlorophyll, and iii) the analysis of the plant responses upon Fe-fertilization, discussing the phases of leaf chlorosis recovery and the control of other leaf nutritional parameters.

1. Introduction

The most prevalent nutritional disorder in fruit tree crops growing in calcareous soils is Fe deficiency (see reviews in [1,2]). The main symptom of Fe deficiency in plants is leaf yellowing, which is usually called leaf chlorosis; this occurs both in growth chamber and field-grown plants (*e.g.*, in sugar beet and peach trees, respectively; Fig. 1). In field conditions, chlorosis in the orchards is often heterogeneous, with individual trees affected to different extents. Images of fruit tree field orchards affected by Fe-chlorosis are shown in Fig. 2 (A: peach tree orchard; B: pear tree orchard). Iron-deficient, chlorotic tree orchards are usually fertilized with Fe every year, because chlorosis causes decreases in tree vegetative growth, a shortening of the orchard lifespan as well as losses in fruit yield [2] and changes in fruit quality [3,4]. The diagnosis of Fe deficiency, conversely to what happens with other nutrient disorders, cannot be adequately assessed using leaf elemental composition, because Fe-deficient field-grown leaves often have Fe concentrations as high as that of Fe-sufficient ones (the "chlorosis paradox"; [5]). This is possibly associated to an accumulation of Fe in or near the vascular system [6,7]. Therefore, leaf chlorophyll (Chl) concentrations (generally monitored using a hand-held device) are used most of the times to assess the Fe nutritional status.

Iron fertilization in trees can be carried out in several ways, including the addition to the soil or irrigation water of Fe-containing compounds [8], as well as providing Fe directly to the plant by spraying tree canopies or injecting trunks or branches with Fe-compounds in solid or liquid forms [1]. There is a very large number (several hundred) of Fe-containing fertilizers, many of them containing the same active principles and others consisting of a mixture of Fe-compounds [8,9]. These Fe-fertilizers often have different degrees of effectiveness due to many different factors [1,8,10]. Therefore, it is necessary to compare the efficiencies of Fe-fertilizers, and many studies are published every year assessing and comparing Fe-containing products (*e.g.*, see [11,12]). In particular, any new Fe-fertilizer must be assessed using this type of studies. The recovery after Fe-fertilization is generally monitored using the leaf Chl concentration, for the reasons explained above, although leaf Fe concentrations are still sometimes used. However, divergences in specific methodological details could be found in the literature, and

Abbreviations: Chl, chlorophyll; EDDHA, ethylenediamine-N-N′bis(*o*-hydroxyphenylacetic) acid; SPAD, Soil and Plant Analyzer Development.

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4.1. The three phases of leaf chlorosis recovery

When considering the corrective effects of Fe-fertilizers, three different response parameters can be assessed: rapidity, maximal intensity and persistence [37]. When using soil applications to fruit trees, once the Fe-fertilizer is applied, there is usually an approximately one-week lag phase where no changes in leaf Chl are observed. Then, leaf Chl concentrations begin to increase, and the rapidity of this phase of the response can vary among different fertilizers during the first month after Fe-application (Fig. 6A). These differences likely reflect different Fe uptake and transport rates, which may depend on the specific product used. After approximately 1–1.5 months the maximal intensity of the response is usually observed, and this may also differ among Fe-fertilizers (Fig. 6B). Finally, the persistence of the response can be observed in the following months, with some Fe-fertilizers leading to a sustained leaf Chl concentration, whereas in others the effects are weakened with time, and trees progressively develop Fe-chlorosis symptoms (Fig. 6C). This is likely the result of the growth effects caused by the correction of Fe deficiency, which inevitably lead to a further increase in tree Fe demand that cannot be met adequately by some fertilizers or dosages. In fact, some fertilization practices such as branch solid injections may be efficient one or several years after Fe-application [38]. In the case of foliar sprays, several applications per year may be needed (for a review, see [10]).

