Referencia: **AGL2006-01416/AGR**

MINISTERIO DE EDUCACIÓN Y CIENCIA

DIRECCIÓN GENERAL DE INVESTIGACIÓN

PROYECTOS I+D, ACCIONES ESTRATÉGICAS Y ERANETS

INFORME FINAL

Investigador Principal:	Anunciación Abadía
Título del Proyecto:	Bases para un uso racional de los fertilizantes de micronutrientes en la nutrición de especies frutales
Organismo:	Consejo Superior de Investigaciones Científicas
Centro:	Estación Experimental de Aula Dei
Departamento:	Nutrición Vegetal
Fecha de Inicio:	01-10-2006
Fecha de Finalización:	31-12-2010

Fecha: 26 de Marzo de 2010

Ilmo. Sr. Subdirector General de Proyectos de Investigación
C/ALBACETE 5. 28071 MADRID.

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.

Objetivo 1. Estudio de las necesidades reales de micronutrientes en melocotonero.

Se proponía evaluar los balances de nutrientes en melocotonero, analizando las salidas de nutrientes debidas a madera de poda, flores, frutos y hojas, y los nutrientes inmovilizados en madera y raíces. Asimismo, se proponía el diseño de un modelo de predicción de demanda de nutrientes.

Se han recogido los datos sobre flores, hojas, frutos y madera de poda como se había propuesto inicialmente. Se ha alargado la toma de muestras hasta la poda y extracción de árboles efectuada entre enero y febrero de 2010, ya que la primera toma de muestra se realizó con las flores de 2007. De esta manera se dispondrá de tres años enteros de datos. Asi pues, los datos finales de este Objetivo no están completos y el modelo final no se obtendrá hasta que no se hayan analizado y procesado todas las muestras recogidas. Ya se han efectuado los primeros estudios estadísticos para hallar las ecuaciones para relacionar diámetro, marco de plantación y biomasa.

A pesar de que ya se propuso el objetivo con la idea de obtener el modelo al final del proyecto, se han presentado algunos resultados obtenidos sobre el balance de nutrientes (resultados **C-6** y **C-15**) y sobre el diseño del modelo de demanda de nutrientes (**C-9** y **C-14**). Se defendió una Tesis de Master en Enero 2009 con datos de pérdidas de nutrientes recogidos durante casi 2 años (**T-4**). En esta Tesis se presentó un modelo preliminar de demanda de nutrientes. Hay una Tesis Doctoral en curso que incluirá este objetivo (**T-3**).

Además se ha trabajado sobre la localización de los sitios de acumulación de Fe y otros nutrientes en materiales deficientes (**AC-5**). Se han empezado a explorar distintas técnicas de imagen para el estudio de metales en plantas (**C-18**), objetivo que se seguirá desarrollando en posteriores proyectos. Se han hecho los primeros estudios de microanálisis elemental en melocotonero mediante SEM/EDXA en plantas con deficiencia de Fe (**C-16** y **AD-3**). Se ha seguido trabajando sobre relaciones de nutrientes y prognosis, estudiando relaciones entre contenidos minerales en distintos tejidos en melocotonero y peral (**C-12**).

Objetivos 2 y 3. Estudio de los mecanismos de acción de los fertilizantes de micronutrientes y estudio de la permanencia de los fertilizantes de micronutrientes y su interacción con otros micronutrientes.

En el Objetivo 2 se proponía el estudio del efecto de diferentes quelatos en la actividad Fe-reductasa de raíz y hoja, la cinética de las reacciones de intercambio de ligando entre los fitosideróforos y los diferentes quelatos sintéticos, la caracterización de la desactivación de mecanismos de respuesta tras la aplicación de fertilizantes y los cambios en xilema y apoplasto tras la fertilización. En el objetivo 3 se proponía la determinación de la presencia de fertilizantes en matrices de agua, suelo y planta, después de desarrollar los métodos adecuados y examinar los efectos de los agentes quelantes en la disponibilidad de otros elementos tanto en planta como en suelo.

Se ha hecho una revisión sobre las técnicas analíticas aplicadas al estudio de los efectos biológicos y ambientales de la fertilización con quelatos férricos (**AC-7**). Se ha seguido trabajando en la optimización de metodologías de extracción y caracterización de quelatos férricos (especialmente Fe(III)-*o,o*EDDHA) en matrices complejas como distintos tejidos vegetales (hoja, raíz y fruto) de varias especies remolacha, tomate, melocotonero, peral y naranjo) para permitir su análisis por dicho método (**AC-10**, **C-4**, **C-5**, **C-13** y **AD-1**). Se ha estudiado el uso de diferentes estándares internos con la misma molécula marcada isotópicamente con Fe (Fe^{54} y Fe^{57}) ó con una molécula estructuralmente análoga desarrollando y validando un método específico para la extracción y determinación del quelato Fe(III)-*o,o*EDDHA en una diversidad de tejidos de diferentes especies vegetales (**AC-8**). El mecanismo de acción del quelato férrico Fe(III)-EDDHA se ha abordado sintetizando el quelato férrico con isótopos estables de Fe (Fe^{54} y Fe^{57}) , alimentando plantas de remolacha deficientes en Fe con dichos compuestos y midiendo la adquisición y distribución del Fe dentro de la planta (**AC-12**, **C-10** y **C-17**). Se ha estudiado la identificacion de impurezas en compuestos de Fe (**AC-10** y **C-21**). Se ha efectuado la caracterización de la desactivación de mecanismos de respuesta tras la aplicación de fertilizantes foliares y a la solución hidropónica en cámara de cultivo (**C-7**). Se ha caracterizado el metabolismo de ácidos orgánicos en una planta modelo afectada por deficiencia (**AC-6**) y el contenido en ácidos

orgánicos y Fe en xilema de plantas de remolacha deficientes en Fe y después de adición de este elemento (**AC-9**). Se ha hecho una revisión sobre el transporte a larga distancia de Fe en plantas (**CI-2**). Se ha caracterizado la formación de complejos metal-nicotianamina, importantes en el transporte de metales en plantas (**AC-3**). Se ha caracterizado el complejo transportador de Fe en xilema (**AC-11** y **C3**). Se han puesto a punto las técnicas de caraterización de proteoma y metaboloma de xilema, hojas y puntas de raíz en plantas modelo: i, caracterización del metaboloma de xilema de plantas de tomate y altramuz (**C-8**); ii, proteoma y metaboloma de raíces de plantas de remolacha (**CI-4**, **C-11 y AC-13**) en ambos casos en plantas control, deficientes en Fe y fertilizadas con quelatos férricos; y iii, metabolómica de distintos tejidos en caso de deficiencia de Fe (**C-20**). Se han efectuado las primeras tomas de muestra de savia de xilema en melocotoneros afectados por distintos grados de clorosis y se están llevando a cabo los correspondientes análisis. También se han tomado muestras de xilema en árboles tratados con productos conteniendo Fe que están siendo analizadas. Se ha caracterizado la excrección y acumulación de riboflavina y sus derivados inducida por deficiencia de Fe en una planta modelo (**C-2** y **C-22**).

Se presentarán dos tesis Doctorales durante 2010 cuyos trabajos se enmarcan en estos objetivos (**T1** y **T2**)

Objetivo 4. Estudio de la optimización de la aspersión foliar como método de corrección de clorosis.

Se proponía un estudio sobre la aspersión foliar y los efectos en planta de la aplicación de distintas formulaciones así como sobre su mecanismo de penetración.

Se han establecido los avances recientes en formulaciones de aspersiones foliares de Fe (**AC-4, AD-2** y **CI-1**). Se ha caracterizado en campo y sobre melocotonero el comportamiento de diversas mezclas de productos conteniendo hierro ($FeSO_4.7H_2O$, Fe(III)-citrato, Fe(III)-EDTA, Fe(III)-DTPA y Fe(III)-IDHA) y varios surfactantes, comprobándose que hay interacciones interesantes entre los diversos compuestos, que serán objeto de estudio en próximas investigaciones (**AC-1**, **AC-2** y **C-1**). También se han puesto de manifiesto los efectos que los diferentes tratamientos causan en el balance nutricional de las hojas de melocotonero (**AC-2**). Por otra parte, se han caracterizado las cutículas de hojas de peral y melocotonero en condiciones de campo en materiales control y afectados por deficiencia de Fe, tanto a nivel de microscopía, óptica y electrónica de barrido, como de peso de ceras, transpiración y apertura estomática (**AC-2**). Se presentó una ponencia invitada en relación a este objetivo en el VI International ISHS Symposium on Mineral Nutrition of Fruit Crops en Faro, Portugal (**CI-3**). Se ha puesto a punto el método de obtención de secciones transversales en hoja para el estudio de gradientes de clorofila y nutrientes en remolacha después de una fertilización foliar (**C-19**) y se está analizando la composición de dichas secciones. Se está trabajando en la posibilidad de adecuar esta técnica a hojas de melocotonero.

Diseminación

Se ha llevado a cabo la diseminación según se había propuesto inicialmente. Así:

✯ Se han presentado ponencias en diferentes reuniones nacionales e internacionales en temas relacionados con el proyecto.

✯ Se han redactado varias publicaciones en revistas científicas incluidas en ISI.

✯ Se ha incluido información sobre los resultados del proyecto (publicaciones y presentaciones a congresos) en la página web del grupo (http://www.stressphysiology.com).

CONGRESOS (ORALES)

C-1) 2007 Isafruit Meeting. Bologna, Italia. Abadía J, Álvarez-Fernández A, Fernández V, Abadía A. Recent developments in fruit tree Fe-fertilization: foliar spray formulations and Fe-chelate analysis.

C-2) 2008 14th International Symposium on Iron Nutrition and Interactions in Plants (14th ISINIP), Beijing, China. 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*.

C-3) 2009 XVIII Reunión de la Sociedad Española de Fisiología Vegetal-X Congreso Hispano-Luso de Fisiología Vegetal. Zaragoza, España. 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.

CONGRESOS (PANELES)

C-4) 2006 XIIth Symposium on Sample Handling for Environmental and Biological Analysis, Zaragoza, España. Orera I, Álvarez- Fernández A, Abadía J, Abadía A. Determination of Fe(III)-chelates used as fertilizers in agricultural matrices.

C-5) 2007 Complexing Agents between Science, Industry, Authorities and Users (CASIAU), Zurich, Suiza. Orera I, Álvarez-Fernández A, Abadía J, Abadía A. Developing an extraction procedure of Fe(III)-EDDHA from plant tissues suitable for its determination by HPLC-ESI/MS.

C-6) 2008 VI International ISHS Symposium on Mineral Nutrition of Fruit Crops, Faro, Portugal. El-Jendoubi H, Hammami S, del Río V, Abadía J, Abadía A. Macro and micronutrient demand in peach trees.

2008 14th International Symposium on Iron Nutrition and Interactions in Plants (14th ISINIP), Beijing, China.

- **C-7)** López-Millán AF, Moussaoui S, Fernández V, Abadía J, Abadía A. Deactivation of sugar beet root responses to iron deficiency upon foliar iron application.
- **C-8)** 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.
- **C-9)** El-Jendoubi H, del Río V, Scandellari F, Abadía J, Tagliavini M, Abadía A. Nutrient (including iron) demand model in peach trees.
- **C-10)** 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,oEDDHA isomers.
- **C-11)** 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.

C-12) 2008 XXII Simposio Ibérico de Nutrición Mineral de las Plantas, Granada, España. 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 diversos materiales.

2008 Isafruit Meeting. Gerona, España.

- **C-13)** 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.
- **C-14)** El-Jendoubi H, Scandellari F, Moreno MA, Abadía J, Tagliavini M, Abadía A. Nutrient demand prediction model in peach trees.
- **C-15)** El-Jendoubi H, Hammami S, Abadía J, A Abadía. Macro and micronutrient demand in peach trees.

2009 XVI International Plant Nutrition Colloquium. Sacramento, California, USA.

- **C-16**) Vazquez S, Pinto F, Abadía A, Abadía J. Elemental microanalysis in leaf transversal sections of peach by SEM/EDXA: Influence of iron nutritional status.

2009 XVIII Reunión de la Sociedad Española de Fisiología Vegetal-X Congreso Hispano-Luso de Fisiología Vegetal. Zaragoza, España.

