Estudio de la homeostasis de Zn y Cd en plantas superiores

Tesis Doctoral

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Estudio de la homeostasis de Zn y Cd en plantas superiores

Memoria presentada por Ruth Sagardoy Calderón, Licenciada en Bioquímica, para optar al grado de Doctor en Ciencias.

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2. Sagardoy R, Morales F, López-Millán AF, Abadía A, Abadía J (2009). Effects of zinc toxicity in sugar beet (*Beta vulgaris* L.) plants grown in hydroponics. Plant Biology 11: 339-350.
3. Sagardoy R, Vázquez S, Florez-Sarasa ID, Albacete A, Ribas-Carbó M, Flexas J, Abadía J, Morales F (2010). Stomatal and mesophyll conductance to CO_2 are the main limitations to photosynthesis in sugar beet plants treated with Zn excess. New Phytologist 187 (1): 145-158.
4. Sagardoy R, Morales F, Rellán-Álvarez R, Abadía A, Abadía J, López-Millán AF (2011). Carboxylate metabolism in sugar beet plants grown with excess Zn. Journal of Plant Physiology 168: 730-733.

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La presentación en la modalidad de compendio de publicaciones de la Tesis Doctoral titulada **"Estudio de la homeostasis de Zn y Cd en plantas superiores"**, presentada por la Licenciada en Bioquímica Dª RUTH SAGARDOY CALDERÓN, para optar al Grado de Doctor por la Universidad de Zaragoza, y certifica que ha sido realizada bajo su dirección en la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas.

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Que los coautores de los artículos que integran esta Tesis poseen todos el grado de Doctor,a excepción de Dª María Solanas, y que ésta última renuncia a incluir el artículo titulado "Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics" en una futura Tesis por compendio de artículos, como consta en la renuncia adjunta a continuación.

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A Carlos, mi constante

"Las obras no se acaban, se abandonan"
Paul Ambroise Valéry (1871-1945, escritor francés)

ÍNDICE GENERAL

Resumen

Antecedentes

El estudio de la homeostasis de nutrientes en plantas trata de entender los procesos de adquisición, transporte y distribución de los nutrientes esenciales, y los mecanismos que se utilizan para mantener la concentración necesaria de esos nutrientes en el organismo para un correcto funcionamiento, evitando una acumulación excesiva que lo dañe. El Zn es un elemento esencial tanto para las plantas como para animales, sin embargo en exceso provoca alteraciones en procesos esenciales para el desarrollo del organismo. El Cd es un metal pesado sin ninguna función biológica y, por tanto, tóxico incluso a bajas concentraciones pero, al tener unas características fisico-químicas parecidas a otros metales esenciales, la planta lo absorbe por los mismos canales llegando a interferir con la entrada, transporte y utilización de varios macro y microelementos. De hecho, la similitud entre el Zn y el Cd hace que los síntomas de toxicidad observados en las plantas sean comunes; se reduce la biomasa en general, se detiene la elongación de las raíces principales y se oscurecen, se desarrollan raíces o pelos secundarios, se observa clorosis en las hojás jóvenes y pueden llegar a necrosar en casos extremos. Internamente, como respuesta a la exposición a metales pesados, la relación clorofila/carotenoides disminuye, se reduce la apertura estomática, la fotosíntesis y la transpiración, y aumenta la concentración de nicotianamina y glutatión (GSH). La toxicidad por metales pesados se debe en parte al estrés oxidativo producido por las especies reactivas de oxígeno (ROS), generadas a través de diferentes mecanismos dependiendo del metal de que se trate. El Zn^{2+} y el Cd^{2+} no experimentan cambios redox pero pueden actuar como pro-oxidantes a través de la reducción del contenido de GSH, necesario para la síntesis de fitoquelatinas, disminuyendo así su disponibilidad para la defensa antioxidante. Así se produce también peroxidación lipídica y daños en las proteínas por formación de grupos carbonilo.

Las plantas son la base de la cadena alimentaria y de ahí la importancia de conocer cuáles son los mecanismos de toxicidad del metal en la planta, así como los mecanismos de defensa de la misma. La contaminación ambiental por Zn y Cd ha aumentado como consecuencia del incremento de la actividad industrial del último siglo. Así, los altos niveles de estos metales encontrados en suelos, aguas de

riego y fertilizantes agrícolas suponen un peligro por su caracter no biodegradable, la toxicidad que ejercen sobre los diferentes cultivos y su biodisponibilidad. La sensibilidad de los cultivos a la presencia de los metales pesados es muy variable, así como la tendencia a acumular los mismos en partes que luego pasen al consumo animal o humano. A pesar de los avances realizados durante las últimas décadas en este tema, casi toda la información que tenemos proviene de estudios realizados en plantas hiperacumuladoras, y aún es muy poco lo que se conoce de la fisiología de especies de interés agronómico sometidas a estrés por metales pesados.

Objetivos de la investigación

El trabajo de investigación desarrollado en esta tesis se ha basado en el estudio de dos especies de interés comercial: el tomate y la remolacha. Las plantas han sido crecidas en cámaras de cultivo en medio hidropónico y exceso de Zn o Cd, añadidos de forma inorgánica a la solución de crecimiento.

El objetivo general consisitió en el estudio, descriptivo y detallado, de los cambios fisiológicos inducidos por las toxicidades de Zn y Cd, con el fin de determinar las causas de los mismos así como los mecanismos de transporte y tolerancia de especies de interés agronómico. Los objetivos concretos se detallan a continuación:

1. Caracterizar los cambios en el crecimiento y composición mineral a consecuencia de la toxicidad de Cd (10 y 100 μM) en plantas de tomate.
2. Caracterizar los cambios en el crecimiento y composición mineral a consecuencia del exceso de Zn (50, 100 y 300 μM) en plantas de remolacha.
3. Estudiar los efectos de la toxicidad de Cd en tomate y el exceso de Zn en remolacha sobre la fotosíntesis, composición pigmentaria y fluorescencia de clorofila.
4. Estudiar las limitaciones en el intercambio gaseoso, fotoquímica y bioquímica del proceso fotosintético a consecuencia del exceso de Zn en plantas de remolacha.
5. Estudiar los cambios en el metabolismo de ácidos orgánicos y la concentración de los mismos en diferentes tejidos de remolacha y tomate crecidos en exceso de Zn y Cd, respectivamente.

6. Caracterizar las concentraciones de Fe, Zn y Cd en el xilema de plantas de remolacha y tomate crecidas en exceso de Zn y Cd, respectivamente.

Metodología utilizada y resultados principales

Efectos de la toxicidad de Cd en plantas de tomate crecidas en solución nutritiva en cámara de cultivo.

Las plantas de tomate crecieron en medio hidropónico con concentraciones de 0, 10 y 100 μM de $CdCl_2$. Las estimaciones realizadas *in silico* con el programa MINTEQA2 para predecir las especies químicas en solución indicaron que cerca del 90% del Cd se encuentra en forma de Cd^{2+}, siendo así fácilmente asimilable por las raíces. Los parámetros de crecimiento de las plantas se redujeron en ambos tratamientos y se oscurecieron las raíces. Las hojas mostraron clorosis cuando crecieron con 10 μM Cd y zonas necróticas cuando lo hicieron con 100 μM Cd. El análisis mineral realizado con diferentes métodos espectrofotométricos (FAAS, FES y método del vanadato-molibdato) mostró que el Cd altera la homeostasis de Fe, Zn y Cu, aumentando su concentración en raíz y disminuyendo la de Mn. Además, la concentración de Cd se incrementó en todas las partes de la planta al aumentar la concentración del metal en la solución nutritiva. La estrategia de detoxificación del tomate resultó similar a la de otras especies, acumulando el Cd preferiblemente en la raíz a bajas concentraciones (tratamiento 10 μM Cd) y removilizándolo a la parte aérea cuando la concentración de metal en solución nutritiva es muy alta (tratamiento 100 μM Cd). Los ensayos enzimáticos realizados en extractos de hojas y raíces mostraron cambios en la actividad de PEPC y enzimas pertenecientes al ciclo de Krebs. En las raíces de tomate de ambos tratamientos de Cd la actividad de la PEPC (involucrada en la fijación anaplerótica de CO_2) aumentó, al igual que la actividad de la CS, ICDH y fumarasa. En las hojas de tomate crecido con 100 μM Cd la actividad de las enzimas CS, ICDH y MDH aumentó mientras que la actividad de la fumarasa disminuyó. Los parámetros fotosintéticos obtenidos con el medidor de intercambio gaseoso CIRAS mostraron un progresivo descenso en la asimilación de CO_2 y la concentración de pigmentos fotosintéticos analizada por HPLC también desciende, siendo este efecto más marcado en el tratamiento de 10 que en el de 100 μM.

Efectos de la toxicidad de Zn en plantas de remolacha crecidas en solución nutritiva en cámara de cultivo.