New Fe-fertilizers for fruit trees should preferentially be aimed to improve overall intensity and persistence rather than rapidness of recovery, which would be less important considering that major effects on fruit quality and yield would be expected to occur only in the following growth season.

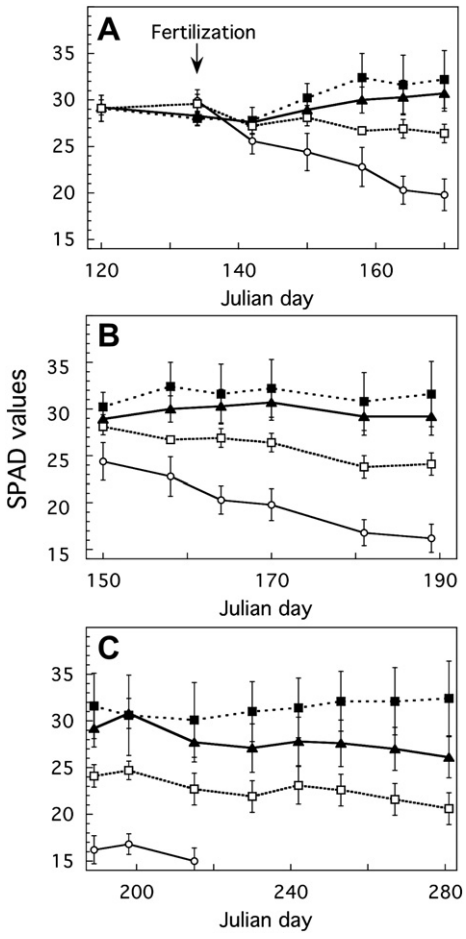

Fig. 6. Time-course of leaf SPAD values after soil Fe-fertilization with different Fe-products in field-grown *Prunus persica* trees showing the three phases of response to Fe-fertilizers. Untreated trees are represented as white circles, the reference product (Fe–EDDHA) as black triangles and two different commercial products as black and white squares (in all cases $n = 4$ trees). The rapidity of the response can be estimated in the first weeks after the Fe-fertilizer application (A). The intensity of the response can be estimated in the approximately 1–1.5 month after the Fe-fertilizer application (B). The persistence of the response can be estimated during the following months (C).

4.2. Control of other leaf nutritional parameters

In field experiments there is always a possibility that other biotic or environmental factors could result in decreases in leaf Chl contents. These include pathogens [39], as well as other nutrient deficiencies such as those of N [40], Zn [41,42] and Mn [43,44]. In plant species other than fruit trees, several metal toxicities [45,46], have been reported to decrease Chl concentrations. Therefore, it is mandatory to have a previous knowledge of the orchard where the experiment will be carried out (*e.g.*, for at least two years), to reduce the possibilities that such interfering factors could be present. The best way to assure that leaf chlorosis is due to Fe deficiency is to use the so-called "biological diagnosis", using local applications of Fe salts (*via* leaf sprays, petiole treatment or leaf injection) to check that re-greening occurs [27,47]. It is always advisable to analyse mineral concentrations in leaf samples at the beginning and at the end of the treatment, as well as on the standard mineral analysis dates, 60 or 120 days after flower full bloom. These analyses constitute an additional and very useful monitoring tool, since they permit monitoring other parameters (Fe and K concentrations, K/Ca and P/Fe ratios, etc.) that also change with the tree Fe nutrition status [48–51].

5. Concluding remarks

When assessing the effectiveness of Fe-fertilizers, it is necessary to use sound practices based in the state-of-the art knowledge on the physiology and biochemistry of Fe deficiency [13]. This includes using appropriate choosing of experimental orchards and individuals (taking special care in assuring the presence of Fe deficiency and the homogeneity of chlorosis) as well as an adequate methodology to measure leaf Chl concentrations. It should be always taken into account that the effectiveness of a given Fe-fertilizer will depend on the specific conditions imposed in the particular study, and in many cases a positive result will not grant efficiency in other scenarios.

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