- **C-17**) Orera I, Abadía A, Abadía J, Álvarez-Fernández A. Study of the plant iron fertilization with synthetic ferric chelates by mass spectrometry.
- **C-18**) Vázquez S, Abadía A, Abadía J. Image techniques: New approaches in metal homeostasis.
- **C-19**) El-Jendoubi H, Lastra M, Vázquez S, Abadía A. Effects of foliar Fe-application on chlorophyll concentration, mineral composition and Fe distribution in sugar beet leaves.
- **C-20**) Rellán-Álvarez R, Rodriguez-Celma J, López-Millán AF, Fiehn O, Álvarez-Fernández A, Abadía A, Abadía J. Plant iron deficiency metabolomics.
- **C-21**) Orera I, Orduna J, Abadía J, Álvarez-Fernández A. Identification of Fe-containing impurities in commercial fertilizars by collision induced dissociation tandem mass spectrometry.
- **C-22**) 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.*

B. RESULTADOS MÁS RELEVANTES ALCANZADOS EN EL PROYECTO (máximo 60 palabras).

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

- Desarrollo de métodos de MS para la caracterización de metabolitos o xenobioticos en plantas (i.e. identificación por primera vez de un complejo Fe-citrato en xilema ó uso de isótopos estables en estudios en plantas).

- Estudio de las diferentes técnicas de microscopía que pueden ayudar en el estudio de nutrición vegetal (i.e. superficies de las hojas de interés en fertilización foliar).

C. RESUMEN DE LOS RESULTADOS DEL PROYECTO.

C1. Formación del personal Nº

Personal formado (3 licenciados o ingenieros)

Personal formado o en formación que se ha transferido al sector industrial:

Doctores ( ) Titulados Superiores ( ) Técnicos ( )

C2. Tesis doctorales	(3) 1 en redacción final, 1 en escritura, 1 en tercer año			
C3. Artículos científicos en revistas	( )	nacionales	(11)	internacionales
C4. Artículos de divulgación en revistas	(1)	nacionales	(2)	internacionales
C5. Artículos de revisión en revistas	( )	nacionales	( )	internacionales
C6. Libros, capítulos de libros y monografías	( )	nacionales	( )	internacionales
C7. Conferencias en congresos (por invitación)	( )	nacionales	(4)	internacionales
C8. Patentes y otros títulos de propiedad industrial	( )	registrados	( )	en explotación
	( )	España	( )	extranjero

C1. FORMACIÓN DE PERSONAL EN EL PROYECTO, describir brevemente.

Dentro de este proyecto se encuentra parcialmente el trabajo experimental (ya terminado) de dos personas que defenderán su Tesis Doctoral en 2010 (I. Orera y R. Rellán). Hay un becario más (H. El-Jendoubi) realizando su Tesis Doctoral enmarcada en objetivos de este proyecto.

Además, 2 técnicos contratados por el proyecto han recibido formación en el ámbito de la Nutrición Vegetal en el tiempo que han durado sus respectivos contratos.

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.

TESIS DE DOCTORALES

A defender en 2010:

T-1) *Desarrollo y aplicación de nuevas metodologías analíticas para el estudio de fertilizantes férricos.* **Irene Orera Utrilla**. Dpto. de Química Analítica de la Universidad de Zaragoza.

T-2) *Transporte de hierro a larga distancia y metabolómica de la deficiencia de hierro en plantas*. **Rubén Rellán Álvarez.** Dpto. de Biología Vegetal de la Universidad Autónoma de Madrid.

A presentar en 2011-2012:

T-3) *Título a determinar***. Hamdi El-Jendoubi**. ETSIA Lleida.

TESIS DE MASTER

T-4) *Necesidades de nutrientes minerales en melocotonero (Prunus persica L. Batsch).* **Hamdi El-Jendoubi,** Enero de 2009, IAMZ-CIHEAM/Universidad de Lérida.

C3. ARTÍCULOS CIENTÍFICOS EN REVISTAS

Indicar: Autor(es), título, referencia de la publicación, **(adjuntar en formato digital la primera página y aquella en la que se mencione a las entidades financiadoras del proyecto)**

Ver: http://www.stressphysiology.com

AC-1) Fernández V, del Río V, Pumariño L, Abadía J, Abadía A (2008) Foliar Fertilization of

peach (*Prunus persica* (L.) Bastch) with different iron formulations: Effects on re-greening, iron concentration and mineral composition in treated and untreated leaf surfaces. Sci Hort, 117: 241-248.

AC-2) Fernández V, Eichert T, del Río V, López-Casado G, Heredia-Guerrero J, Abadía A, Heredia A, Abadía J (2008) Leaf changes associated with iron deficiency chlorosis in fieldgrown pear and peach: physiological implications. Plant Soil, 311:161-172.

AC-3) 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 ionization time-of-flight mass spectrometry, Rapid Commun Mass Sp, 22:1553-1562.

AC-4) Fernández V, I Orera, J Abadía, A Abadía (2009) Foliar iron fertilisation of fruit trees: present and future perspectives. J Hortic Sci Biotech, 84:1-6.

AC-5) Jiménez S, Morales F, Abadía A, Abadía J, Moreno MA, Gogorcena Y (2009) Uptake, transport, and 2D mapping of within-leaf nutrient variability in the GF 677 peach almond hybrid. Preferential sites of iron accumulation in conducting tissues under iron deficiency. Plant Soil, 315:93-106.

AC-6) López-Millán A-F, Morales F, Gogorcena Y, Abadía A, Abadía J (2009) Organic acid metabolism in iron deficient tomato plants. Journal of Plant Physiology, 166:375-384.

AC-7) 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.

AC-8) Orera I, Abadía A, Abadía J, Álvarez-Fernández A (2010) 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.

AC-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.

AC-10) Orera I, Orduna J, Abadía J, Álvarez-Fernández A (2010) Electrospray-Collision-Induced Dissociation Mass Spectrometry: a Tool to Characterize Synthetic Polyaminocarboxilate Ferric Chelates used as Fertilizers. Rapid Commun Mass Sp, 24: 109-119.

AC-11) Rellán-Álvarez R, Giner-Martínez-Sierra J, Orduna J, Orera I, Rodríguez-Castrillón JA, García-Alonso JI, Abadía J, Álvarez-Fernández A (20010) 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 Physiology, 51: 91-102.

Artículos en revisión ó en preparación

AC-12) Orera I, Abadía A, Abadía J, Álvarez-Fernández A. Iron and Fe(III)-chelate uptake and distribution in plants grown in hydroponics and treated with racemic and meso Fe(III)-o,oEDDHA isomers in the nutrient solutions. En prep.

AC-13) Rellán-Álvarez R, Andaluz S, Rodríguez-Celma J, Wohlgemuth G, Zocchi G, Álvarez-Fernández A, Fiehn O, López-Millán AF, Abadía J. Changes in the proteomic and metabolic profiles of *Beta vulgaris* root tips in response to iron deficiency and resupply. BMC Plant Biology, en revisión.

C4. ARTÍCULOS DE DIVULGACIÓN EN REVISTAS

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

AD-1) Orera I, Abadía J, Abadía A, Álvarez-Fernández A (2009) Nuevas metodologías aplicadas a la investigación de quelatos de hierro sintéticos. Vida Rural, 1 septiembre 2009, 60-64.

AD-2) Toselli M, Scudellari D, Fernández V, Abadía J (2009) La nutrizione fogliare delle colture arboree da frutto. Italus Hortus 13, 17-23.

AD-3) 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, http://www.escholarship.org/uc/item/9mc2h13p).

C5. ARTÍCULOS DE REVISIÓN
Indicar: Autor(es), título, referencia de la publicación, **(adjuntar primera página en formato digital).**

C6. LIBROS, CAPÍTULOS DE LIBROS Y MONOGRAFÍAS
Indicar: Autor(es), título, referencia de la publicación, **(adjuntar en formato digital portada e índice donde figure la información).**

C7. CONFERENCIAS EN CONGRESOS, SIMPOSIOS Y REUNIONES (POR INVITACIÓN)
Indicar: Autor(es), nombre del congreso, lugar de celebración, año.

CI-1) 2006 XIV Congreso del "Grupo de Fertilización Foliar" Würzburg, Alemania. Fernández V, Pumariño L, Del Río V, Abadía J, Abadía A. Re-greenning of chlorotic peach leaves: effect of different iron sources and surfactants.

CI-2) 2007 X Congreso Hispano-Luso de Fisiología Vegetal. Alcalá de Henares, Madrid. Abadía J, Álvarez-Fernández A, AF López-Millán, Orera I, Rellán R, Abadía A. Long-distance metal transport in plants.

CI-3) 2008 VI International ISHS Symposium on Mineral Nutrition of Fruit Crops, Faro, Portugal. Fernández V, Abadía J, Abadía A. Foliar fertilisation: a reliable strategy to control plant nutrient deficiencies?

CI-4) 2009 XVI International Plant Nutrition Colloquium. Sacramento, California, USA. 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.

C8. PATENTES Y OTROS TÍTULOS DE PROPIEDAD INDUSTRIAL
Indicar: Autor(es), título, registro, entidad titular de la patente, año, países, clase.
Indicar cuales están en explotación.

C9. OTROS RESULTADOS EXTRAORDINARIOS NO INCLUIDOS EN LOS APARTADOS ANTERIORES
Indicar Naturaleza y Autor (es). Descríbalo brevemente en un máximo de 50 palabras.

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

( ) 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.

Directamente relacionadas con el proyecto hay varias colaboraciones en curso, algunas ya iniciadas en proyectos anteriores:

• con el grupo de Massimo Tagliavini (Universidad de Bolzano, Italia) en el estudio de balances de nutrientes. Con este grupo se colabora en el proyecto ISAFRUIT financiado por la Comisión Europea (ref 016279, 2006-2010) (**C-9** y **C-14**).

• con el grupo de Maribela Pestana (Universidad del Algarve, Portugal) en el estudio de balances nutricionales y fertilización en materiales afectados por clorosis. La IP del proyecto (A. Abadía) ha participado en dos proyectos financiados por la FCT-Portugal (ref PTDC/AGR-ALI/66065/2006, 2007-2009, finalizado), y (ref PTDC-AGR-AAM/100115/2008, 2009-2011, en curso) tambien en relación con clorosis férrica.

• con el grupo de Monji Msallem (Instituto de Olivicultura, Túnez) en el estudio de nutrición clásica y aspersión foliar. Con este grupo se ha conseguido financiación por medio de

proyectos AECID (ref A/8333/07 y A/017280/08, 2008-2010), y con miembros de este grupo se continúa publicando (**AC-9**).

• con el grupo de José Ignacio García Alonso (Universidad de Oviedo) en la utilización de isótopos estables en metrología química. Se ha mandado una publicación con miembros de este grupo (**AC-11** y **C-3**) y otra está en proceso de redacción (**AC-12** y **C-10**).

• con el grupo de Antonio Heredia (Universidad de Málaga) en el estudio de cutículas foliares. Se ha publicado un papel con actividades conjuntas (**AC-2**).

• con el grupo de José Manuel Andrés, (ICB-CSIC, de Zaragoza) en el estudio por microscopía electrónica de las superficies cuticulares de peral y melocotonero. Con este grupo se mantuvo un proyecto financiado por DGA (ref PM003-2006, 2006-2008).

• con la Dra. Damaris Ojeda de la Universidad de Chihuahua (Méjico) en el tema de nutrición mineral. Esta persona ha efectuado dos estancias (2008 y 2009) en nuestro grupo, ambas de la Fundación Carolina (AECID).

También se han iniciado colaboraciones con otros grupos de investigación, en las que una parte de los trabajos que se están desarrollando se refieren específicamente a este proyecto:

-para análisis de metabolitos con el Laboratorio de Metabolómica en el Genome Center (Dr. Fiehn), UC Davis, USA (**AC-13, C-8, C-11** y **C-20**).

-para algunas técnicas específicas de microscopía con el Research Institute for Bioresources (Dr.Ma), Okayama University, Japón.

-para estudios de ESI-MS/MS (Q-TOF) con el ICMA-CSIC (Dr. Orduna, Zaragoza, España). Se han mandado dos publicaciones conjuntas (**AC-10**, **AC-11**, **C-2**, **C-3**, **C-21** y **C-22**).

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.

En 2009 hemos comenzado la participación en un proyecto ERANET KBBE (Euroinvestigacion EUI 2008-03618), relacionado parcialmente con el proyecto en algunos trabajos complementarios. En este proyecto participan los Drs. Philippar (Universidad de München), Briat (Universidad de Montpellier), von Wirén (Universidad de Hohenheim) y García-Mina (Roullier Group) y está liderado por la Universidad de München. El objetivo del proyecto es el estudio de los procesos reguladores de la relación en la homeostasis de Fe en el cloroplasto y el citosol de células vegetales con los mecanismos de adquisición y transporte de Fe. Este proyecto ha sido evaluado en la UE, y ha recibido financiación del Plan Nacional del MICINN.

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.

G. RELACIONES O COLABORACIONES CON DIVERSOS SECTORES

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

1. Describa en detalle la relación mantenida con los EPO's, y la participación concreta de éstos 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).

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.

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

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.

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.

Las distintas presentaciones sobre el tema del proyecto en Congresos y publicaciones han dado lugar a la firma de varios convenios o contratos con empresas del sector. En todos los casos se ha tratado de empresas extranjeras y dedicadas al sector de fertilizantes.