Las plantas de remolacha se crecieron en medio hidropónico usando las siguentes concentraciones: 1,2 (control), 50, 100 y 300 μM de $ZnSO_4$. La estimación de las especies iónicas en solución nutritiva con el programa MINTEQA2 indicó que entre el 91-93% del Zn se encuentra en forma de Zn^{2+}. El peso fresco y seco de las plantas, así como su contenido en agua, se reducen progresivamente al aumentar la concentración de Zn en la solución nutritiva. Se redujo el número de hojas y su área, y mostraron bordes arrugados y síntomas de clorosis. Las raíces se oscurecieron y acortaron, apareciendo raíces laterales y algunas puntas amarillas, signo de deficiencia de Fe. El análisis mineral por FAAS, FES y método del vanadato-molibdato mostró que el contenido de N, K, Mg y P desciende al aumentar el Zn aunque se mantienen en unos niveles suficientes para la planta. Las hojas tienen menos Fe y Mn que en las plantas controles y no hay cambios en el Cu. Los ensayos enzimáticos en extractos de puntas de raíz para medir la actividad de la reductasa férrica, que aumenta en deficiencia de Fe, mostraron ligeros incrementos en el tratamiento de 50 μM Zn. Se midieron los parámetros fotosintéticos con el intercambiador gaseoso CIRAS, la fluorescencia clorofílica con el fluorímetro PAM2000 y la concentración de pigmentos fotosintéticos fue analizada por HPLC. No hay diferencias significativas en los parámetros de intercambio gaseoso (P_N, E, g_s, y C_i) en las plantas crecidas con 50 y 100 μM Zn comparadas con las controles, pero en las de 300 μM Zn la transpiración (E), conductancia estomática (g_s), y concentración de CO_2 subestomática (C_i) descienden en un 73, 82 y 24%, respectivamente. Los parámetros de fluorescencia tampoco varían significativamente en las plantas de 50 y 100 μM Zn, aunque el quenching no fotoquímico (NPQ) aumentó en los tratamientos de 100 y 300 μM Zn, y se observó una disminución de la eficiencia del fotosistema II en el tratamiento de 300 μM Zn. Los pigmentos fotosintéticos disminuyen progresivamente al aumentar la concentración de Zn en la solución de crecimiento. La activación del ciclo de las xantofilas (ciclo VAZ) en las plantas de 50 μM y, principalmente, en las de 100 μM Zn, mostró que había estrés fotosintético. La remolacha es un buen modelo para el estudio de la homeostasis de Zn ya que ésta se encuentra finamente regulada:

la concentración del metal se incrementa en todas las partes de la planta cuando aumentamos la concentración de Zn en la solución nutritiva pero en hoja se dan pocos cambios entre los diferentes tratamientos, manteniéndose alrededor de los 250 $\mu g.g^{-1}$ de peso seco. Además, aunque a 50 μM de Zn exista una moderada deficiencia de Fe, en los tratamientos de 100 y 300 μM los efectos en la reducción de la fotosíntesis y la traspiración observados no parecen estar relacionados directamente con esta deficiencia.

Estudio de las limitaciones fotosintéticas en plantas de remolacha crecidas con exceso de Zn.

Para investigar las causas de la reducción de la fotosíntesis que encontramos en remolacha en exceso de Zn se crecieron plantas con 1,2 (control), 100 y 300 μM de $ZnSO_4$. Las medidas de fluorescencia clorofílica e intercambio gaseoso se realizaron con un LiCor-6400 y la intensidad luminosa utilizada en este trabajo es superior a la de experimentos anteriores. Siguiendo los modelos de Farquhar y de Grassi y Magnani se calculó la aportación de los diferentes componentes que participan en la fotosíntesis y en qué medida afectan a la reducción de la misma: conductancia estomática, conductancia del mesófilo y características bioquímicas. Tras conocer que la principal limitación de la fotosíntesis es causada por un descenso del 76% en la conductancia estomática, se realizaron una serie de estudios para seguir la respuesta de los estomas a señales químicas (añadiendo ácido abscísico-ABA) e hidraúlicas (cambios en la humedad relativa y desecamiento foliar) y ver cómo afectan estos cambios a la fotosíntesis, usando el modelo de Ball-Woodrow-Berry. Además, se recogieron muestras de xilema para analizar la concentración de ABA por HPLC-MS. Estos estudios mostraron que los estomas no responden a las señales hidraúlicas y que la concentración de ABA en el xilema es menor que en las plantas control, lo que hace pensar en cambios de tipo estructural en los estomas. Utilizando técnicas de microscopía electrónica de barrido (SEM y LT-SEM) se observó que la fisiología de las hojas de remolacha crecidas con exceso de Zn difiere de las controles. En las hojas de 300 μM de Zn hay menor densidad estomática y los estomas son más pequeños y de forma redondeada. En muchos casos la abertura estomática aparecía sellada con una sustancia sin identificar pero que, tras unos ensayos con disolventes orgánicos,

mostró ser algún tipo de cera. También se observaron cambios en la estructura interna de la hoja que pueden explicar la reducción de la conductancia en el mesófilo. Adicionalmente, se midió la actividad enzimática de la anhidrasa carbónica (CA) y la respiración en las hojas de remolacha. La CA incrementa su actividad en un 80% en las plantas de 300 μM quizá intentando compensar la reducción de la conductancia en el mesófilo. La respiración, medida con un espectrómetro de masas de relaciones isotópicas, aumenta el doble en ambos tratamientos de Zn; posiblemente la planta utilice esa energía para aumentar la capacidad de compartimentar el Zn o de los mecanismos de exclusión.

Estudio del metabolismo de los carboxilatos en plantas de remolacha crecidas con exceso de Zn.

El estudio se realizó en remolacha crecida con 1,2 (control), 50, 100 y 300 μM de $ZnSO_4$. Los ensayos de actividad de diferentes enzimas involucradas en el metabolismo de carboxilatos se realizaron en extractos de raíz y hojas y se analizó el xilema por HPLC-TOFMS. Los extractos de raíces de plantas crecidas con 50 y 100 μM Zn mostraron incrementos en citrato sintasa (CS) y fosfo*enol*piruvato carboxilasa (PEPC) comparadas con extractos de controles. Las concentraciones de ácidos cítrico y málico aumentan en las de 100 μM, pero no en plantas crecidas con 50 μM Zn. En xilema, ambos tratamientos aumentan su concentración de ácidos cítrico y málico comparados con los controles y en hojas encontramos también incrementados estos ácidos y un aumento en la actividad de CS y fumarasa. Sólo en 50 μM Zn también aumenta la isocitrato deshidrogenasa (ICDH). En plantas crecidas con 300 μM de Zn, la única enzima cuya actividad aumenta en raíz es la CS, el resto de enzimas disminuyen su actividad al compararla con la de las controles. La concetración de ácido cítrico en las raíces de estas plantas también aumenta. En xilema encontramos mayores concentraciones de ácidos cítrico y málico que en controles pero el flujo de C total hacia la parte aérea es similar al de controles, debido a las bajas tasas de transpiración de estas plantas. Con los datos reunidos se propuso un modelo del metabolismo de carboxilatos en plantas de remolacha crecidas en exceso de Zn. Los cambios observados en el metabolismo de las raíces de las plantas de 50-100 μM Zn muestran: (i) un aumento de la fijación anaplerótica de C asociado al incremento de

la actividad de la CS y PEPC; (ii) alteraciones en el ciclo de Krebs debido al descenso de la actividad de ICDH (en 100 μM Zn) y fumarasa (en 50 μM Zn); (iii) incremento en el flujo de carboxilatos desde la raíz a las hojas *via* xilema, según muestran los incrementos en las concentraciones del total de carboxilatos en xilema frente a la concentración sin cambios de los mismos en raíces. Este mecanismo de sintetizar carboxilatos en raíces para transportarlos a hojas puede estar paliando el déficit en la síntesis de los mismos en hoja debido a la redución de la actividad fotosintética. En las plantas crecidas con 300 μM de Zn se observó un comportamiento totalmente diferente ya que la reducción en la actividad de la PEPC en raíz indica que no existe esa fijación de C.

Efecto de la toxicidad de Zn y Cd en la concentración de otros metales en el xilema de tomate y remolacha y análisis de ácidos orgánicos en xilema de tomate.

El tomate y la remolacha son capaces de acumular metales pesados en raíces y traslocarlos a las hojas *via* xilema, esto produce alteraciones en la homeostasis de otros nutrientes esenciales, como por ejemplo una deficiencia inducida de Fe. Para conocer la concentración de estos metales (Fe, Zn y Cd) en el xilema de nuestras plantas modelo se crecieron en hidroponía: (i) remolacha con 10 y 50 μM de Zn para ver los efectos del exceso de Zn, (ii) remolacha y tomate en 10 y 50 μM de Cd para estudiar los efectos de la toxicidad de Cd en ambas especies. El aislamiento de xilema se realizó a los 4 y 8 días de iniciar los tratamientos de toxicidad y las muestras se analizaron por ICP/OES y ICP/MS. El xilema de tomate también fue analizado por HPLC-TOFMS para conocer su concentración de ácidos orgánicos. En remolacha crecida en exceso de Zn, la concentración de Zn encontrada en xilema fue entre 2 y 8 veces mayor (en 10 μM Zn y 50 μM Zn, respectivamente) a la encontrada en xilema de plantas controles mientras que las concentraciones de Fe no se vieron significativamente afectadas. El tratamiento con Cd incrementa la concentración de este metal en el xilema, pero el tiempo de exposición al metal afecta de forma muy diferente a cada especie. En remolacha el aumento es progresivo mientras que el tomate absorbe rápidamente el metal (muestreo a los 4 días) para disminuir posteriormente su concentración en xilema (muestreo a los 8 días). Con la toxicidad de Cd la concentración de Fe en xilema aumenta en la remolacha y decrece

en tomate, mientras que el Zn no sufre cambios en remolacha y se incrementa en tomate. En xilema de tomate encontramos que el ácido málico se incrementa con la concentración de Cd y el tiempo de exposición al metal. Sabemos que la actividad de varias enzimas del ciclo de Krebs está aumentada, entre ellas MDH y CS en raíz, lo que concuerda con el aumento de ácido málico encontrado en xilema. Estas plantas muestran además síntomas de deficiencia de Fe debido a la competición entre el Cd y el Fe en la absorción por las raíces, que se refleja en un menor transporte de Fe en el xilema. Estos datos sugieren que el tomate sometido a exceso de Cd responde fijando CO_2 en las raíces compensando la reducción en la fotosíntesis que sufren. Resumiendo, la absorción de Cd en la remolacha aumenta con la dosis y el tiempo de exposición, mientras que el tomate alcanza en los primeros días concentraciones 10 veces mayores de las medidas en remolacha. Esto explicaría la mayor tolerancia de la remolacha a la toxicidad por Cd comparada con el tomate, ya que la cinética de adquisición del Cd en remolacha es progresiva y no compite con la absorción de Fe y Zn. Este trabajo sienta las bases para futuros estudios sobre posibles ligandos de Cd y Zn en xilema por LC-MS.