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	31.497,56
Costes de ejecución	24.511,58
TOTAL GASTO REALIZADO	56.009,14

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)

-Víctor del Río, Titulado Medio-grupo 2	10/01/2007 al 31/08/2008	apoyo tareas campo
-Mónica Lastra, Titulado Medio-grupo 2	01/02/2009 al 31/12/2010	apoyo tareas campo
-Irene Orera, Titulado Superior-grupo 1	02/06/2009 al 05/07/2009	objetivos 2 y 3

Total 78.990,39 €

2. Material inventariable (describir brevemente el material adquirido)

Se ha comprado un Medidor de Clorofila SPAD, un ordenador portatil para la toma de parámetros de diversos aparatos y un tensiómetro para medidas de tensión superficial.

Total 11.531,41 €

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

La financiación se ha utilizado como se propuso inicialmente para compra de reactivos, gases, diverso fungible de HPLC, MS y microscopía, mantenimiento de cultivos, material de plástico o vidrio, etc.

Total 53.465,50 €

4. Viajes y dietas (describir brevemente)

Los viajes han sido los propuestos inicialmente, con las salvedades detalladas en informes anteriores.

Total 8.746,57 €

5. Otros gastos (describir brevemente)

En otros gastos se incluyen actividades de difusión, análisis externos, trabajos externos de campo, etc.

Total 12.266,14 €

---

6. Costes indirectos

21 % del total de gastos directos

Total 34.650,00 €

---

7. Dotación adicional o complementos salariales, si procede

Total 0 €

TOTAL GASTOS EJECUTADOS DEL PROYECTO **199.650,01 €**

CON ESTE INFORME DEBERÁ ADJUNTARSE:

Fotocopia de reingreso al Tesoro Público, si procede, de los fondos no utilizados.
Nota: *Debe cumplimentarse este apartado independientemente de la justificación*

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 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 sólo 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 MEC.

						Si no incluido en solicitud original:		
Apellido 1	**Apellido 2**	**Nombre**	**NIF/NIE**	**Catgª Profesional**	**Incluido solicitud original**	**Función en el proyecto**	**Fecha de Alta**	**Observaciones**
Abadía	Bayona	Anunciación	17854929Y	Prof. Investigación	S			
Álvarez	Fernández	Ana María	09392534R	Científico Titular	S			
Vazquez	Reina	Saúl	50205647M	Postdoctoral JAE		Obj.1, 2 y 4	27-03-08	
Orera	Utrilla	Irene	25187707Q	Becaria DGA	S			Finalización beca 31-12-08, baja proyecto 11-2-09
Orera	Utrilla	Irene	25187707Q	Temporal		Obj. 2 y 3	02-06-09	Finalización 05-07-09
El Jendoubi		Hamdi	X7799529-E	Becario FPI		Realización Tesis	01-08-07	Beca asociada al proyecto
Lastra	Hernández	Monica	13931192T	Temporal		Técnico apoyo campo	01-02-09	Resolución contrato 31-12-09
Fernández	Fernández	Victoria	11835405L	Contrato JdlC	S			Baja solicitada (4-2-09), denegada (24-2-09). No ha participado en ninguna actividad del proyecto desde Abril de 2008

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" expuestas en la página web del MEC.

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 sólo en el caso de que este tipo de gasto **no estuviera previsto en la solicitud inicial**

Sin cambios sobre petición inicial

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.**

Sin cambios sobre petición inicial

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.

Sin cambios sobre petición inicial

FIN DEL INFORME FINAL

AC-1

Scientia Horticulturae 117 (2008) 241–248

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Foliar fertilization of peach (*Prunus persica* (L.) Batsch) with different iron formulations: Effects on re-greening, iron concentration and mineral composition in treated and untreated leaf surfaces

Victoria Fernández [a,1,*], Victor Del Río [a,1], Lorena Pumariño [a], Ernesto Igartua [b], Javier Abadía [a], Anunciación Abadía [a]

[a] Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 13034, E-50080 Zaragoza, Spain
[b] Departamento de Genética y Producción Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 13034, E-50080 Zaragoza, Spain

ARTICLE INFO

Article history:
Received 27 December 2007
Received in revised form 7 May 2008
Accepted 9 May 2008

Keywords:
Foliar fertilization
Iron chelates
Iron chlorosis
Iron deficiency
Leaf penetration
Foliar uptake
Surfactants

ABSTRACT

A trial to assess the effects of applying several Fe-containing formulations on Fe-deficient (chlorotic) peach leaves was carried out under field conditions. Solutions consisting of an Fe-containing compound ($FeSO_4 \cdot 7H_2O$, Fe(III)–citrate, Fe(III)–EDTA, Fe(III)–DTPA or Fe(III)–IDHA) and one of five different surfactant treatments (no surfactant, an organo-silicon, an ethoxylated oil, a non-ionic alkyl polyglucoside and a household detergent) were applied to one half of the leaf *via* dipping, first at the beginning of the trial and then after 4 weeks. The re-greening of treated and untreated leaf areas was estimated with a SPAD apparatus, on a weekly basis, during 8 weeks. At the end of the experimental period, leaves were detached, and tissue Fe, N, P, K, Ca, Mg, Mn, Zn and Cu concentrations were determined in Fe-treated and untreated leaf areas. Treatment with Fe-containing solutions always resulted in leaf chlorophyll (Chl) increases, which however significantly depended on the Fe-source, the surfactant-type and the combination between both formulation components. Untreated leaf zones experienced a Chl increase only in some cases, and this depended on the type of surfactant used. Iron application significantly increased the Fe concentration of treated and untreated leaf areas, especially with some formulations. Foliar treatment with Fe-containing solutions induced significant changes in the concentration of several nutrients as compared to those found in Fe-deficient peach leaves, with changes being similar in treated and untreated leaf areas, although in some elements the extent of the changes was of a different magnitude in both materials. This indicates that some leaf mineral composition changes typical of chlorotic leaves are dependent on leaf Fe concentration rather than on leaf Chl levels. Results obtained are relevant to help understand the factors involved in the penetration and bioavailability of leaf-applied Fe, and to assess the potential of foliar Fe fertilization to control Fe deficiency in fruit trees.

1. Introduction

Iron (Fe) deficiency chlorosis can impair fruit quality and yield and lead to early tree death (Álvarez-Fernández et al., 2003, 2006). To ensure adequate economic agricultural returns in areas where soil conditions induce Fe chlorosis, plants must be treated with Fe-containing compounds on a regular basis. Iron chelate supply *via* fertigation or soil treatment is currently the most effective method to control Fe chlorosis under field conditions (Lucena, 2006). However, foliar Fe fertilization could also be an economical and target-oriented strategy to cure plant Fe chlorosis, although recent reviews have shown that the response to Fe sprays could be variable, depending on plant species and experimental conditions (Fernández and Ebert, 2005; Fernández et al., 2005). The lack of understanding of the many factors relating to penetration, translocation and bioavailability of leaf applied, Fe-containing solutions has hindered the development of suitable spray formulations.

Aerial plant organs are covered by a continuous hydrophobic cuticle, which constitutes the interface between the plant and the surrounding environment (Schönherr, 2006). The cuticle is a chemically heterogeneous membrane of variable structure and composition, depending on many factors (Jeffree, 2006). It consists

* Corresponding author. Tel.: +34 976716069; fax: +34 976716145.
E-mail address: victoria.fernandez@gmx.de (V. Fernández).
[1] These authors contributed equally to this work.

0304-4238/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2008.05.002

5. Conclusion

All Fe-compounds can be effective in promoting re-greening, provided an adequate surface-active agent is used in the formulation. In fact, the type of surfactant is a key factor in the efficiency of the Fe foliar fertilizer, and three of them, i.e. the organo-silicon, the ethoxylated oil and the non-ionic alkyl polyglucoside, showed some efficacy depending on the Fe-compound used. Foliar Fe application increased Fe concentrations both in treated and untreated areas, whereas re-greening occurred preferentially in treated *versus* untreated areas. Iron application changed dramatically the mineral composition of Fe-deficient leaves, both in Fe-treated and untreated areas, leading to element concentrations fairly similar to those of Fe-sufficient leaves. Given the complex scenario ruling the performance of Fe spray formulations, more research efforts should be carried out in the future to elucidate the mechanisms of foliar penetration, Fe allocation and its metabolism within the plant.

Acknowledgements

Study supported by the Spanish Ministry of Science and Education (Projects AGL2004-0194 and AGL2006-01416, co-financed with FEDER), the European Commission (ISAFRUIT project, Thematic Priority 5-Food Quality and Safety of the 6th Framework RTD Programme, Contract no. FP6-FOOD-CT-2006-016279), and the Aragón Government (group A03). V. Fernández was on a CSIC "I3P" post-doctoral contract co-financed by the European Social Fund. We would like to thank F. Lahoz (Institute of Materials Sciences CSIC-University of Zaragoza) for his support to estimate the size of Fe(III)-chelates. Thanks to Lanxess, Degussa and Cognis, for providing free sample products for experimental purposes.