Conclusiones

1. Concentraciones elevadas de Zn añadido en forma de sal en solución nutritiva reducen el crecimiento, la tasa fotosintética y la transpiración de plantas de remolacha.
2. La remolacha es una especie tolerante al exceso de Zn. Es capaz de transportar y almacenar el metal en la parte aérea pero mantiene una concentración de Zn en hoja constante frente al aumento de metal en solución, posiblemente gracias a mecanismos de exclusión en la raíz.
3. Las plantas de remolacha cultivadas con un exceso moderado de Zn, exhiben síntomas de una deficiencia moderada de Fe. Si bien la toma y transporte *via* xilema del Fe no se ven afectados, su concentración en hoja se reduce a la mitad, indicando un posible mecanismo de competición en la descarga a hoja entre ambos metales.

4. A concentraciones elevadas de Zn, las plantas de remolacha no presentan clorosis, sino que muestran una fuerte reducción del crecimiento y de la tasa fotosintética. La tasa de respiración se dobla, probablemente para aumentar la capacidad de la compartimentación/exclusión del metal.
5. En remolacha, la principal limitación de la fotosíntesis en condiciones de elevado exceso de Zn es la reducción de las conductancias estomática y del mesófilo. En estas condiciones los cambios fisiológicos en los estomas y la estructura interna del mesófilo dificultan la difusión del CO_2.
6. El tomate es una especie sensible a la toxicidad de Cd. En plantas cultivadas con bajas concentraciones de Cd la estrategia de detoxificación consiste en la acumulación de Cd en las raíces, mientras que a concentraciones altas se produce una movilización masiva de Cd a las hojas. El exceso de Cd disminuye el crecimiento y la tasa fotosintética.
7. El exceso de Cd en tomate altera la homeostasis de otros nutrientes, interfiriendo el paso de Fe al xilema y facilitando el de Zn.
8. Tanto la toxicidad de Zn en remolacha como la de Cd en tomate provocan un aumento de la fijación anaplerótica de C en la raíz en forma de ácidos orgánicos, que tiende a compensar la carencia de esqueletos carbonados asociada con la disminución de la fotosíntesis.
9. En ambas especies, la toxicidad de Zn y la de Cd aumentan la actividad del ciclo de Krebs y la respiración, aumentando la disponibilidad de poder reductor para hacer frente al exceso de metal y los daños oxidativos producidos en la célula.
10. Se han definido las condiciones óptimas de toxicidad de Cd y Zn en tomate y remolacha que permiten obtener xilema en concentraciones adecuadas para abordar la especiación de estos elementos en dicho compartimento.

Capítulo 1

Introducción

Durante las últimas décadas, numerosos estudios en el campo de la nutrición vegetal han tratado de desentrañar cómo las plantas asimilan y distribuyen los nutrientes que necesitan para un correcto crecimiento y desarrollo (Marschner, 1995; Mengel *et al.*, 2001; Karley & White, 2009; Miller *et al*., 2009; Miwa *et al.*, 2009; Puig & Peñarrubia, 2009; White & Broadley, 2009). Además de oxígeno, dióxido de carbono y agua, las plantas necesitan de al menos 14 elementos para una nutrición adecuada, que se obtienen generalmente del suelo (Marschner,1995; Mengel *et al*., 2001). La deficiencia de alguno de estos elementos en las zonas agrícolas reduce el crecimiento y la producción de los cultivos, mientras que el exceso de alguno de ellos también provoca daños en las cosechas (MacNicol & Beckett, 1985; Marschner, 1995; Mengel *et al*., 2001). Por ejemplo, en suelos ácidos podemos encontrar toxicidad por Mn y Al, en suelos sódicos existe toxicidad por B y Na, y en suelos salinos se presentan toxicidades de Na y Cl. En la agricultura de regadío hay problemas con Na, B, y Cl y desequilibrios de Ca, Mg y K, y en campos encharcados e inundados toxicidades de Mn y Fe. Además, hay terrenos cuya composición es rica en Ni, Co, Cr o Se, y otros donde la influencia del hombre ha llevado a la acumulación de metales como Zn, Cu, Cd, Hg y Pb, y que o son o pueden resultar tóxicos debido a su exceso (White & Brown, 2010).

En la siguiente página (Tabla 1) se puede ver una relación de los elementos esenciales tanto para vegetales como para animales. En el bloque superior encontramos los macroelementos (N, K, P, Ca, Mg y S) y a continuación los microelementos, llamados así por ser necesarios en menores cantidades. En la mitad inferior de la tabla se incluyen elementos que no son necesarios para su crecimiento, pero son considerados como beneficiosos para las plantas al favorecerlo en ciertas condiciones ambientales. El rango de concentración que se considera normal se define como aquél que permite que el crecimiento del cultivo alcance el 90% del rendimiento máximo. La concentración considerada tóxica es la medida en un cultivo cuyo crecimiento disminuye en más de un 10% (White & Brown, 2010). La amplitud que existe en los rangos de concentración se debe a que hay una alta variabilidad dependiendo tanto de la especie estudiada, como del genotipo, el tejido (aunque aquí nos limitamos a datos en hoja) y las condiciones de crecimiento.

Tabla 1. *Concentraciones críticas de elementos minerales medidas por peso seco de hoja en cultivos no tolerantes (modificada de White & Brown, 2010).*

	Esencial		Concentración en hoja (mg g^{-1} peso seco)	
Elemento	Plantas	Animales	Normal	Toxicidad
Nitrógeno (N)	Si	Si	15 - 40	
Potasio (K)	Si	Si	5 - 40	>50
Fósforo (P)	Si	Si	2 - 5	>10
Calcio (Ca)	Si	Si	0.5 - 10	>100
Magnesio (Mg)	Si	Si	1.5 - 3.5	>15
Azufre (S)	Si	Si	1.0 - 5.0	
Cloro (Cl)	Si	Si	0.1 - 6.0	4.0 - 7.0
Boro (B)	Si	sugerido	5 - 100 × 10^{-3}	0.1 - 1.0
Hierro (Fe)	Si	Si	50 - 150 × 10^{-3}	>0.5
Manganeso (Mn)	Si	Si	10 - 20 × 10^{-3}	0.2 - 5.3
Cobre (Cu)	Si	Si	1 - 5 × 10^{-3}	15 - 30 × 10^{-3}
Cinc (Zn)	Si	Si	15 - 30 × 10^{-3}	100 - 300 × 10^{-3}
Níquel (Ni)	Si	sugerido	0.1 × 10^{-3}	20 - 30 × 10^{-3}
Molibdeno (Mo)	Si	Si	0.1 - 1.0 × 10^{-3}	1
Sodio (Na)	beneficioso	Si	–	2 - 5
Selenio (Se)	beneficioso	Si	–	10 - 100 × 10^{-3}
Cobalto (Co)	beneficioso	Si	–	10 - 20 × 10^{-3}
Yodo (I)	–	Si	–	1 - 20 × 10^{-3}
Fluor (F)	–	sugerido	–	0.1
Litio (Li)	–	sugerido	10 - 200 × 10^{-3}	
Plomo (Pb)	–	sugerido	–	10 - 20 × 10^{-3}
Arsénico (As)	–	sugerido	–	1 - 20 × 10^{-3}
Vanadio (V)	–	sugerido	–	1 - 10 × 10^{-3}
Cromo (Cr)	–	sugerido	–	1 - 2 × 10^{-3}
Silicio (Sí)	beneficioso	sugerido	–	–
Aluminio (Al)	beneficioso	–	–	40 - 200 × 10^{-3}
Cadmio (Cd)	–	–	–	5 - 10 × 10^{-3}
Mercurio (Hg)	–	–	–	2 - 5 × 10^{-3}

La absorción de metales pesados por las plantas es, generalmente, el primer paso de su entrada en la cadena alimentaria. Las plantas han desarrollado mecanismos específicos para absorber, traslocar y almacenar nutrientes (Lasat, 2000), pero algunos metales no esenciales pueden igualmente ser asimilados por los vegetales al presentar un comportamiento químico similar al de otros microelementos esenciales. La absorción y posterior acumulación de un metal depende de:

1- la movilidad del metal desde el suelo a la superficie de la raíz,

2- el paso del metal a través de las membranas de las células de raíz,

3- transporte desde las células corticales al xilema, desde donde se podrá traslocar a tallos y hojas,

4- la posible movilización del metal desde la hoja a otros tejidos de almacenamiento que sean fuente de alimento (semillas, tubérculos, frutos) por el floema.

El rango fisiológico entre deficiencia y toxicidad para los metales esenciales de transición es muy estrecho, y su homeostasis está finamente regulada para ajustarse a las fluctuaciones de la disponibilidad de micronutrientes (Figura 1; Marschner, 1995). También existe una tolerancia basal a los metales no esenciales, y se han descrito síntomas y respuestas de las plantas cuando se exponen a concentraciones superiores. Sin embargo, poco se sabe aún de los mecanismos que desarrollan las plantas para mantener su crecimiento en presencia de concentraciones de metales potencialmente tóxicas o para transportar y almacenar el exceso de metal. Tampoco se conoce mucho sobre la señalización y activación de genes de respuesta a estrés por metales pesados. Todos estos procesos determinan el grado de eficiencia que tiene cada especie y genotipo para enfrentarse a la toxicidad por metales pesados (Krämer & Clemens, 2005; Clemens, 2006).

Las plantas constituyen una parte muy importante de la dieta humana, por lo que la contaminación de verduras, cereales o frutas debido a la contaminación de los suelos de cultivo supone un alto riesgo para la calidad y seguridad de la alimentación (Muchuweti *et al*., 2006). La alta concentración de metales pesados en plantas comestibles está relacionada con la prevalencia de distintas enfermedades, como cáncer gastrointestinal en humanos (Turkdogan *et al.*, 2002; Kocasoy & Sahin, 2007).

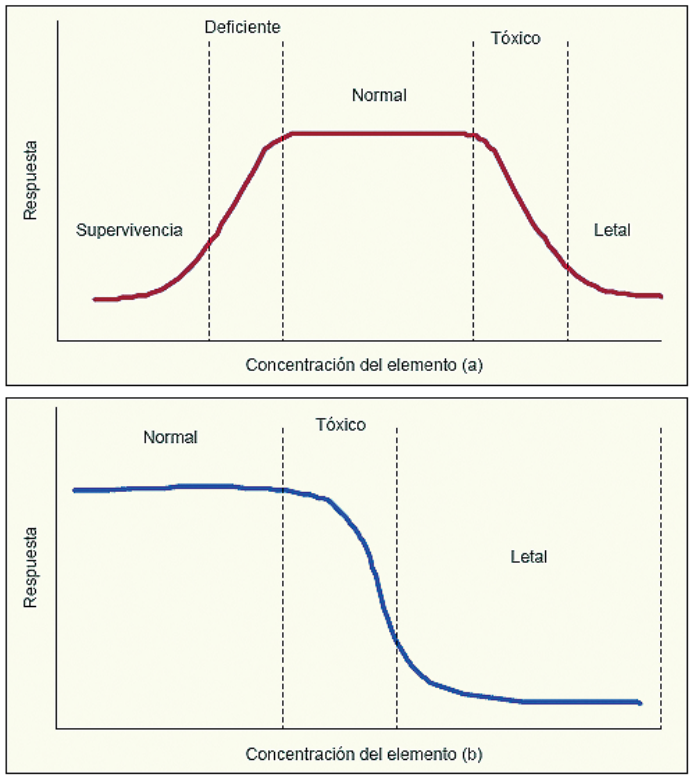

Figura 1. *Relación entre concentración y respuesta para elementos esenciales (a) y tóxicos (b). Modificado de Chen (2000).*

Son principalmente fuentes antropogénicas, tales como las actividades mineras, los residuos de incineración y de procesos industriales y la fertilización de cultivos las responsables de la acumulación de metales pesados en plantas y animales y, por tanto, de la introducción de metales pesados en la cadena trófica (Wilson & Pyatt, 2007).

En respuesta a este problema, las investigaciones en esta área han centrado sus esfuerzos en entender la naturaleza de los mecanismos de adquisición, tolerancia y toxicidad en las plantas. Algunos de los avances más importantes alcanzados han sido desarrollar variedades tolerantes de especies que puedan crecer en suelos contaminados y el descubrimiento de nuevas variedades que pueden extraer los metales del suelo, con el objetivo de que vuelva a ser útil para la agricultura, una práctica que se conoce como fitorremediación.

METALES PESADOS

Se consideran metales pesados aquellos elementos con una densidad igual o superior a 5 $g.cm^{-3}$ (Wild, 1993) o de número atómico mayor a 20 (excepto los pertenecientes a los grupos I y II de la tabla periódica). Se encuentran como elementos traza en la corteza terrestre, en un porcentaje menor al 0,1%, generalmente por debajo de 0,01%, excepto en el caso del hierro (Fe) que se encuentra en un 5%. Los metales pesados más comunes son cromo (Cr), manganeso (Mn), níquel (Ni), cobre (Cu), cinc (Zn), molibdeno (Mo), cadmio (Cd), mercurio (Hg) y plomo (Pb). El arsénico (As) y el selenio (Se) se incluyen frecuentemente dentro de esta categoría aún siendo no metales, ya que presentan orígenes y comportamientos asociados (Brady & Weil, 2002; García & Dorronsoro, 2005).

Los metales pesados se clasifican en dos grupos:

- Oligoelementos o micronutrientes: requeridos en pequeñas cantidades por plantas y animales, el organismo no puede crecer ni puede completar su ciclo biológico sin un suministro adecuado de los mismos. Son potencialmente tóxicos si los niveles de exposición son lo suficientemente altos. Dentro de este grupo encontramos: As, B, Co, Cr, Cu, Mo, Mn, Ni, Fe, Se y Zn.
- Metales pesados sin función biológica conocida, cuya presencia en el organismo a bajas dosis ya pueden desencadenar efectos tóxicos. Son, entre otros: Cd, Hg, Pb, Sb, Bi, Sn, Tl, etc...

En el primer caso, estos elementos participan en funciones esenciales para la vida, por ejemplo en la estabilización de membranas y en la regulación de la actividad de numerosas enzimas. Los problemas aparecen cuando los niveles en tejido se encuentran por encima o por debajo de las concentraciones fisiológicas requeridas por el organismo (Figura 1). Los metales pesados más peligrosos debido a sus características de toxicidad, persistencia y bioacumulación son, por este orden, Hg, Cd y Pb, seguidos a bastante distancia por Cu, Zn, Cr y Ni (Kennisch, 1996; Azcúe, 1993). La consecuencia más grave de que los metales no sean biodegradables es su acumulación en las cadenas tróficas, porque los organismos que se encuentran en la parte superior de las mismas, entre ellos el hombre, pueden encontrarse expuestos a elevadas concentraciones de estos elementos (Neff, 2002; Wang, 2002).

Presencia de metales pesados en el suelo

En ecosistemas agrícolas, la mayoría de los metales pesados están incluidos en ciclos biogeoquímicos en los cuales los dos componentes fundamentales son el suelo y la planta. El contenido de metales pesados en el suelo debería ser únicamente función de la composición del material original y de los procesos edafogenéticos, pero la actividad humana ha incrementado el contenido de estos metales en cantidades considerables, y de hecho la entrada de metales pesados en el suelo ha ido aumentando desde que comenzó la industrialización (García & Dorronsoro, 2005). Las actividades antropogénicas han provocado el enriquecimiento de los metales en el medio ambiente, originándose una acumulación de 100 a 1000 veces más alta en relación con su proporción natural en la corteza terrestre, con la excepción del Fe y el Hg (Wedepohl, 1991). Además de la industrialización, las prácticas agrícolas constituyen una importantísima fuente de metales en el suelo en muchas partes del mundo, especialmente en zonas de actividad intensa (Alloway, 1995). Las principales fuentes son:

- Impurezas en los fertilizantes: Cd, Cr, Mo, Pb, U, V y Zn.
- Aguas y lodos residuales: especialmente Cd, Ni, Cu, Pb y Zn.
- Estiércoles de producción animal intensiva: Cu, As y Zn.
- Plaguicidas: Cu, As, Hg, Pb, Mn y Zn.
- Residuos derivados de fertilizantes orgánicos: Cd, Cu, Ni, Pb y Zn.

Los metales pesados llegan al suelo por vía aérea (aerosoles, partículas minerales, polvos suspendidos y transportados por el aire,...) y terrestre (fertilizantes, plaguicidas, residuos sólidos, etc.) y, por otra, se pierden ya sea por la absorción por las plantas, o bien por lixiviación o erosión. Algunos factores que influyen en la movilización de metales pesados en el suelo, según Sauquillo *et al*. (2003), son:

1. Capacidad de intercambio catiónico del suelo. Es función de la cantidad y tipos de materia orgánica y de arcilla que posea el suelo, cuanto mayor sea la capacidad de intercambio mayor será la inmovilización ejercida sobre los metales.
2. pH. Por norma general, una disminución de pH aumenta el riesgo de paso a la solución del suelo: una unidad de incremento en el pH hace descender 100

veces los niveles de Cd, Cu, Ni y Zn en la solución del suelo.

3. Potencial redox. El estado redox de un suelo afecta a la especiación metálica y a la solubilidad de los cationes. Un suelo con condiciones oxidantes favorecerá la oxidación y disolución de sulfuros insolubles. En condiciones reductoras (suelos encharcados y de alta compactación), metales como el Fe y Mn se encuentran como cationes divalentes y aumenta su disponibilidad para las plantas. En cambio, la forma reducida de Cu es muy inestable y puede precipitar, dando lugar a deficiencias de dicho elemento.
4. Efectos sinérgicos y antagónicos entre elementos. En caso de sinergia, dos elementos en combinación pueden producir efectos distintos a los que se obtendrían sumando las efectos de cada uno por separado. También pueden darse antagonismos que, orientados de forma adecuada, pueden resultar útiles para limitar los riesgos originados por la presencia de un cierto elemento. Así, el problema del Cd puede ser regulado por el Zn, con una relación Zn/Cd elevada en suelo.