References

Abadía, J., Nishio, J.N., Monge, E., Montañés, L., Heras, L., 1985. Mineral composition of peach leaves affected by iron chlorosis. J. Plant Nutr. 8, 697–707.
Abadía, J., Álvarez-Fernández, A., Morales, F., Sanz, M., Abadía, A., 2002. Correction of iron chlorosis by foliar sprays. Acta Hortic. 594, 115–122.
Álvarez-Fernández, A., Paniagua, P., Abadía, J., Abadía, A., 2003. Effects of Fe deficiency chlorosis on yield and fruit quality in peach (*Prunus persica* L. Batsch). J. Agric. Food Chem. 51, 5738–5744.
Álvarez-Fernández, A., García-Laviña, P., Fidalgo, J., Abadía, J., Abadía, A., 2004. Foliar fertilization to control iron chlorosis in pear (*Pyrus communis* L.) trees. Plant Soil 263, 5–15.
Álvarez-Fernández, A., Abadía, J., Abadía, A., 2006. Iron deficiency, fruit yield and quality. In: Abadía, J., Barton, L.L. (Eds.), Iron Nutrition and Interactions in Plants. Springer, Dordrecht, The Netherlands, pp. 85–101.
Bargel, H., Koch, K., Cerman, Z., Neihuis, C., 2006. Structure-function relationship of the plant cuticle and cuticular waxes—a smart material? Funct. Plant Biol. 33, 893–910.
Belkhodja, R., Morales, F., Sanz, M., Abadía, A., Abadía, J., 1998. Iron deficiency in peach trees: effects on leaf chlorophyll and nutrient concentrations in flowers and leaves. Plant Soil 203, 257–268.
Beyer, M., Lau, S., Knoche, M., 2005. Studies on water transport through the sweet cherry fruit surface. IX. Comparing permeability in water uptake and transpiration. Planta 220, 474–485.
Bienfait, H.F., Scheffers, M.R., 1992. Some properties of ferric citrate relevant to the iron nutrition of plants. Plant Soil 143, 141–144.
Domínguez, E., Heredia, A., 1999. Water hydration in cutinized cell walls: a physico-chemical analysis. Biochim. Biophys. Acta 1426, 168–176.
Eichert, T., Kurtz, A., Goldbach, H., 2006. Investigations on the contribution of the stomatal pathway to foliar uptake. Acta Hortic. 721, 307–312.
Eichert, T., Goldbach, H., 2008. Equivalent pore radii of hydrophilic foliar uptake routes in stomatous and astomatous leaf surfaces—further evidence for a stomatal pathway. Physiol. Plant. 132, 491–502.
Fernández, V., 2004. Investigations on Foliar Iron Application to Plants—A New Approach. Shaker Verlag, Aachen, Germany, 171 pp.
Fernández, V., Ebert, G., 2005. Foliar iron fertilization—a critical review. J. Plant Nutr. 28, 2113–2124.
Fernández, V., Ebert, G., Winkelmann, G., 2005. The use of microbial siderophores for foliar iron application studies. Plant Soil 272, 245–252.
Fernández, V., Del Río, V., Abadía, J., Abadía, A., 2006. Foliar iron fertilization of peach (*Prunus persica* (L.) Batsch): effects of iron compounds, surfactants and other adjuvants. Plant Soil 289, 239–252.
Furia, T.E., 1972. Sequestrants in Foods. CRC Handbook of Food Additives. CRC Press, Inc., Boca Raton, FL, USA.
Haefs, R., Schmidt-Eiberger, M., Mainx, H.G., Mittelstaedt, W., Noga, G., 2002. Studies on a new group of biodegradable surfactants for glyphosate. Pest Manag. Sci. 58, 825–833.
Heredia, A., 2003. Biophysical and biochemical characteristics of cutin, plant biopolymer. Biochim. Biophys. Acta 1620, 1–7.
Igartua, E., Grasa, R., Sanz, M., Abadía, A., Abadía, J., 2000. Prognosis of iron chlorosis from the mineral composition of flowers in peach. J. Hortic. Sci. Biotechnol. 75, 111–118.
Jeffree, C.E., 2006. The fine structure of the plant cuticle. In: Riederer, M., Müller, C. (Eds.), Biology of the Plant Cuticle. Annual Plant Reviews, vol. 23. Blackwell Publishing Ltd., Oxford, UK, pp. 11–125.
Kerstiens, G., 2006. Water transport in plant cuticles: an update. J. Exp. Bot. 57, 2493–2499.
Liu, Z., 2004. Effects of surfactants on foliar uptake of herbicides—a complex scenario. Colloids Surf. B: Biointerfaces 35, 149–153.
Lucena, J.J., 2006. Synthetic iron chelates to correct iron deficiency in plants. In: Abadía, J., Barton, L.L. (Eds.), Iron Nutrition and Interactions in Plants. Springer, Dordrecht, The Netherlands, pp. 103–128.
Luque, P., Gavara, R., Heredia, A., 1995a. A study of the hydration process of isolated cuticular membranes. New Phytol. 129, 283–288.
Luque, P., Bruque, S., Heredia, A., 1995b. Water permeability of isolated cuticular membranes: a structural analysis. Arch. Biochem. Biophys. 317, 417–422.
Matas, J.A., López-Casado, G., Cuartero, J., Heredia, A., 2005. Relative humidity and temperature modify the mechanical properties of isolated tomato fruit cuticles. Am. J. Bot. 92, 462–468.
Morales, F., Grasa, R., Abadía, A., Abadía, J., 1998. The iron "chlorosis paradox" in fruit trees. J. Plant Nutr. 21, 815–825.
Neumann, M., Prinz, R., 1975. The reduction by surfactants of leaf burn resulting from foliar sprays and a salt-induced inhibition of the effect. J. Sci. Food Agric. 26, 909–914.
Perkins, M.C., Roberts, C.J., Briggs, D., Davies, M.C., Friedmann, A., Hart, C., Bell, G., 2005. Macro and microthermal analysis of plant wax/surfactant interactions: plasticizing effects of two alcohol ethoxylated surfactants on an isolated cuticular wax and leaf model. Appl. Surf. Sci. 243, 158–165.
Popp, C., Burghardt, M., Friedmann, A., Rieder, M., 2005. Characterization of hydrophilic and lipophilic pathways of *Hedera helix* L. cuticular membranes: permeation of water and uncharged organic compounds. J. Exp. Bot. 56, 2797–2806.
Rich, H.W., Morel, F.M.M., 1990. Availability of well-defined iron colloids to the marine diatom *Thdassiosiru weissflogii*. Limnol. Oceanogr. 35, 652–662.
Schlegel, T., Schönherr, J., Schreiber, L., 2005. Size selectivity of aqueous pores in stomatous cuticles of *Vicia faba* leaves. Planta 221, 648–655.
Schlegel, T., Schönherr, J., Schreiber, L., 2006. Rates of foliar penetration of chelated Fe(III): role of light, stomata, species, and leaf age. J. Agric. Food Chem. 54, 6809–6813.
Schönherr, J., Fernández, V., Schreiber, L., 2005. Rates of cuticular penetration of chelated Fe^{III}: role of humidity, concentration, adjuvants, temperature and type of chelate. J. Agric. Food Chem. 53, 4484–4492.
Schönherr, J., 2006. Characterisation of aqueous pores in plant cuticles and permeation of ionic solutes. J. Exp. Bot. 57, 2471–2491.
Schreiber, L., 2006. Review of sorption and diffusion of lipophilic molecules in cuticular waxes and the effects of accelerators on solute mobilities. J. Exp. Bot. 57, 2515–2523.
Tamura, H., Knoche, M., Bukovac, M.J., 2001. Evidence for surfactant solubilization of plant epicuticular wax. J. Agric. Food Chem. 49, 1809–1816.
Van Dam, R.A., Porterc, N.A., Ahokasa, J.T., Holdwayb, D.A., 1999. Stability of DTPA and iron(III)–DTPA under laboratory ecotoxicological conditions. Water Res. 33, 1320–1324.
Wang, C.J., Liu, Z.Q., 2007. Foliar uptake of pesticides—present status and future challenge. Pestic. Biochem. Physiol. 87, 1–8.
Weichert, H., Knoche, M., 2006. Studies on water transport through the sweet cherry fruit surface. 11. $FeCl_3$ decreases water permeability of polar pathways. J. Agric. Food Chem. 54, 6294–6302.
Westwood, M.N., 1978. Temperate-zone Pomology. W.H. Freeman & Co., San Francisco, USA, pp. 142–155.

Plant Soil (2008) 311:161–172
DOI 10.1007/s11104-008-9667-4

AC-2

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

Received: 7 April 2008 / Accepted: 21 May 2008 / Published online: 20 June 2008

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

Responsible Editor: Ismail Cakmak.

V. Fernández (✉) · V. Del Río · A. Abadía · J. Abadía
Plant Nutrition Department, Estación Experimental de Aula Dei, CSIC,
P.O. Box 13034, 50080 Zaragoza, Spain
e-mail: victoria.fernandez@gmx.de

T. Eichert
Plant Nutrition Department, Institute of Crop Science and Resource Conservation, University of Bonn,
Karlrobert-Kreiten-Strasse 13,
53115 Bonn, Germany

G. López-Casado · J. A. Heredia-Guerrero · A. Heredia
Grupo de Caracterización y Síntesis de Biopolímeros Vegetales, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga,
29071 Málaga, Spain

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

Acknowledgements This study was supported by the Spanish Ministry of Science and Education (MEC, grants AGL2006-01416 and AGL2007-61948, co-financed with FEDER), the European Commission (ISAFRUIT project, Thematic Priority 5-Food Quality and Safety of the 6th Framework Programme of RTD; Contract no. FP6-FOOD-CT-2006-016279) and the Aragón Government (group A03). V.F. was supported by a "Juan de la Cierva"-MEC post-doctoral contract, co-financed by the European Social Fund. T.E. was supported by the CAI Europa XXI for a short term stay at the EEAD-CSIC. We would like to thank I. Tacchini and J.M. Andrés (ICB-CSIC, Zaragoza, Spain), F. Pinto (ICA-CSIC, Madrid, Spain) and R. Jordana (University of Navarra, Pamplona, Spain) for support with SEM techniques, L. Cistué for support with optical microscopy and image analysis. Thanks to Novozymes, for providing free sample products for experimental purposes.

References

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

Abadía A, Ambard-Bretteville F, Tremolières A (1988) Iron-deficiency in pea leaves: effect on lipid composition and synthesis. Physiol Plant 72:713–717

Álvarez-Fernández A, Grasa R, Abadía A, Sanz M, Abadía J (2003) Evaluación agronómica de nuevos quelatos de hierro. Phytoma 146:30–36

Álvarez-Fernández A, García-Laviña P, Fidalgo J, Abadía J, Abadía A (2004) Foliar fertilization to control iron chlorosis in pear (*Pyrus communis* L.) trees. Plant Soil 263:5–15

Álvarez-Fernández A, Abadía J, Abadía A (2006) Iron deficiency, fruit yield and quality. In: Abadía J, Barton LL (eds) Iron nutrition and interactions in plants. Springer, Dordrecht, pp 85–101

Anderson CA (1984) Development of leaf water deficits in detached green and lime-chlorotic leaves of seedlings from populations of *Eucalyptus obliqua* L'Hérit. Plant Soil 77:171–181

Bukovac MJ, Flore JA, Baker EA (1979) Peach leaf surfaces: changes in wettability, retention, cuticular permeability, and epicuticular wax chemistry during expansion with especial reference to spray application. J Am Soc Hortic Sci 104:611–617

Briat JF (2007) Iron dynamics in plants. In: Kader JC, Delseny M (eds) Advances in botanical research, vol 46: Incorporating advances in plant pathology. Academic, London, UK, pp 138–169. ISBN: 9780123737052

Conrad ME, Umbreit JN (2000) Iron absorption and transport—an update. Am J Hematol 64:287–298

DeMichele DW, Sharpe PJH (1973) An analysis of the mechanics of guard cell motion. J Theor Biol 41:77–96

Eichert T, Burkhardt J (2001) Quantification of stomatal uptake of ionic solutes using a new model system. J Exp Bot 52:771–781

Eichert T, Goldbach HE (2008) Equivalent pore radii of hydrophilic foliar uptake routes in stomatous and astomatous leaf surfaces—further evidence for a stomatal pathway. Physiol Plant 132:491–502

Eichert T, Goldbach HE, Burkhardt J (1998) Evidence for the uptake of large anions through stomatal pores. Bot Acta 111:461–466

Fernández V, Del Río V, Abadía J, Abadía A (2006) Foliar iron fertilization of peach (*Prunus persica* (L.) Batsch): effects of iron compounds, surfactants and other adjuvants. Plant Soil 289:239–252

Heredia A (2003) Biophysical and biochemical characteristics of cutin, plant biopolymer. Biochim Biophys Acta 1620: 1–7

Holloway PJ (1969) The effects of surface wax on leaf wettability. Ann Appl Biol 63:145–153

Hutchinson TC (1970) Lime chlorosis as a factor in seedling establishment on calcareous soils. II. The development of leaf water deficits in plants showing lime-chlorosis. New Phytol 69:143–157

Iwanoff L (1928) Zur Methodik der Transpirationsbestimmung am Standort. Ber Dtsch Bot Ges 46:306–310

Jeffree CE (2006) The fine structure of the plant cuticle. In: Riederer M, Müller C (eds) Biology of the plant cuticle. Annual plant reviews, vol. 23. Blackwell, Oxford

Kaiser H, Legner N (2007) Localization of mechanisms involved in hydropassive and hydroactive stomatal responses of *Sambucus nigra* to dry air. Plant Physiol 143:1068–1077

Kerstiens G (2006) Water transport in plant cuticles: an update. J Exp Bot 57:2493–2499

Lange OL, Führer G, Gebel J (1986) Rapid field determination of photosynthetic capacity of cut spruce twigs (*Picea abies*) at saturation ambient CO_2. Trees (Berl) 1:70–77

Larbi A, Morales F, Abadía J, Abadía A (2003) Effect of branch solid Fe implants on Fe xylem transport in peach and pear: changes in organic acid and Fe concentrations and pH. J Plant Physiol 160:1473–1482

Larbi A, Abadía A, Abadía J, Morales F (2006) Down co-regulation of light absorption, photochemistry, and carboxylation in Fe-deficient plants growing in different environments. Photosynth Res 89:113–126

Lucena JJ (2006) Synthetic iron chelates to correct iron deficiency in plants. In: Abadía J, Barton LL (eds) Iron nutrition and interactions in plants. Springer, Dordrecht, pp 103–128

Maldonado-Torres R, Etchevers-Barra JD, Alcántar-González G, Rodriguez-Alcazar J, Colinas-León MT (2006) Morphological changes in leaves of Mexican lime affected by iron chlorosis. J Plant Nutr 29:615–628

Monge E, Pérez C, Pequerul A, Madero P, Val J (1993) Effect of iron chlorosis on mineral nutrition and lipid composition of thylakoid biomembrane in *Prunus persica* (L.). Bastch Plant Soil 154:97–102

Morales F, Grasa R, Abadía A, Abadía J (1998) Iron chlorosis paradox in fruit trees. J Plant Nutr 21:815–825

Norris RF (1974) Penetration of 2,4-D in relation to cuticle thickness. Am J Bot 61:74–79

Norris RF, Bukovac MJ (1968) Structure of the pear leaf cuticle with special reference to cuticular penetration. Am J Bot 55:975–983

Powles JE, Buckley TN, Nicotra AB, Farquhar GC (2006) Dynamics of stomatal water relations following leaf excision. Plant Cell Environ 29:981–992

Raschke K (1970a) Leaf hydraulic system: rapid epidermal and stomatal responses to changes in water supply. Science 167:189–191

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2008; **22**: 1553–1562
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3523

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

Rubén Rellán-Álvarez, Javier Abadía and Ana Álvarez-Fernández*
Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 202, 50080 Zaragoza, Spain

Received 16 October 2007; Revised 18 February 2008; Accepted 3 March 2008

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. Copyright © 2008 John Wiley & Sons, Ltd.

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

**Correspondence to*: A. Álvarez-Fernández, Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 202, 50080 Zaragoza, Spain.
E-mail: ana.alvarez@eead.csic.es
Contract/grant sponsor: Spanish Ministry of Education and Science (MEC; co-financed with FEDER); contract/grant number: AGL2006-1416 and AGL2007-61948.