Efectos de los metales pesados en las plantas

En general, las plantas son mucho más resistentes a los incrementos en la concentración que a la insuficiencia de un elemento esencial dado. Distinto es el caso de un elemento no esencial, donde el nivel de daño suele aumentar conforme lo hace la concentración disponible del elemento (Figura 1). Cuando un metal se encuentra en exceso dentro de un organismo puede unirse a grupos funcionales que contienen átomos de azufre, nitrógeno u oxígeno o puede desplazar a otros metales de características fisico-químicas similares. Si además el metal tiene actividad redox, como el Cu, Fe o Mn, pueden intervenir en procesos de transferencia de electrones y catalizar la formación de radicales libres (Pilon *et al.,* 2009). Esto implica:

1. Inactivación de proteínas y enzimas. La unión del metal a grupos funcionales de una proteína y/o el desplazamiento de cationes que actúan como co-factores provocan el bloqueo del centro activo de la enzima o cambios estructurales que interrumpen el funcionamiento normal de estas moléculas.
2. Procesos oxidativos. Las especies reactivas de oxígeno (ROS), como los radicales hidroxilo $OH^{\cdot}$ y superóxido O^{2-} y el peróxido de hidrógeno H_2O_2,

se generan en procesos metabólicos que implican al oxígeno, como la fotosíntesis y la respiración. La actividad redox de algunos metales, como el Fe en la reacción de Fenton, favorece la formación de radicales libres.

Reacción de Haber-Weiss

$$H_2O_2 + OH\cdot \rightarrow H_2O + O^{2-} + H^+$$

$$H_2O_2 + O^{2-} \rightarrow O_2 + OH^- + OH\cdot$$

Reacción de Fenton

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

Los ROS formados reaccionan con los lípidos de membrana, produciendo peroxidaciones que debilitan las membranas celulares aumentando su permeabilidad. Dichos compuestos también dan lugar a oxidaciones en los grupos tiol y amino de las proteínas inactivándolas y pueden provocar mutaciones en los ácidos nucleicos o alterar su síntesis y reparación. Por ello, la toxicidad de los metales pesados en los organismos se debe a una combinación de estos factores, ya que al daño oxidativo generado en todo tipo de moléculas hay que sumar la interferencia con los procesos metabólicos de protección y detoxificación de la célula.

Respuestas de las plantas al exceso de metales pesados

Las plantas tienen un sistema de homeostasis altamente regulado que les permite mantener la concentración de los elementos esenciales en niveles óptimos donde los necesiten (citosol, orgánulos celulares) y, simultáneamente, protegerse de una posible toxicidad. Al contrario ocurre con los elementos tóxicos, para los cuales no existe un sistema de homeostasis sino de detoxificación, por lo que las plantas no han de regular tan finamente las concentraciones de los elementos tóxicos y generalmente utilizan moléculas quelantes que acomplejan el metal y del almacenamiento en vacuola.

Cuando las plantas se encuentran en presencia de un metal que puede resultar dañino, bien por su naturaleza tóxica o por su alta concentración si es un metal esencial, pueden utilizar diferentes estrategias. Unas basan su resistencia a los metales ya sea en una exclusión eficiente del metal, o bien almacenándolo en las vacuolas de las células radiculares, restringiendo así su transporte a la parte aérea.

Otras prefieren acumular el metal en la parte aérea en formas no tóxicas para evitar que puedan causar daño oxidativo a las células. La planta puede protegerse formando complejos metálicos estables no tóxicos con quelantes (como fitoquelatinas, ácidos orgánicos, aminoácidos, metalotioneínas o compuestos fenólicos) y/o secuestrando los metales desde zonas con un metabolismo activo (citoplasma) hacia el interior de vacuolas o en la pared celular, donde no puedan ocasionar efectos tan adversos (Krämer *et al*., 1996; Tolrà *et al.*, 1996; Hall, 2002; Schat *et al.*, 2002). Un caso extremo de la estrategia de acumulación es el de las plantas hiperacumuladoras, que pueden superar en 100 ó más veces los valores normales de metales encontrados en parte aérea. Estas plantas son especies muy tolerantes a uno o más metales pesados y a menudo su distribución está restringida a suelos ricos en metales, pues no son competitivas en zonas no contaminadas (Baker, 1981).

Por lo tanto, cada metal y cada especie vegetal interactúan de un modo específico, dependiendo de las condiciones en que se encuentren el metal y la especie vegetal a estudiar. Se han realizado diferentes clasificaciones de especies de interés agronómico según su sensibilidad a ciertos metales pesados, tanto esenciales como tóxicos. Se sabe que la lechuga, espinaca, acelga, escarola o remolacha son cultivos de alta bioacumulación de metales en las partes comestibles (Tabla 2; Boixadera & Teira, 2001; Larbi *et al*., 2002). La bioacumulación consiste en la capacidad de los organismos de acumular selectivamente metales en determinados tejidos u órganos respecto de las concentraciones existentes en el medio en que habitan. Pero de nuevo esa acumulación es variable, así que la asimilación de Cr y Pb es pequeña y los metales son bloqueados en la raíz, mientras que Cd y Hg pueden acumularse en los tejidos vegetales en concentraciones tóxicas para los animales sin que ello produzca efecto adverso para la planta (Tabla 3; Boixadera & Teira, 2001).

Tabla 2. *Bioacumulación relativa en las partes comestibles de diferentes cultivos. (Extraído de Boixadera & Teira, 2001).*

Alta	**Moderada**	**Baja**	**Muy baja**
Lechuga	Col rizada	Col	Judía
Espinaca	Nabo (raíces)	Maíz dulce	Guisante
Acelga	Rábano	Brócoli	Melón
Escarola	Mostaza	Coliflor	Tomate
Endibia	Patata	Col de Bruselas	Pimienta
Berro		Apio	Berenjena
Nabos (hojas)		Bayas	Árboles frutales
Remolacha			
Zanahoria			

Tabla 3. *Acumulación relativa por vegetales: Cd y Pb en fracciones comestibles; Cu, Ni y Zn en hojas (Extraído de Boixadera & Teira, 2001).*

	Alta	**Baja**
Cd	Lechuga, espinaca, apio, col.	Patata, maíz, judía, guisante.
Pb	Col rizada, centeno, apio.	Determinadas variedades de cebada, patata, maíz.
Cu	Remolacha azucarera, determinadas variedades de cebada.	Puerro, col, cebolla.
Ni	Remolacha azucarera, centeno, remolacha forrajera, nabo.	Maíz, puerro, cebada, cebolla.
Zn	Remolacha azucarera, remolacha forrajera, espinaca, raíz de remolacha.	Patata, puerro, tomate, cebolla.

CINC

El Zn ocupa el puesto número 23 entre los elementos más abundantes de la corteza terrestre y su contenido en la litosfera está en torno a 70 $mg.kg^{-1}$ (Adriano, 2001). Forma gran variedad de sales (cloratos, cloruros, sulfatos y nitratos) solubles en agua, mientras que los óxidos, carbonatos, fosfatos, silicatos y sulfuros son relativamente insolubles (Adriano, 2001). El ion Zn^{2+} es incoloro, divalente en todos sus compuestos y existe en forma hidratada en soluciones acuosas neutras y ácidas; sin embargo, en solución alcalina precipita como hidróxido. Su contenido en el suelo depende de la naturaleza de la roca madre (Kabata-Pendias & Pendias, 1992), de la materia orgánica, de la textura y del pH (Adriano, 2001). En suelos no contaminados se encuentra ampliamente distribuido a concentraciones de elemento traza (10-300 $mg.kg^{-1}$) con un promedio de 50 $mg.kg^{-1}$ (Lindsay, 1979) y una movilidad geoquímica relativamente baja, que decrece al aumentar el pH (Zauner *et al.*, 1999). El uso del Zn no ha dejado de aumentar a lo largo de la historia, y las numerosas aplicaciones que tienen tanto el metal como sus compuestos han llevado a que en la actualidad esté presente en residuos y emisiones contaminantes.

Importancia bioquímica del Zn

La primera vez que se demostró la necesidad del Zn fue por Raulin en 1869, que observó que el moho del trigo común (*Aspegillus niger*) no era capaz de crecer en ausencia de Zn. Desde entonces se ha comprobado que el Zn es esencial para el crecimiento, desarrollo y diferenciación de microorganismos, plantas y animales, es decir, es un elemento esencial para todos los organismos (Vallee & Galdes, 1984; Arrivault *et al.*, 2006). Es el segundo metal de transición más abundante en los seres vivos después del Fe, y el único que está presente en enzimas de todos los tipos de la clasificación que las divide en oxidoreductasas, transferasas, hidrolasas, liasas, isomerasas y ligasas (Enzyme Commission number, EC 1-6) (Webb, 1992; Broadley *et al.*, 2007). Aproximadamente 300 enzimas contienen sitios de unión a Zn en su estructura (Vallee & Galdes, 1984; Brandão-Neto *et al.*, 1995), siendo algunos ejemplos la NADH-deshidrogenasa o la alcohol deshidrogenasa que cataliza el paso de etanol a acetaldehído en la fermentación alcohólica. El Zn también forma parte del centro catalítico de otras enzimas, como las 5 isoformas de anhidrasa carbónica

(CA) encontradas hasta ahora, que acelera la hidratación reversible del CO_2 a bicarbonato en los tejidos fotosintéticos (Evans *et al*., 2009). Junto al Cu, el Zn está presente en algunos tipos de superóxido dismutasas (SOD), enzimas localizadas en el citoplasma y diferentes orgánulos celulares, involucradas en la defensa frente a los radicales superóxido. Las proteínas más numerosas capaces de unir Zn son las que poseen dominios del tipo "zinc finger" o "dedos de Zn"; estas enzimas regulan la transcripción del DNA por unión directa a esta molécula, siendo determinantes en la estructura de la cromatina, la estabilidad de los ribosomas, el metabolismo del RNA y las interacciones proteína-proteína (Klug, 1999; Englbrecht *et al.*, 2004). En el genoma humano se estima que alrededor del 10% de las proteínas tienen dominios de unión a Zn (Andreini *et al.*, 2006), y son muchas más implicadas en su transporte y homeostasis (Beyersmann & Haase, 2001).

El Zn en el suelo

Para el crecimiento adecuado de la mayoría de los cultivos se establece que la concentración de Zn en hoja debe estar entre 15 y 20 mg $Zn.kg^{-1}$ de peso seco (Marschner, 1995). La deficiencia de Zn es la más extendida de entre todos los micronutrientes, especialmente en suelos de pH alto (Graham *et al.*, 1992; White & Zasoski, 1999; Cakmak, 2002, 2004; Alloway, 2004). Aunque la toxicidad de Zn es menos común, los suelos con un pH bajo favorecen la movilidad del catión, y esto es especialmente peligroso en suelos contaminados por actividades antropogénicas como:

- zonas de áreas mineras y de fundición.
- suelos agrícolas tratados con fertilizantes, aguas o lodos residuales, pesticidas, etc.
- zonas urbanas donde se dan deposiciones aéreas provenientes de las zonas industriales (industria metalúrgica, química, microelectrónica).

En estas situaciones el metal puede ser altamente biodisponible y cuando las plantas se exponen a un exceso de Zn pueden desarrollar síntomas de toxicidad tales como hojas cloróticas e inhibición del crecimiento radicular (Woolhouse, 1983). Estos síntomas comienzan a ser visibles cuando la concentración de Zn en hoja supera los 300 mg $Zn.kg^{-1}$ de peso seco, aunque algunos cultivos los muestran ya a

concentraciones por debajo de 100 mg $Zn.kg^{-1}$ de peso seco (Tabla 1). Los rangos de concentraciones adecuadas o tóxicas del metal son muy variables dependiendo de la especie vegetal e incluso entre variedades de la misma especie.

Transporte de Zn en la planta

El Zn se adquiere del suelo principalmente en su forma catiónica como ión Zn^{2+} libre (Grotz & Guerinot, 2006; Broadley *et al*., 2007) y entra al citoplasma de las células radiculares por medio de transportadores pertenecientes a la familia ZIP (ZRT/IRT-like Protein; Pence *et al*., 2000; Assunção *et al*., 2001; Colangelo & Guerinot, 2006; Palmgren *et al*., 2008). Los transportadores ZIP han sido caracterizados en *Arabidopsis thaliana* (Grotz *et al*., 1998), *Medicago truncatula* (López-Millán *et al.*, 2004), *Glycine max* (Moreau *et al*., 2002) y *Oryza sativa* (Ramesh *et al*., 2003; Ishimaru *et al.,* 2005) y su expresión se induce en situaciones de deficiencia de Zn (Grotz & Guerinot, 2006). Las gramíneas, que exudan fitosideróforos (PS) en respuesta a la deficiencia de Fe (estrategia II), son capaces de usar esta estrategia de quelación para obtener Zn en forma de complejo Zn-PS además de como ión libre, como se demostró en el caso del maíz por von Wirén *et al.* (1996). Las proteínas YSL -Yellow Stripe Like- catalizan la absorción de Zn acomplejado por PS en estas plantas (Schaaf *et al*., 2004, 2005; Suzuki *et al*., 2006; Haydon & Cobbett, 2007). Los transportadores de la familia ZIP y las proteínas YSL se localizan en la membrana plasmática de las células de raíz y hojas y también están implicados en el flujo de Zn y los complejos Zn-PS, respectivamente, por el sistema vascular, el transporte lateral a floema y desde el floema a los tejidos sumidero (Haydon & Cobbett, 2007; Puig *et al.*, 2007; Waters & Grusak, 2008). El Zn también puede entrar en las células a través de canales de Ca^{2+} permeables a Zn^{2+} de la membrana plasmática según estudios de Demidchik *et al*. (2002) en *Arabidopsis thaliana*.

Una vez en el interior de la célula, el transporte del Zn a la vacuola está mediado por las proteínas de tolerancia a metales MTP1 y MTP3, que se inducen en exceso de Zn y se encuentran tanto en *Arabidopsis thaliana* como en las hiperacumuladoras *Arabidopsis halleri* y *Thlaspi goesingense* (Drager *et al.*, 2004; Kim *et al*., 2004; Desbrosses-Fonrouge *et al*., 2005; Arrivault *et al*., 2006), y pertenecen a la familia CDF (Cation Diffusion Facilitator). Haydon & Cobbett (2007) localizaron en

tonoplasto de *A. thaliana* el transportador ZIF1 -Zinc-Induced Facilitator- y sugirieron su implicación en el transporte de Zn al interior de la vacuola. Los transportadores NRAMPs -Natural Resistance-Associated Macrophage Protein- implicados en la removilización de Fe desde la vacuola (Thomine *et al*., 2003) también podrían removilizar Zn (Curie *et al*., 2000; Persans & Salt, 2000; van de Mortel *et al*., 2006; Maestri *et al*., 2010).

En el transporte de Zn a larga distancia, se han encontrado en *Arabidopsis thaliana* miembros de la familia de transportadores de metales pesados P_{1B}-ATPasa, llamados HMA (Heavy Metal Associated) esenciales para la homeostasis de Zn. AtHMA2 y AtHMA4 se expresan en el tejido vascular de raíces, tallos y hojas y se localizan en la membrana plasmática. Están implicados en la descarga de Zn al floema y al xilema y, por tanto, en el transporte desde las raíces a las hojas y viceversa (Williams & Mills, 2005; Sinclair *et al*., 2007; Blindauer & Schmid 2010). En la membrana vacuolar se localiza AtHMA3, que proporciona tolerancia frente a niveles elevados de Zn, Cd, Co y Pb, probablemente mediando el paso de estos metales a la vacuola (Morel *et al*., 2009). AtHMA1 se encuentra en la envoltura del cloroplasto y su ausencia provoca el aumento de Zn en su interior, lo que ha sugerido una función de bombeo de Zn^{2+} fuera del cloroplasto para prevenir efectos tóxicos (Kim *et al.*, 2009). De ese modo, en *A. thaliana* diferentes miembros de la familia P_{1B}-ATPasas son usados para enfrentarse tanto al exceso de Zn (HMA1 y 3) como a su deficiencia (HMA2 y 4). PCR2, una proteína perteneciente a la familia de transportadores denominadas PCR (Plant Cadmium Resistance), tendría una función similiar a HMA2 y HMA4, según Song *et al.* (2010).

En el citoplasma de las células vegetales abundan proteínas capaces de unir Zn, y además el Zn puede estar unido a nicotianamina o ácidos orgánicos, por lo que la concentración del metal libre se supone que es muy reducida (Broadley *et al*., 2007; Krämer *et al*., 2007; Palmgren *et al*., 2008). En el xilema de la hiperacumuladora *T. caerulescens* el Zn se ha encontrado como ión libre en un 80%, estando el resto unido a citrato según Salt *et al*. (1999). Tanto en esta planta como en *A. halleri* más del 80% del Zn de la hoja es soluble en agua o en ácidos débiles (Tolrà *et al.*, 1996; Zhao *et al*., 1998, 2000; Ma *et al*., 2005; Ueno *et al*., 2008). En las vacuolas podría

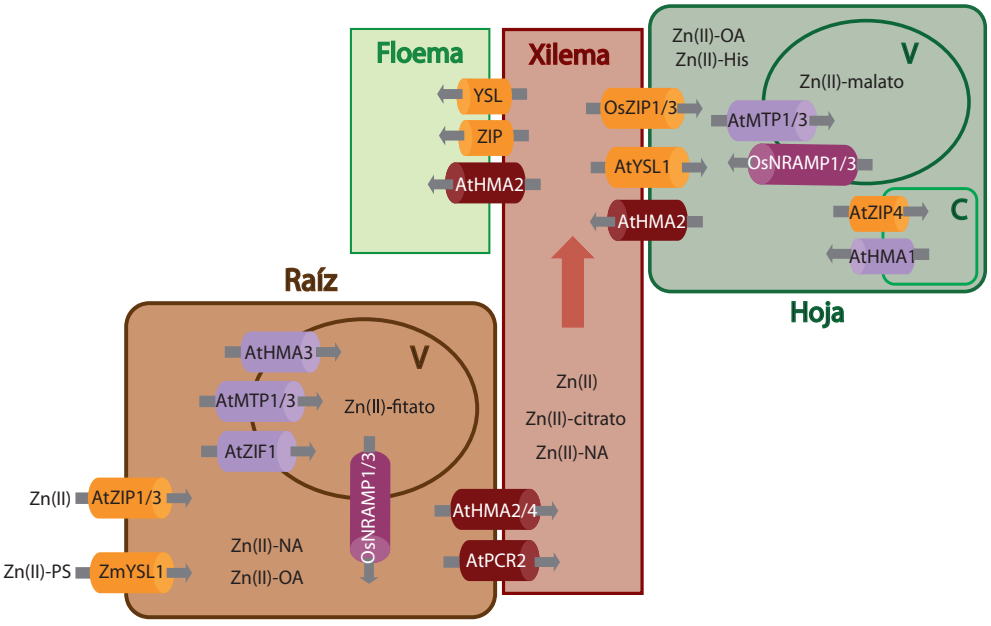

Figura 2. *Adquisición, transporte y almacenamiento de Zn en la planta. Los transportadores y posibles quelatos de Zn se representan en raíz, xilema y hojas.*

estar acomplejado por ácidos orgánicos (Broadley *et al*., 2007) o fitatos (Otegui *et al.*, 2002; Mitsuhashi *et al.*, 2005). En la Figura 2 se resume la información sobre absorción y transporte del Zn detallada en este apartado.