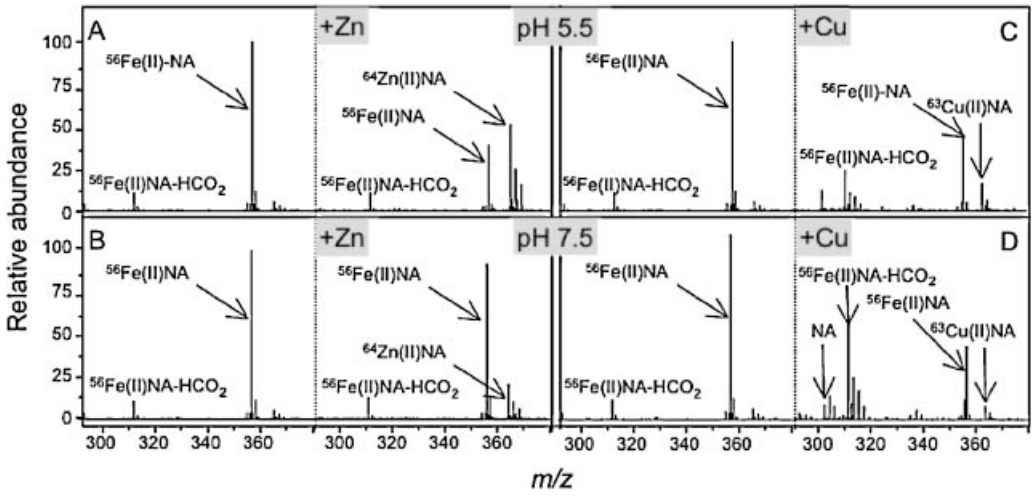

Figure 5. Metal-exchange reactions between Fe(II)-NA and Zn(II) at pH 5.5 (A) and pH 7.5 (B), and between Fe(II)-NA and Cu(II) at pH 5.5 (C) and 7.5 (D). Metal and Fe(II)-NA complex concentrations of the initial solutions were 50 μM.

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/.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Education (Grant Nos. AGL2006-1416 and AGL2007-61948, co-financed with FEDER), the European Commission (EU 6th Framework Integrated Project ISA-FRUIT), and the Aragón Government (group A03). The authors would also like to thank the two reviewers for helpful contributions.

REFERENCES

1. Hall JL. *J. Exp. Bot.* 2002; **53**: 1. DOI: 10.1093/jexbot/53.366.1.
2. Noma M, Noguchi M, Tamaki E. *Tetrahedron Lett.* 1971; 2017.
3. von Wirén N, Klair S, Bansal S, Briat JF, Khodr H, Shioiri T, Leigh RA, Hider RC. *Plant Physiol.* 1999; **119**: 1107.
4. Benes I, Schreiber K, Ripperger H, Kircheiss A. *Experientia* 1983; **39**: 261.
5. Stephan UW, Scholz G. *Physiol. Plant.* 1993; **88**: 522.

Journal of Horticultural Science & Biotechnology (2009) **84** (1) 1–6

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

By V. FERNÁNDEZ, I. ORERA, J. ABADÍA* and A. ABADÍA
Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain
(e-mail: jabadia@eead.csic.es)

(Accepted 22 July 2008)

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.

*Author for correspondence.

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.

This study was supported by the European Commission (ISAFRUIT Project, Thematic Priority 5-Food Quality and Safety, 6th Framework RTD Programme, Contract No. FP6-FOOD-CT-2006-016279), the Spanish Ministry of Science and Education (Projects AGL2006-01416 and AGL2007-61948, co-financed by FEDER), and the Aragón Government (Group A03). V. Fernández was supported by a "Juan de la Cierva" MEC post-doctoral Contract, co-financed by the European Social Fund.

REFERENCES

Abadía, J., Alvarez-Fernandez, A., Morales, F., Sanz, M. and Abadía, A. (2002). Correction of iron chlorosis by foliar sprays. *Acta Horticulturae*, **594,** 115–122.

Abadía, J., Álvarez-Fernández, A., Rombolà, A. D., Sanz, M., Tagliavini, M. and Abadía, A. (2004). Technologies for the diagnosis and remediation of Fe deficiency. *Soil Science and Plant Nutrition*, **50,** 965–971.

Álvarez-Fernández, A., Paniagua, M. P., Abadía, J. and Abadía, A. (2003). Effects of Fe deficiency-chlorosis on yield and fruit quality in peach (*Prunus persica* L. Batsch). *Journal of Agricultural and Food Chemistry*, **51,** 5738–5744.

Álvarez-Fernández, A., García-Laviña, P., Fidalgo, J., Abadía, J. and Abadía, A. (2004). Foliar fertilization to control iron chlorosis in pear (*Pyrus communis* L.) trees. *Plant and Soil*, **263,** 5–15.

Álvarez-Fernández, A., Abadía, J. and Abadía, A. (2006). Iron deficiency, fruit yield and quality. In: *Iron Nutrition and Interactions in Plants*. (Abadía, J. and Barton L. L., Eds.). Springer, Dordrecht, The Netherlands. 85–101.

Álvarez-Fernández, A., Orera, I., Abadía, J. and Abadía, A. (2007). Determination of synthetic ferric chelates used as fertilizers by liquid chromatography-electrospray mass spectrometry in agricultural matrices. *Journal of the American Society for Mass Spectrometry*, **18,** 37–47.

Barthlott, W. and Neinhuis, C. (1997). Purity of the sacred lotus, or escape from contamination in biological surfaces. *Planta*, **202,** 1–8.

Cerdán, M., Alcañiz, S., Juárez, M., Jordá, J. D. and Bermúdez, D. (2007). Kinetic behavior of Fe(*o,o*-EDDHA)-humic substance mixtures in several soil components and in calcareous soils. *Journal of Agricultural and Food Chemistry*, **55,** 9159–9169.

Chamel, A. (1996). Foliar uptake of chemicals studied with whole plants and isolated cuticles. In: *Plant Growth and Leaf-Applied Chemicals*. (Neumann, P. M., Ed.). CRC Press Inc., Boca Raton, FL, USA. 27–48.

Currier, H. B. and Dybing, C. D. (1959). Foliar penetration of herbicides. Review and present status. *Weeds*, **7,** 195–213.

Eichert, T. and Burkhardt, J. (2001). Quantification of stomatal uptake of ionic solutes using a new model system. *Journal of Experimental Botany*, **52,** 771–781.

Eichert, T. and Goldbach, E. (2008). Equivalent pore radii of hydrophilic foliar uptake routes in stomatous and astomatous leaf surfaces – further evidence for a stomatal pathway. *Physiologia Plantarum*, **132,** 491–502.

Eichert, T., Kurtz, A. and Goldbach, E. (2006). Investigations on the contribution of the stomatal pathway to foliar uptake. *Acta Horticulturae*, **721,** 307–312.

Fernández, V. (2004). *Investigations on Foliar Iron Application to Plants – a New Approach*. Shaker Verlag, Aachen, Germany. 171 pp.

Fernández, V. and Ebert, G. (2005). Foliar iron fertilization – a critical review. *Journal of Plant Nutrition*, **28,** 2113–2124.

Plant Soil
DOI 10.1007/s11104-008-9735-9

AC-5

REGULAR ARTICLE

Elemental 2-D mapping and changes in leaf iron and chlorophyll in response to iron re-supply in iron-deficient GF 677 peach-almond hybrid

S. Jiménez · F. Morales · A. Abadía · J. Abadía · M. A. Moreno · Y. Gogorcena

Received: 15 February 2008 / Accepted: 21 July 2008

Abstract Iron is an essential micronutrient for plant growth and development, involved in key cellular processes. However, the distribution of Fe in plant tissues is still not well known. In the so-called Fe chlorosis paradox, leaves of fruit trees grown in the field usually have high concentrations of Fe but still are Fe-deficient. Leaves of the *Prunus* rootstock GF 677 (*P. dulcis*×*P. persica*) grown in hydroponics have been used to carry out two-dimensional (2-D) nutrient mapping by synchrotron radiation-induced X-ray fluorescence. Iron-deficient leaves accumulated more Fe in the midrib and veins, with Fe concentration being markedly lower in mesophyll leaf areas. The effects of Fe deficiency and Fe re-supply on leaf chlorophyll concentration and on the distribution of Fe and other nutrients within different plant tissues have been investigated in the same plants. After Fe re-supply, leaf Fe concentrations increased largely in all leaf types. However, whereas re-greening was almost completely achieved in apical leaves, in some expanded leaves the increase in chlorophyll concentration was only moderate. Therefore, after Fe re-supply Fe-deficient expanded leaves of the *Prunus* rootstock GF 677 had significant increases in Fe concentration but were still chlorotic. This is similar to what occurs in leaves of peach trees in field conditions, opening the possibility that this system could be used as a model to study the Fe chlorosis paradox.

Keywords Inactive iron · Iron chlorosis · Iron deficiency · Iron re-supply · Leaf iron distribution · *Prunus*

Abbreviations

Chl	chlorophyll
EDTA	ethylenediaminetetracetic acid
DW	dry weight
μ-SRXF	synchrotron radiation-induced X-ray fluorescence

Introduction

Iron deficiency is one of the major abiotic stresses affecting fruit tree crops growing in calcareous soils in the Mediterranean area. The most obvious effect of

Responsible Editor: Jian Feng Ma.

S. Jiménez and F. Morales contributed equally to this work

S. Jiménez · M. A. Moreno · Y. Gogorcena (✉)
Department of Pomology, Aula Dei Experimental Station,
Consejo Superior de Investigaciones Científicas (CSIC),
Apdo. 13034,
E-50080 Zaragoza, Spain
e-mail: aoiz@eead.csic.es

F. Morales · A. Abadía · J. Abadía
Department of Plant Nutrition,
Aula Dei Experimental Station,
Consejo Superior de Investigaciones Científicas (CSIC),
Apdo. 13034,
E-50080 Zaragoza, Spain

physiologically inactive Fe pools in the apoplast of chlorotic leaves.

Growing plants under Fe deficiency conditions for some time and then re-supplying Fe in controlled growth chambers could be a good method to mimic the "Fe chlorosis paradox" in laboratory conditions. After 2 weeks of Fe deficiency, there was a gradient of leaf Chl concentrations from fully yellow apical youngest leaves, through expanded leaves with typical interveinal Fe chlorosis symptoms, and then to basal leaves, including some leaves (leaves 5 to 7) slightly affected by Fe deficiency and other (leaves 8 to 11) fully green. Apical leaves became green quickly after Fe re-supply, whereas fully chlorotic leaves re-greened very fast (data not shown). However, expanded leaves having a mild chlorosis did not regreen completely, confirming previous results of Alcántara et al. (2000). Upon Fe re-supply, all leaf types had large increases in Fe concentrations. Therefore, some leaves were still chlorotic but had high Fe concentrations (mimicking the "Fe chlorosis paradox"). Lack of re-greening of expanded leaves upon Fe re-supply could be explained by difficulties in recovering damaged structures or in using newly acquired Fe in cells (Bohórquez et al. 2001).

In conclusion, Fe was located preferentially in the midrib and veins in Fe-deficient leaves, and this could explain why chlorosis is often interveinal in field-grown chlorotic materials. This could be among the causes leading to the appearance of the "Fe chlorosis paradox". Growing plants under Fe deficiency conditions for some time and then re-supplying Fe could be a good method to mimic the "Fe chlorosis paradox" in controlled growth chambers, to better explain how plants transport Fe in different plant tissues.

Acknowledgements Supported by grants from the Spanish MEC (AGL2005-05533 and AGL2006-1416) co-financed by FEDER, PETRI (PTR1995-0580) and DGA (A44 and A03). S. Jiménez was supported by an I3P-CSIC predoctoral fellowship (cofinanced by FSE). We gratefully acknowledge P. Chevallier and E. Foy for their help during the work with μ-SRXF, R. Giménez for her help with figures and A. F. López-Millán and A. Álvarez-Fernández for valuable discussion.