Los genes que codifican muchas de las proteínas responsables de la absorción, acomplejamiento y redistribución en las plantas muestran sobreexpresión en situaciones de deficiencia de Zn. Entre ellos están los genes *ZIPs, HMAs, YSLs, ZIF1, NAS*-nicotianamine synthase-, así como otros genes que codifican enzimas involucradas en la síntesis de fitosideróforos (Pence *et al.*, 2000; Wintz *et al*., 2003; Papoyan & Kochian, 2004; Arrivault *et al*., 2006; Filatov *et al*., 2007; Hammond *et al*., 2006; Talke *et al*., 2006; Suzuki *et al.*, 2006; van de Mortel *et al*., 2006; Haydon & Cobbett, 2007; Krämer *et al*., 2007; Milner & Kochian, 2008). En consonancia con esto, los genes que codifican los transportadores antes mencionados (*ZIPs, MTPs, NRAMPs, HMAs* y *YSL*) muestran en las plantas hiperacumuladoras una alta sobreexpresión constitutiva (Klein *et al*., 2008; Roosens *et al*., 2008).

Efectos del exceso de Zn

La toxicidad de Zn produce síntomas parecidos a las toxicidades de Cd y Pb (Foy *et al.*, 1978; Larbi *et al.*, 2002; Fodor *et al.*, 2005). El primer síntoma que presentan la mayoría de las especies es la clorosis de hojas jóvenes y, en casos graves, zonas necróticas. La raíz principal se acorta, aparecen pequeñas raíces laterales y se observa un amarilleamiento general. No sólo se reduce el crecimiento y cambia la morfología de hojas y raíces dando lugar a plantas raquíticas, sino que también se ha observado que tienen un menor contenido en agua (Horler *et al.*, 1980; Bonnet *et al*., 2000; Broadley *et al*., 2007). El transporte de otros elementos en el xilema se ve alterado y se reduce tanto la transpiración como la fotosíntesis (Robb *et al*., 1980; Schuerger *et al*., 2003; Vaillant *et al*., 2005), posiblemente asociado a una mayor resistencia del mesófilo a la difusión del CO_2 y a una menor conductancia estomática (Van Assche *et al*., 1980; Prasad & Strzalka, 1999). Se ha sugerido que la clorosis de las hojas jóvenes en estas plantas pueda estar causada por deficiencias de Fe o Mg, bien porque el exceso de Zn interfiere con su asimilación o porque el Zn es capaz de desplazar esos iones de los centros activos de ciertas proteínas, quedando estas inactivas (Pilon *et al*., 2009). En el fotosistema II (PSII), el transporte electrónico fotosintético y el desprendimiento de oxígeno quedarían inhibidos, debido a la sustitución competitiva del Mn por Zn en el sitio de la fotólisis del agua (Van Assche & Clijsters, 1986; Ralph & Burchett, 1998). De hecho, hay trabajos que confirman descensos en la eficiencia del PSII y en la fotofosforilación cuando hay un exceso de Zn (Bonnet *et al*., 2000; Schuerger *et al.*, 2003). La alta concentración de Zn también favorece la producción de ROS y la actividad de enzimas como superóxido dismutasa (SOD), ascorbato peroxidasa (APX) y glutatión reductasa (GSR) se ve incrementada (Prasad *et al*., 1999).

Respuestas de la planta al exceso de Zn

La capacidad de las plantas hiperacumuladoras para transportar y almacenar metales en altas concentraciones las ha convertido en un buen modelo de estudio. Sin embargo, muchos de los datos obtenidos en estas especies no son extrapolables a plantas no-hiperacumuladoras. La mayoría de las especies no son hiperacumuladoras y se enfrentan de diferente modo al exceso de metales dependiendo de si son genotipos

tolerantes o sensibles. Muchas especies toleran las elevadas concentraciones de metal en el suelo porque restringen la absorción por la raíz y/o la translocación hacia las hojas, lo que les permite mantener concentraciones relativamente bajas en la biomasa aérea, dentro de un intervalo amplio de concentraciones de metales en el suelo. Las especies sensibles son aquellas que se ven significativamente afectadas por altas concentraciones del metal. Por lo tanto, esta clasificación hace referencia a la eficiencia de uso que hacen del metal y que las lleva a sufrir en menor o mayor medida los efectos de su toxicidad. En el caso del Zn, una vez que ha entrado en la raíz la planta tiene dos alternativas:

1. Inmovilizar el Zn en las raíces. En la raíz se pueden encontrar fracciones de Zn asociadas a las paredes celulares como en *Agrostis tenuis*, en forma de fosfatos como en *Phaseolus vulgaris* (Sarret *et al*., 2001) y como oxalato, como en *Silene vulgaris* (Mathys, 1977). Por otro lado, el almacenamiento de Zn en las vacuolas de células radiculares reduce la disponibilidad del metal y su paso hacia el xilema.
2. Transportar el Zn *via* xilema a la parte aérea. El metal puede inmovilizarse en los tricomas (Clemens & Manceau, 2006; Sarret *et al*., 2006) o en las vacuolas. En el caso de las plantas hiperacumuladoras, pueden transportar a la parte aérea y almacenar en hojas altos niveles de metal, normalmente en células de la epidermis. Además, estas plantas son capaces de mantener una concentración cuatro veces menor en las células del mesófilo, protegiendo el proceso de la fotosíntesis que se lleva a cabo principalmente en estas células (Küpper *et al*., 1999).

El término "hiperacumuladora" fue acuñado para referirse a plantas desarrolladas en campo capaces de acumular >1.000 mg Ni kg^{-1} de materia seca en algún tejido de su biomasa aérea (Brooks *et al*., 1977). De forma general, las hiperacumuladoras alcanzan concentraciones de metales en hojas entre 10 y 100 veces las concentraciones "normales" (Chaney *et al.*, 2000). Actualmente se utiliza el término hiperacumuladora de metales para designar plantas que acumulan >10.000 mg kg^{-1} de Mn y Zn, >1.000 mg kg^{-1} de Co, Cu, Pb, Ni, As y Se y >100 mg kg^{-1} de Cd.

Una especie sensible como *Thlaspi arvense* almacena aproximadamente 2,5 veces

más Zn en las vacuolas de raíz si la comparamos con su equivalente hiperacumuladora *Thlaspi caerulescens*. Ademas, el xilema de *T. caerulescens* contiene unas 5 veces más concentración de Zn que *T. arvense* cuando ambas plantas crecen en la misma concentración del metal (Milner & Kochian, 2008).

Una vez el Zn llega a la parte aérea, es probable que las formas de almacenamiento del metal sean diferentes entre hiperacumuladoras y no hiperacumuladoras. Al contrario que para otros metales tóxicos, las fitoquelatinas (PCs) no parecen jugar un papel importante en la unión a Zn en las hiperacumuladoras ni conferir tolerancia al Zn en general (Küpper *et al*., 2000; Clemens, 2001; Callahan *et al.*, 2006; Wójcik *et al.*, 2006). La relevancia de muchos ácidos orgánicos, aminoácidos, péptidos y proteínas (como metalotioneínas o fitosideróforos derivados de nicotianamina) que podrían unir Zn en las hiperacumuladoras aún no se conoce (Callahan *et al*., 2006). Lo que sí se ha observado es una correlación positiva entre cationes inorgánicos y ácidos carboxílicos (malato, citrato, oxalato) y la abundancia de aminoácidos en el material vegetal analizado (Zhao *et al*., 1998, 2000; Salt *et al*., 1999; Küpper *et al*., 2004).

CADMIO

El Cd es un elemento divalente, con masa atómica 112,41. Su comportamiento químico es parecido al del Zn, si bien mucho más afín al S y más móvil en ambientes ácidos. Es uno de los metales pesados más tóxicos debido a su alta movilidad en el suelo y a los daños que produce en los organismos incluso a bajas concentraciones (Barceló & Poschenrieder, 1990). Debido a su potencial de bioacumulación es uno de los metales contaminantes de mayor importancia (USEPA, 1998). Desde hace décadas se conocen los daños que puede producir su ingestión, y por ejemplo se ha observado que los animales alimentados con plantas contaminadas terminan por desarrollar tanto daños renales y pulmonares (Ryan *et al*., 1982) como ciertos tipos de cáncer (Huff *et al*., 2007).

El Cd no tiene ninguna función biológica conocida pero se acumula de forma activa por las plantas, debido a que en su forma catiónica más habitual (Cd^{2+}) este elemento presenta grandes analogías químicas con otros elementos esenciales. Esta similitud explica por qué su transporte y almacenamiento en el interior de la planta

está íntimamente ligado a proteínas transportadoras específicas de otros cationes como Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+} y Ni^{2+} (Conn & Gilliham, 2010). Una acumulación excesiva de Cd altera la absorción y distribución de otros metales esenciales para la planta por medio de un mecanismo de competición (Ramos *et al*., 2002). De este modo, es responsable de deficiencias y desequilibrios nutricionales, así como de reducir el crecimiento de la planta al alterar sus procesos metabólicos. Es capaz, por ejemplo, de desplazar al Zn de algunos de sus sitios activos o competir con el Ca en ciertos sistemas biológicos. De acuerdo con esto, los posibles efectos tóxicos del Cd se pueden reducir mediante un tratamiento preventivo de los suelos con Zn o con Ca, cuyo caracter protector se basa en la inhibición de la absorción y transporte del Cd por competición (Das *et al.*, 1997; Perfus-Barbeoch *et al*., 2002; He *et al*., 2005).