References

Abadía J, Abadía A (1993) Iron and plant pigments. In: Barton LL, Hemming BC (eds) Iron chelation in plants and soil microorganisms. Academic, San Diego, pp 327–343

Alcántara E, Romera FJ, Cañete M, de la Guardia MD (2000) Effects of bicarbonate and iron supply on Fe(III) reducing capacity of roots and leaf chlorosis of the susceptible peach rootstock "Nemaguard". J Plant Nutr 23:1607–1617

Álvarez-Fernández A, Abadía J, Abadía A (2006) Iron deficiency, fruit yield and fruit quality. In: Barton LL, Abadía J (eds) Iron Nutrition in Plants and Rizospheric Microorganisms. Springer, Dordrecht, pp 85–101

Andaluz S (2005) Estudio de los cambios inducidos por la deficiencia de hierro en el proteoma de las plantas. PhD thesis. University of Zaragoza, Zaragoza

A.O.A.C. (1990) Official methods of analysis of the association of official analytical chemists. In: Hedrich K (ed) Academic, Washington DC, p 1141

Becker R, Fritz E, Manteuffel R (1995) Subcellular localization and characterization of excessive iron in the nicotianamine-less tomato mutant *chloronerva*. Plant Physiol 108:269–275

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

Bienfait HF, Van der Briel W, Mesland-Mul NT (1985) Free space iron pools in roots. Generation and mobilization. Plant Physiol 78:596–600

Bohórquez JM, Romera FJ, Alcántara E (2001) Effect of Fe^{3+}, $Zn^{2+}$and Mn^{2+}on ferric reducing capacity and regreening process of the peach rootstock Nemaguard (*Prunus persica* (L.) Batsch). Plant Soil 237:157–163

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

Chen Y, Shi J, Tian G, Zheng S, Lin Q (2004) Fe deficiency induces Cu uptake and accumulation in *Commelina communis*. Plant Sci 166:1371–1377

Cinelli F, Tamantini I, Iacona C (2004) Nutritional (Fe-Mn) interactions in 'Big Top' peach plants as influenced by the rootstock and by the soil $CaCO_3$ concentration. Soil Sci Plant Nutr 50:1097–1102

Curie C, Briat J-F (2003) Iron transport and signaling in plants. Annu Rev Plant Biol 54:183–206

Fodor F, Gáspár L, Morales F, Gogorcena Y, Lucena JJ, Cseh E, Kröpfl K, Abadía J, Sárvári É (2005) Effects of two iron sources on iron and cadmium allocation in poplar (*Populus alba*) plants exposed to cadmium. Tree Physiol 25:1173–1180

Gogorcena Y, Abadía J, Abadía A (2000) Induction of in vivo root ferric chelate reductase activity in fruit tree rootstock. J Plant Nutr 23:9–21

Gogorcena Y, Abadía J, Abadía A (2004) A new technique for screening iron-efficient genotypes in peach rootstocks: elicitation of root ferric chelate reductase by manipulation of external iron concentrations. J Plant Nutr 27:1701–1715

González-Vallejo EB, Morales F, Cistué L, Abadía A, Abadía J (2000) Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. Plant Physiol 122:337–344

Gruber B, Kosegarten H (2002) Depressed growth of non-chlorotic vine grown in calcareous soil is an iron deficiency symptom prior to leaf chlorosis. J Plant Nutr Soil Sci 165:111–117

Journal of Plant Physiology **166** (2009) 375—384

Available online at www.sciencedirect.com

ScienceDirect

JOURNAL OF PLANT PHYSIOLOGY

www.elsevier.de/jplph

Metabolic responses in iron deficient tomato plants

Ana Flor López-Millán[a], Fermín Morales[a], Yolanda Gogorcena[b], Anunciación Abadía[a], Javier Abadía[a,*,1]

[a]*Department of Plant Nutrition, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), P.O. Box 13034, E-50080 Zaragoza, Spain*
[b]*Pomology Department, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), P.O. Box 13034, E-50080 Zaragoza, Spain*

Received 18 April 2008; received in revised form 13 June 2008; accepted 13 June 2008

KEYWORDS
Iron;
Organic anions;
Phospho*enol*pyruvate carboxylase;
Strategy I;
Tomato

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.

*Corresponding author. Tel.: 34 976 716056; fax: 34 976 716145.
E-mail address: jabadia@eead.csic.es (J. Abadía).
[1]Courier address: Av. Montañana 1005, Campus de Aula Dei, E-50059 Zaragoza, Spain.

0176-1617/$ - see front matter
doi:10.1016/j.jplph.2008.06.011

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.

Acknowledgements

This study was supported by the Spanish Ministry of Science and Education (projects AGL2006-1416 and AGL2007-61948, co-financed with FEDER), the European Commission (Thematic Priority 5–Food Quality and Safety, 6th Framework RTD Programme, Contract no. FP6-FOOD–CT-2006-016279) and the Aragón Government (group A03). We thank M. Ribas-Carbó for his help in interpreting O_2 consumption data.

References

Abadía J. Leaf responses to Fe deficiency – a review. J Plant Nutr 1992;15:1699–713.

Abadía J, López-Millán AF, Rombolà A, Abadía A. Organic acids and Fe deficiency: a review. Plant Soil 2002; 241:75–86.

Andaluz S, López-Millán AF, Peleato ML, Abadía J, Abadía A. Increases in phosphoenolpyruvate carboxylase activity in iron-deficient sugar beet roots: analysis of spatial localization and post-translational modification. Plant Soil 2002;241:43–8.

Bialczyk J, Lechowski Z. Chemical-composition of xylem sap of tomato grown on bicarbonate containing medium. J Plant Nutr 1995;18:2005–21.

Chaney RL, Brown JC, Tiffin LO. Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol 1972;50:208–13.

Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM, et al. The cyanide-resistant oxidase: to inhibit or not to inhibit, that is the question. Plant Physiol 1996;110:1–2.

De Nisi P, Zocchi G. Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. J Exp Bot 2000;51:1903–9.

Durrett TP, Gassmann W, Rogers EE. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. Plant Physiol 2007;177: 197–205.

Eide D, Broderius M, Fett J, Guerinot ML. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. P Natl Acad Sci USA 1996;93:5624–8.

Franceschi VR, Nakata PA. Calcium oxalate in plants: formation and function. Annu Rev Plant Biol 2005;56: 41–71.

González-Vallejo EB, González-Reyes JA, Abadía A, López-Millán AF, Yunta F, Lucena JJ, et al. Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet. Aus J Plant Physiol 1999;26:601–11.

Kim SA, Guerinot ML. Mining iron: iron uptake and transport in plants. FEBS Lett 2007;581:2273–80.

Kobayashi T, Nishizawa NK, Mori S. Molecular analysis of iron-deficient graminaceous plants. In: Barton LL, Abadía A, editors. Iron nutrition in plants and rhizospheric microorganisms. Dordrecht: Springer; 2006. p. 395–436.

Landsberg EC. Function of rhizodermal transfer cells in the iron stress response mechanisms of *Capsicum annum* L. Plant Physiol 1986;82:511–7.

Lennon AM, Neuenschwander UH, Ribas-Carbó M, Giles L, Ryals JA, Siedow JN. The effects of salicylic acid and tobacco mosaic virus infection on the alternative oxidase of tobacco. Plant Physiol 1997; 115:783–91.

López-Millán AF, Morales F, Abadía A, Abadía J. Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport. Plant Physiol 2000a;124:873–84.

López-Millán AF, Morales F, Andaluz S, Gogorcena Y, Abadía A, De las Rivas J, et al. Responses of sugar beet roots to iron deficiency. Changes in carbon assimilation and oxygen use. Plant Physiol 2000b;124:885–97.

López-Millán AF, Morales F, Abadía A, Abadía J. Iron-deficiency-associated changes in the composition of the leaf apoplastic fluid from field-grown pear (*Pyrus communis* L.) trees. J Exp Bot 2001;52:1489–98.

Marschner H. Mineral nutrition of higher plants. London: Academic Press; 1995.

Marschner H, Römheld V, Kissel M. Different strategies in higher-plants in mobilization and uptake of iron. J Plant Nutr 1986;9:695–713.

Matsumura H, Miyachi S. Cycling assay for nicotinamide adenine dinucleotides. Methods Enzymol 1980;69: 465–70.

Journal of Horticultural Science & Biotechnology (2009) **84** (1) 7–12

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

By I. ORERA, J. ABADÍA, A. ABADÍA and A. ÁLVAREZ-FERNÁNDEZ*
Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain
(e-mail: ana.alvarez@eead.csic.es) *(Accepted 22 July 2008)*

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'-

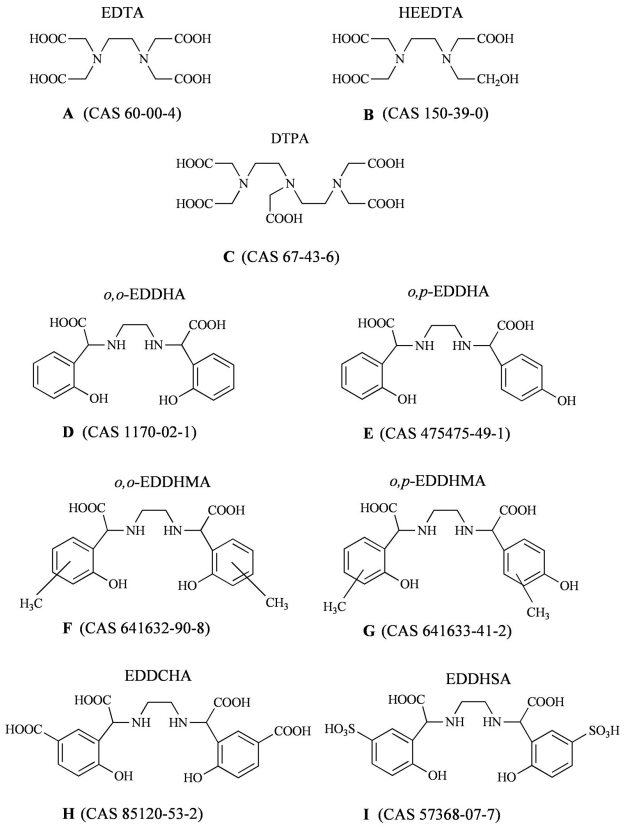

FIG. 1
Chemical structures and abbreviated names of Fe(III)-chelating agents (Panels A–I) allowed by current EU Commission Regulation No. 162/2007 in Fe-fertilisers (Anon, 2007). CAS Numbers of the compounds are indicated below the formulae.

*Author for correspondence.

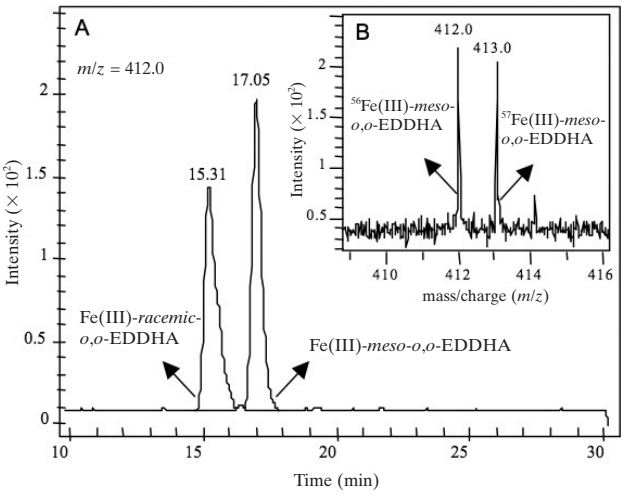

FIG. 3.
Chromatogram (Panel A) and mass spectrum (inset, Panel B) of a xylem sap sample extracted from tomato plants treated with Fe(III)-*o,o*-EDDHA. The inset (Panel B) shows a zoomed mass spectrum (409-416 *m/z*) at a retention time of 17.05 min.

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.

This study was supported by the European Commission (ISAFRUIT Project, Thematic Priority 5-Food Quality and Safety, of the 6th Framework Programme of RTD; Contract No. FP6-FOOD-CT-2006-016279), the Spanish Ministry of Science and Education (Projects AGL2006-1416 and AGL2007-61948, co-financed by FEDER), and the Aragón Government (Group A03). Irene Orera was supported by a CONAID-DGA predoctoral grant from the Aragón Government.

REFERENCES

Abadía, J., Álvarez-Fernández, A., Rombolà, A. D., Sanz, M., Tagliavini, M. and Abadía, A. (2004). Technologies for the diagnosis and remediation of Fe deficiency. *Soil Science and Plant Nutrition*, **50,** 965–971.

Álvarez-Fernández, A., Cremonini, M. A., Sierra, M. A., Placucci, G. and Lucena, J. J. (2002). Nature of impurities in fertilisers containing EDDHMA/Fe^{3+}, EDDHSA/Fe^{3+}, and EDDCHA/Fe^{3+} chelates. *Journal of Agricultural and Food Chemistry*, **50,** 284–290.

Álvarez-Fernández, A., García-Marco, S. and Lucena, J. J. (2005). Evaluation of synthetic iron(III)-chelates (EDDHA/Fe^{3+}, EDDHMA/Fe^{3+} and the novel EDDHSA/Fe^{3+}) to correct iron chlorosis. *European Journal of Agronomy*, **22,** 119–130.

Álvarez-Fernández, A., Abadía, J. and Abadía, A. (2006). Iron deficiency, fruit yield and fruit quality. In: *Iron Nutrition in Plants and Rhizospheric Microorganisms*. (Barton, L. L. and Abadía, J., Eds.). Springer, Dordrecht, The Netherlands. 85–101.

Álvarez-Fernández, A., Orera, I., Abadía, J. and Abadía, A. (2007). Determination of synthetic ferric chelates used as fertilisers by liquid chromatography-electrospray/mass spectrometry in agricultural matrices. *Journal of the American Society for Mass Spectrometry*, **18,** 37–47.

Anon (2001a). Comitè Européen de Normalisation. Fertilizers – Treatment with a cation exchange resin for the determination of the chelated micro-nutrient content and of the chelated fraction of micro-nutrients. EN 13366: 2001 E.

Anon (2001b). Comitè Européen de Normalisation. Fertilizers – Determination of chelating agents in fertilizers by ion chromatography – Part 1: EDTA, HEDTA and DTPA. EN 13368-1: 2001 E.

Anon (2001c). Comitè Européen de Normalisation. Fertilizers – Determination of chelating agents in fertilizers by ion chromatography – Part 2: EDDHA and EDDHMA. EN 13368-2: 2001 E.

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2009; **23**: 1694–1702
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4056

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

Irene Orera, Anunciación Abadía, Javier Abadía and Ana Álvarez-Fernández*
Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain

Received 24 February 2009; Revised 27 March 2009; Accepted 28 March 2009

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

**Correspondence to*: A. Álvarez-Fernández, Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain.
E-mail: ana.alvarez@eead.csic.es
Contract/grant sponsor: Spanish Ministry of Science and Innovation; contract/grant number: AGL2006-1416 and AGL2007-61948, co-financed with FEDER.
Contract/grant sponsor: European Commission (Thematic Priority 5–Food Quality and Safety, 6th Framework RTD Programme); contract/grant number: FP6-FOOD–CT-2006-016279.
Contract/grant sponsor: Aragón Government (group A03).

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.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation (MICINN) (projects AGL2006-1416 and AGL2007-61948, co-financed with FEDER), the Commission of European Communities (ISAFRUIT Project, Thematic Priority 5-Food Quality and Safety, of the 6th Framework Programme of RTD; Contract No.FP6-FOOD-CT-2006-016279), and the Aragón Government (Group A03). IO was supported by a CONAID-DGA predoctoral fellowship. Acquisition of the HPLC/MS(TOF) apparatus was cofinanced with FEDER. The authors thank Dr. J.I. García Alonso (University of Oviedo, Oviedo, Spain) for his advice on isotope exchange experiments, A. Poc and A. Calviño for their assistance in growing plants and F. Morales for a careful reading of the manuscript.

REFERENCES

1. Rombolà A, Tagliavini M. *Iron Nutrition in Plants and Rhizospheric Microorganisms*. Springer: Dordrecht, The Netherlands, 2006.
2. Lucena JJ. *Iron Nutrition in Plants and Rhizospheric Microorganisms*. Springer: Dordrecht, The Netherlands, 2006.
3. Tiffin LO, Brown JC. *Plant Physiol.* 1961; **36**: 710.
4. Jeffreys RA, Hale VQ, Wallace A. *Soil Sci.* 1961; **92**: 268.
5. Römheld V, Marschner H. *J. Plant Nutr.* 1981; **3**: 551.
6. Orera I, Abadía J, Abadía A, Álvarez-Fernández A. *J. Hortic. Sci. Biotechnol.* 2009; **84**: 7.
7. Batra PP, Maier RH. *Plant Soil.* 1964; **20**: 105.
8. Jeffreys RA, Wallace A. *Agron. J.* 1968; **60**: 613.
9. Bienfait HF, García-Mina J, Zamareño AM. *Soil Sci. Plant Nutr.* 2004; **50**: 1103.
10. Collins RN. *J. Chromatogr. A* 2004; **1059**: 1.
11. Nowack B. *Environ. Sci. Technol.* 2002; **36**: 4009.
12. Matuszewski BK, Constanzer ML, Chavez-Eng CM. *Anal. Chem.* 2003; **75**: 3019.
13. Cantera RG, Zamarreño AM, García-Mina JM. *J. Agric. Food Chem.* 2002; **50**: 7609.
14. Álvarez-Fernández A, Orera I, Abadía J, Abadía A. *J. Am. Soc. Mass Spectrom.* 2007; **18**: 37. DOI: 10.1016/j.jasms.2006.08.018.
15. Ouerdane L, Mari S, Czernic P, Lebrun M, Lobinski R. *J. Anal. At. Spectrom.* 2006; **21**: 676.
16. Collins RN, Onisko BC, McLaughlin MJ, Merrington G. *Environ. Sci. Technol.* 2001; **35**: 2589.
17. Xuan Y, Scheuermann EB, Meda AR, Hayen H, von Wiren N, Weber G. *J. Chromatogr. A* 2006; **1136**: 73.
18. Stokvis E, Rosing H, Beijnen JH. *Rapid Commun. Mass Spectrom.* 2005; **19**: 401.
19. King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. *J. Am. Soc. Mass Spectrom.* 2000; **11**: 942.
20. Lindegardh N, Annerberg A, White NJ, Day NPJ. *J. Chromatogr. B* 2008; **862**: 227. 10.1016/j.jchromb.2007.12.011.
21. Taylor PJ. *Clin. Biochem.* 2005; **38**: 328. DOI: 10.1016/j.clinbiochem.2004.11.007.
22. Wieling J. *Chromatographia* 2002; **55**: 107.
23. Wang S, Cyronak M, Yang E. *J. Pharm. Biomed. Anal.* 2007; **43**: 701. DOI: 10.1016/j.jpba.2006.08.010.
24. Jemal M, Schuster A, Whigan DB. *Rapid Commun. Mass Spectrom.* 2003; **17**: 1723.
25. Chavez-Eng CM, Constanzer ML, Matuszewski BK. *J. Chromatogr. B* 2002; **767**: 117.
26. Lucena JJ, Barak P, Hernández-Apaolaza L. *J. Chromatogr. A* 1996; **727**: 253.
27. López-Millán AF, Morales F, Abadía A, Abadía J. *Plant Physiol.* 2000; **124**: 873.
28. López-Millán AF, Morales F, Gogorcena Y, Abadía A, Abadía J. *J. Plant Physiol.* 2009; **166**: 375. DOI: 10.1016/j.jplph.2008.06.011.
29. Álvarez-Fernández A, Paniagua P, Abadía J, Abadía A. *J. Agric. Food Chem.* 2003; **51**: 5738.
30. Yunta F, García-Marco S, Lucena JJ, Gómez-Gallego M, Alcázar R, Sierra MA. *Inorg. Chem.* 2003; **42**: 5412.
31. Shi GE. *Rapid Commun. Mass Spectrom.* 2003; **17**: 202.
32. De Liñán C. *Vademecum de productos fitosanitarios y nutricionales*. Agrotécnicas: Madrid, Spain, 2007.
33. Annesley TM. *Clin. Chem.* 2003; **49**: 1041.
34. Matuszewski BK, Constanzer ML, Chavez-Eng CM. *Anal. Chem.* 1998; **70**: 882.
35. Buhrman DL, Price PI, Rudewicz PJ. *J. Am. Soc. Mass Spectrom.* 1996; **7**: 1099.
36. Wallace A. *Ann. NY Acad. Sci.* 1960; **88**: 361.

DOI: 10.1002/rcm

Journal of Plant Physiology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

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

Ajmi Larbi, Fermín Morales, Anunciación Abadía, Javier Abadía *,[1]

Department of Plant Nutrition, Aula Dei Experimental Station (CSIC), P.O. Box 13034, E-50080 Zaragoza, Spain

ARTICLE INFO

Article history:
Received 24 April 2009
Received in revised form
12 August 2009
Accepted 2 September 2009

Keywords:
Apoplastic fluid
Iron deficiency
Resupply
Sugar beet
Xylem sap

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

* Corresponding author. Tel.: +34 976 716056; fax: +34 976 716145.
E-mail address: jabadia@eead.csic.es (J. Abadía).
[1] Courier address: Av. Montañana 1005, Aula Dei Experimental Station (CSIC), Campus de Aula Dei, E-50059 Zaragoza, Spain.

0176-1617/$ - see front matter © 2009 Published by Elsevier GmbH.
doi:10.1016/j.jplph.2009.09.007

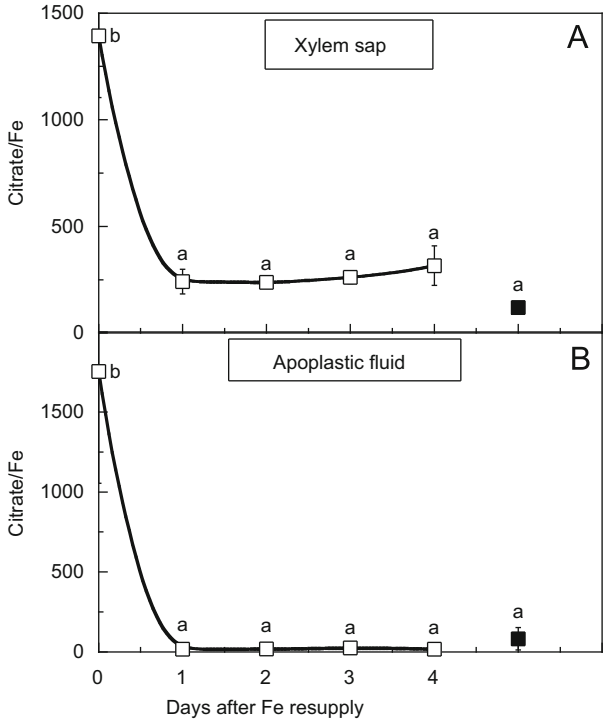

Fig. 6. Citrate:Fe ratios in xylem sap (A) and leaf apoplastic fluid (B) after adding Fe to the nutrient solution. Citrate:Fe xylem sap and apoplastic fluid in Fe-sufficient plants (solid square on the right side), Fe-deficient plants (open square at day 0) and Fe-deficient plants resupplied with Fe (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.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation (MICINN) (projects AGL2006-1416 and AGL2007-61948, co-financed with FEDER), the Commission of European Communities (ISAFRUIT Project, Thematic Priority 5-Food Quality and Safety, of the 6th Framework Programme of RTD; Contract no. FP6-FOOD-CT-2006-016279) and the Aragón Government (Group A03). A.L. was supported by a pre-doctoral fellowship from the Spanish Institute of International Cooperation. The authors gratefully acknowledge the technical assistance of Aurora Poc in growing the plants and Rebeca Tamayo in organic acid analysis.

References

Abadía J. Leaf responses to Fe deficiency – a Review. J Plant Nutr 1992;15:1699–713.

Abadía J, López-Millán AF, Rombolà A, Abadía A. Organic acids and Fe deficiency: a review. Plant Soil 2002;241:75–86.

Bialczyk J, Lechowski Z. Chemical-composition of xylem sap of tomato grown on bicarbonate containing medium. J Plant Nutr 1995;18:2005–21.

Brüggemann W, Maaskantel K, Moog P. Iron uptake by leaf mesophyll cells – the role of the plasma membrane-bound ferric-chelate reductase. Planta 1993;190:151–5.

Dannel F, Pfeffer H, Marschner H. Isolation of apoplasmic fluid from sunflower leaves and its use for studies on influence of nitrogen supply on apoplasmic pH. J Plant Physiol 1995;50:208–13.

De la Guardia MD, Alcántara E. Ferric chelate reduction by sunflower (*Helianthus annuus* L.) leaves: influence of light, oxygen, iron deficiency and leaf age. J Exp Bot 1996;47:669–75.

De Nisi P, Zocchi G. Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. J Exp Bot 2000;51:1903–9.

González-Vallejo EB, González-Reyes JA, Abadía A, López-Millán AF, Yunta F, Lucena JJ, Abadía J. Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet. Aust J Plant Physiol 1999;26:601–11.

Please cite this article as: Larbi A, et al. Changes in iron and organic acid concentrations in xylem sap and apoplastic fluid of iron-deficient *Beta vulgaris* plants in response to iron resupply. J Plant Physiol (2009), doi:10.1016/j.jplph.2009.09.007

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2010; **24**: 109–119
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4361

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

Irene Orera[1], Jesús Orduna[2], Javier Abadía[1] and Ana Álvarez-Fernández[1]*

[1]Department of Plant Nutrition, Aula Dei Experimental Station, CSIC, P.O. Box 13034, E-50080 Zaragoza, Spain
[2]New Organic Materials Unit, Institute of Materials of Aragón, CSIC-University of Zaragoza, c/ Pedro Cerbuna 12, E-50009 Zaragoza, Spain

Received 26 June 2009; Revised 30 October 2009; Accepted 31 October 2009

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

*Correspondence to: A. Álvarez-Fernández, Department of Plant Nutrition, Aula Dei Experimental Station, CSIC, P.O. Box 13034, E-50080 Zaragoza, Spain.
E-mail: ana.alvarez@eead.csic.es

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

SUPPORTING INFORMATION

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

Acknowledgements

Study supported by the Spanish Ministry of Science and Innovation (MICINN) (projects AGL2006-1416 and AGL2007-61948, co-financed with FEDER), the Commission of European Communities (ISAFRUIT Project, Thematic Priority 5-Food Quality and Safety, of the 6th Framework Programme of RTD; Grant number FP6-FOOD-CT-2006-016279), and the Aragón Government (Groups A03 and E39). IO was supported by a CONAID-DGA predoctoral fellowship. Acquisition of the HPLC/MS(TOF), HPLC/MS(QTOF) and ion trap devices was co-financed with FEDER.

REFERENCES

1. Marschner H. *Mineral Nutrition of Higher Plants*. Society for General Microbiology: London, 1995.
2. Fageria NK, Baligar VC, Clark RB. *Advances in Agronomy*. Academic Press: San Diego, 2002.
3. Lucena JJ. *Iron Nutrition in Plants and Rhizospheric Microorganisms*. Springer: Dordrecht, 2006.
4. Nowack B. *Environ. Sci. Technol.* 2002; **36**: 4009.
5. Knepper TP. *Trac-Trends Anal. Chem.* 2003; **22**: 708.
6. ANON. *Off. J. Eur. Union* 2007; **L51**: 7.
7. ANON. *Off. J. Eur. Union* 2003; **L34**: 1.
8. ANON. *Off. J. Eur. Union* 2004; **L359**: 25.
9. ANON. *Comité Européen de Normalisation* 2006; CEN/TS 15452:2006 E.
10. ANON. *Comité Européen de Normalisation* 2001; EN 13368-2: 2001 E.
11. ANON. *Comité Européen de Normalisation* 2001; EN 13366:2001 E.
12. ANON. *Comité Européen de Normalisation* 2001; EN 13368-1: 2001 E.
13. ANON. *Comité Européen de Normalisation* 2006; CEN/TS 15451:2006 E.
14. Kroll H, Knell M, Powers J, Simonian J. *J. Am. Chem. Soc.* 1957; **79**: 2024.
15. Hernández-Apaolaza L, Barak P, Lucena JJ. *J. Chromatogr. A* 1997; **789**: 453.
16. Sierra MA, Gómez-Gallego M, Alcázar-Montero R, Lucena-Marotta JJ, Álvarez-Fernández A, Yunta-Mezquita F. *EU Patent* 02/00604 A1, 2002.
17. Dexter M. *US Patent* 2824128, 1958.
18. Petree HE, Myatt HL, Jelenevsky AM. *US Patent* 4130582, 1978.
19. Gómez-Gallego M, Sierra MA, Alcázar R, Ramírez P, Pinar C, Mancheno MJ, García-Marco S, Yunta F, Lucena JJ. *J. Agric. Food Chem.* 2002; **50**: 6395.
20. Hernández-Apaolaza L, Álvarez-Fernández A, Lucena JJ. *J. Plant Nutr.* 2000; **23**: 2035.
21. Álvarez-Fernández A. *PhD thesis*. Universidad Autónoma de Madrid, 2000.
22. García-Marco S. *PhD thesis*. Universidad Autónoma de Madrid, 2005.
23. Cremonini MA, Álvarez-Fernández A, Lucena JJ, Rombolà A, Marangoni B, Placucci G. *J. Agric. Food Chem.* 2001; **49**: 3527.
24. Álvarez-Fernández A, Cremonini MA, Sierra MA, Placucci G, Lucena JJ. *J. Agric. Food Chem.* 2002; **50**: 284.
25. Hernández-Apaolaza L, García-Marco S, Nadal P, Lucena JJ, Sierra MA, Gómez-Gallego M, Ramírez-López P, Escudero R. *J. Agric. Food Chem.* 2006; **54**: 4355.
26. García-Marco S, Cremonini MA, Esteban P, Yunta F, Hernández-Apaolaza L, Placucci G, Lucena JJ. *J. Chromatogr. A* 2005; **1064**: 67.
27. Dodi A, Monnier V. *J. Chromatogr. A* 2004; **1032**: 87.
28. Álvarez-Fernández A, Orera I, Abadía J, Abadía A. *J. Am. Soc. Mass Spectrom.* 2007; **18**: 37.
29. Orera I, Abadía A, Abadía J, Álvarez-Fernández A. *Rapid Commun. Mass Spectrom.* 2009; **23**: 1694. DOI: 10.1002/rcm.4056.
30. Baron D, Hering JG. *J. Environ. Qual.* 1998; **27**: 844.
31. Quintana JB, Reemtsma T. *J. Chromatogr. A* 2007; **1145**: 110.
32. Lucena JJ, Barak P, Hernández-Apaolaza L. *J. Chromatogr. A* 1996; **727**: 253.
33. Bristow T, Constantine J, Harrison M, Cavoit F. *Rapid Commun. Mass Spectrom.* 2008; **22**: 1213.
34. Ojanperä S, Pelander A, Pelzing M, Krebs I, Vuori E, Ojanperä I. *Rapid Commun. Mass Spectrom.* 2006; **20**: 1161.
35. Deeba M, Ford ME, Jonson TA. *US Patent* 4918233, 1990.
36. Schwarz F, Schollhorn H, Thewalt U, Lippert B. *J. Chem. Soc. Chem. Commun.* 1990; 1282.
37. Boichard J, Brossard BP, Gay MLMJ, Janin RMC. *US Patent* 3607924, 1971.
38. García-Marco S, Martínez N, Yunta F, Hernández-Apaolaza L, Lucena JJ. *Plant Soil* 2006; **279**: 31.
39. Rodríguez-Castrillón JA, Moldovan M, García-Alonso JI, Lucena JJ, García-Tome ML, Hernández-Apaolaza L. *Anal. Bioanal. Chem.* 2008; **390**: 579.
40. Rojas CL, Romera FJ, Alcántara E, Pérez-Vicente R, Sariego C, García-Alonso JI, Boned J, Marti G. *J. Agric. Food Chem.* 2008; **56**: 10774.
41. Laghi L, Alcañiz S, Cerdán M, Gómez-Gallego M, Sierra MA, Placucci G, Cremonini MA. *J. Agric. Food Chem.* 2009; **57**: 5143. DOI: 10.1021/jf900116z.

PCP
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

Rubén Rellán-Álvarez[1], Justo Giner-Martínez-Sierra[2], Jesús Orduna[3], Irene Orera[1], José Ángel Rodríguez-Castrillón[2], José Ignacio García-Alonso[2], Javier Abadía[1,*] and Ana Álvarez-Fernández[1]

[1]Department of Plant Nutrition, Aula Dei Experimental Station, CSIC, PO Box 13034, E-50080 Zaragoza, Spain
[2]Department of Physical and Analytical Chemistry, University of Oviedo, c/Julian Clavería 8, E-33006 Oviedo, Spain
[3]New Organic Materials Unit, Institute of Materials Science of Aragón, CSIC-University of Zaragoza, c/Pedro Cerbuna 12, E-50009 Zaragoza, Spain
*Corresponding author: E-mail, jabadia@eead.csic.es; Fax, +34-976716145
(Received September 24, 2009; Accepted November 18, 2009)

Regular Paper

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

Plant Cell Physiol. 51(1): 91–102 (2010) doi:10.1093/pcp/pcp170, available online at www.pcp.oxfordjournals.org

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.

Funding

This work was supported by the Spanish Ministry of Science and Innovation (grant numbers AGL2006-1416, AGL2007-61948 and CTQ2006-05722, co-financed with FEDER); the European Commission (Thematic Priority 5—Food Quality and Safety, 6th Framework RTD Programme, grant number FP6-FOOD-CT-2006-016279) and the Aragón Government (groups A03 and E39). Acquisition of the HPLC–TOFMS apparatus was cofinanced with FEDER. R.R.-A., J.G.-M.-S., I.O. and J.A.R.-C. were supported by FPI-MICINN, FICYT-MICINN, CONAID-DGA and FPU-MICINN grants, respectively.

Acknowledgments

We thank Ade Calviño and Aurora Poc (Aula Dei Experimental Station–CSIC, Zaragoza, Spain) for growing the plants, Professor Jian Feng Ma (Research Institute for Bioresources, Okayama University, Kurashiki, Japan) for the kind supply of DMA and Dr. Ana-Flor López-Millán (Aula Dei Experimental Station–CSIC, Zaragoza, Spain) for critically reviewing the manuscript and helpful suggestions.

References

Abadía, J., López-Millán, A.F., Rombolà, A. and Abadía, A. (2002) Organic acids and Fe deficiency: a review. *Plant Soil* 241: 75–86.

Becke, A.D. (1993) A new mixing of Hartree-Fock and local density-functional theories. *J. Chem. Phys.* 98: 1372–1377.

Briat, J.F., Curie, C. and Gaymard, F. (2007) Iron utilization and metabolism in plants. *Curr. Opin. Plant Biol.* 10: 276–282.

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

Brown, J.C. and Tiffin, L.O. (1965) Iron stress as related to the iron and citrate ocurring in stem exudate. *Plant Physiol.* 40: 395–400.

Canals, I., Oumada, F.Z., Rosés, M. and Bosch, E. (2001) Retention of ionizable compounds on HPLC. 6. pH measurements with the glass electrode in methanol-water mixtures. *J. Chromatogr. A* 911: 191–202.

Clark, R.B., Tiffin, L.O. and Brown, J.C. (1973) Organic-acids and iron translocation in maize genotypes. *Plant Physiol.* 52: 147–150.

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., et al. (2008) Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103: 1–11.

De Laeter, J., Böhlke, J., De Bievre, P., Hidaka, H., Peiser, H., Rosman, K., et al. (2003) Atomic weights of the elements: review 2000. *Pure Appl. Chem.* 75: 683–800.

Deeth, R., Anastasi, A., Diedrich, C. and Randell, K. (2009) Molecular modelling for transition metal complexes: Dealing with d-electron effects. *Coord. Chem. Rev.* 253: 795–816.

Durrett, T.P., Gassmann, W. and Rogers, E.E. (2007) The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiol.* 144: 197–205.

Evans, R.W., Rafique, R., Zarea, A., Rapisarda, C., Cammack, R., Evans, P.J., et al. (2008) Nature of non-transferrin-bound iron: Studies on iron citrate complexes and thalassemic sera. *J. Biol. Inorg. Chem.* 13: 57–74.

Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., et al. (2003) Gaussian 03, Revision B.05. Gaussian, Inc., Pittsburgh, PA, USA.

Gautier-Luneau, I., Merle, C., Phanon, D., Lebrun, C., Biaso, F., Serratrice, G., et al. (2005) New trends in the chemistry of iron(III) citrate complexes: Correlations between X- ray structures and solution species probed by electrospray mass spectrometry and kinetics of iron uptake from citrate by iron chelators. *Chem. Europ. J.* 11: 2207–2219.

González Iglesias, H., Fernández Sánchez, M.L., Rodríguez-Castrillón, J.A., García-Alonso, J.I., Sastre, J.L. and Sanz-Medel, A. (2009) Enriched stable isotopes and isotope pattern deconvolution for quantitative speciation of endogenous and exogenous selenium in rat urine by HPLC-ICP-MS. *J. Anal. Atom. Spectrom.* 24: 460–468.

González-Vallejo, E., González-Reyes, J., Abadía, A., López-Millán, A., Yunta, F., Lucena, J., et al. (1999) Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet. *Aust. J. Plant Physiol.* 26: 601–611.

Hider, R.C., Yoshimura, E., Khodr, H. and von Wiren, N. (2004) Competition or complementation: the iron-chelating abilities of nicotianamine and phytosiderophores. *New Phytol.* 164: 204–208.

Kim, S.A. and Guerinot, M.L. (2007) Mining iron: iron uptake and transport in plants. *FEBS Lett.* 581: 2273–2280.

Küpper, H., Mijovilovich, A., Meyer-Klaucke, W. and Kroneck, P.M. (2004) Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by x-ray absorption spectroscopy. *Plant Physiol.* 134: 748–757.

Larbi, A., Morales, F., Abadía, J. and Abadía, A. (2003) Effects of branch solid Fe sulphate implants on xylem sap composition in field-grown peach and pear: changes in Fe, organic anions and pH. *J. Plant Physiol.* 160: 1473–1481.

Lensbouer, J., Patel, A., Sirianni, J. and Doyle, R. (2008) Functional characterization and metal ion specificity of the metal-citrate complex transporter from *Streptomyces coelicolor*. *J. Bacteriol.* 190: 5616–5623.

Lippard, S.J. (1988) Oxo-bridged polyiron centers in biology and chemistry. *Angew. Chem. Int. Ed.* 27: 344–361.

López-Millán, A., Morales, F., Gogorcena, Y., Abadía, A. and Abadía, J. (2009) Metabolic responses in iron deficient tomato plants. *J. Plant Physiol.* 166: 375–384.

López-Millán, A.F., Morales, F., Abadía, A. and Abadía, J. (2000) Effects of iron deficiency on the composition of the leaf apoplastic fluid and