El Cd en el suelo

La concentración natural de Cd en el suelo es generalmente <1 $mg.kg^{-1}$. En algunos casos, puede existir de forma natural en concentraciones superiores cuando se encuentra asociado a minerales de Zn, o en áreas cercanas a depósitos de Cd. En suelos contaminados, la especie de Cd soluble predominantes es el ion libre Cd^{2+}, y está presente en cantidad creciente donde el pH es mayor que 6,5 (Alloway, 1995). La incorporación natural de Cd al suelo procede, principalmente, de la actividad volcánica, la lixiviación de rocas y los incendios forestales. Las fuentes antropogénicas, por otro lado, son muy variadas. Aunque es un metal relativamente raro, está presente en pequeñas cantidades como impureza en minerales de Zn y por ello aparece como subproducto industrial de la producción de este metal; también existe como residuo en procesos que implican Cu y Pb. En general, se recupera como subproducto de los procesos de fundición y refinamiento de concentrados de Zn en una proporción de 3,0-3,5 $kg.t^{-1}$ de Zn (Adriano, 2001). El Cd también puede sustituir al Ca en la apatita y la calcita, de tal forma que está presente en fertilizantes fosfatados. Además, existen otros procesos antropogénicos que aumentan la contaminación por Cd, entre ellos la incineración de materiales que contienen Cd, los combustibles fósiles, las aguas residuales municipales y las descargas de lodos (Azcúe, 1993; Eisler, 2000; Kirkham, 2006).

Transporte de Cd en la planta

Por ser un metal no esencial se asume que no existen mecanismos de entrada específicos para el Cd. Los estudios moleculares en *Arabidopsis thaliana* y *Oryza sativa* indican que los transportadores de metales esenciales como Fe^{2+} y Zn^{2+} median el paso de Cd^{2+} a las células de raíz, como es el caso de la proteína IRT1, perteneciente a la familia ZIP (Korshunova *et al.*, 1999; Guerinot, 2000; Nakanishi *et al.*, 2006; Morrissey & Guerinot, 2009). También la proteína LCT1 (Low-affinity Cation Transporter) es capaz de transportar no sólo K^+ y Na^+, sino también Ca^{2+} y Cd^{2+} al citosol (Clemens *et al.*, 1998).

Una vez dentro de la célula, el Cd puede coordinarse con ligandos de S como glutatión (GSH) o fitoquelatinas (PCs) y ácidos orgánicos (OA) como el citrato (Domínguez-Solís *et al.*, 2004; Clemens, 2006). Otras posibles moléculas responsables de la quelación del Cd son las metalotioneínas (MTs), también ricas en S. De esta forma los complejos Cd-ligando pueden ser movilizados en el interior de la planta (Shah & Nongkynrih, 2007). Una vez internalizado, la vacuola es el

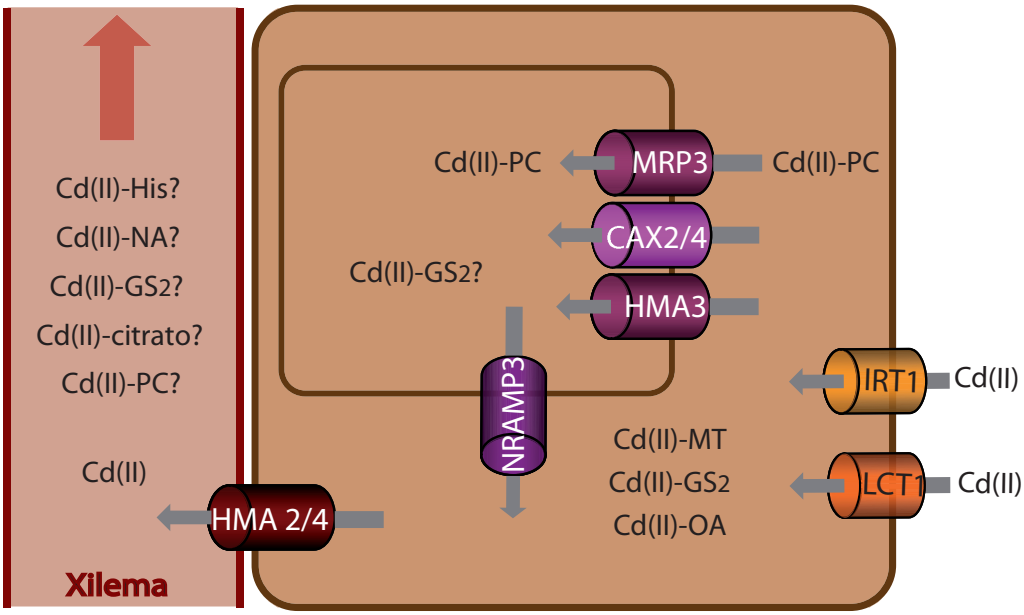

Figura 3. *Representación esquemática de los mecanismos de toma, secuestro y translocación del Cd en raíces.*

principal sitio de acumulación. Los transportadores de cationes CAX2 y CAX4 (de la familia CAX- CAtion eXchangers-), localizados en el tonoplasto e implicados en el transporte de Ca a la vacuola, también transportan otros metales como el Cd (Park *et al.,* 2005; Korenkov *et al*., 2007; Edmond *et al*., 2009; Schneider *et al*., 2009). También HMA3, en la membrana vacuolar de *A. thaliana*, proporciona tolerancia frente a concentraciones elevadas de Cd (Morel *et al*., 2009). La proteína ScYCF1 de *Saccharomyces cerevisiae*, perteneciente a la sub-familia MRP -Multidrug Resistance-associated Protein-, media el transporte de complejos Cd-glutatión del citoplasma a la vacuola (Li *et al.*, 1997) y en *S. pombe* SpHMT1 está implicado en el transporte de complejos Cd-PC (Ortiz *et al*., 1995). El transportador AtMRP3 de *A. thaliana*, homólogo de ambos, está implicado en el transporte y detoxificación de Cd, aunque no se ha podido demostrar que lo transporte unido a un ligando (Bovet *et al*., 2003; Hanikenne *et al*., 2005). En *Arabidopsis*, el transportador de membrana vacuolar AtNRAMP3 transporta Cd y ha sido aislado también en la hiperacumuladora *T. caerulescens* (Maestri *et al.*, 2010). Un estudio reciente en tonoplasto de cebada confirma que proteínas pertenecientes a las familias ABC (transportadores de complejos Cd-PCs) y Nramp se encuentran sobreexpresadas en presencia de 20 μM Cd^{2+} (Schneider *et al*., 2009).

En la Figura 3 se muestra un esquema de la toma del Cd por las células de la raíz, su almacenamiento en vacuola y descarga al xilema. Una vez en el xilema, el metal es transportado a la parte aérea de la planta, concentrándose en orden decreciente en tallos, hojas, frutos y semillas (Clemens *et al*., 2002; Chan & Hale, 2004). El paso de Cd al xilema está mediado por los transportadores HMA2 y HMA4 en *Arabidopsis thaliana* (Verret *et al.*, 2004) y sus homólogos en las especies hiperacumuladoras *Thlaspi caerulescens* y *A. halleri* (Papoyan & Kochian, 2004; Hanikenne *et al*., 2008). No existen estudios definitivos sobre la forma química en que se encuentra el Cd en el xilema. Los análisis de xilema por resonancia magnética nuclear (NMR) de Ueno *et al*. (2008) muestran que la mayoría del Cd se encuentra en forma iónica en la hiperacumuladora *A. halleri*, pero se desconoce qué ocurre con el resto del metal o en otras especies. En un estudio de Senden *et al*. (1994) se encontró que al añadir ácido cítrico a las raíces de plantas de tomate la absorción de Cd^{2+} aumentaba

el doble, y que el transporte desde las raíces a las hojas era entre 6 y 8 veces mayor. Se han propuesto diversos candidatos como transportadores de Cd en el xilema: citrato, histidina, cisteína, glutatión y nicotianamina entre ellos. Salt *et al*. (1995) mostró que el Cd se coordinaba con ligandos de S tipo PCs en raíces y hojas de *B. juncea*, mientras que en el xilema lo hacía con ligandos de O y N. En cambio, Gong *et al.* (2003) encontró evidencias indirectas de que las PCs estaban involucradas en el transporte a larga distancia del metal en *A. thaliana*. En hoja, la distribución de Cd en la hiperacumuladora *T. caerulescens* se da predominantemente en las vacuolas del mesófilo y en las de la epidermis en menor cantidad, mientras que está ausente en el apoplasto, según el microanálisis por rayos X realizado por Wójcik *et al*. (2005). En *A. halleri*, capaz de acumular 1500 $mg.kg^{-1}$ de Cd en hoja, la mayor parte del metal está en la base de los tricomas (Hokura *et al*., 2006) y células del mesófilo (Küpper *et al*., 1999, 2000; Ueno *et al*., 2008). Esta variedad de datos sugieren que las formas químicas en que los metales se acumulan y transportan difieren según la especie vegetal y el metal estudiado (Conn & Gilliham, 2010).

Toxicidad del Cd

Los efectos tóxicos del Cd sobre las plantas han sido ampliamente estudiados (Sanitá di Toppi & Gabbrielli, 1999; Benavides *et al*., 2005). Sin embargo, los mecanismos de su toxicidad aun no se conocen completamente. Se ha comprobado que en general el Cd interfiere en la entrada, transporte y utilización de elementos esenciales y del agua, provocando desequilibrios nutricionales e hídricos en la planta (Poschenrieder *et al*., 1989; Sandalio *et al*., 2001; Singh & Tewari, 2003). Las plantas expuestas a suelos contaminados con Cd presentan modificaciones en la apertura estomática, fotosíntesis y transpiración (Sandalio *et al.,* 2001). Uno de los síntomas más extendidos es la clorosis producida por una deficiencia en Fe (Benavides *et al*., 2005) o Mn (Goldbol & Hutterman, 1985). El tratamiento con Cd produce reducción de la actividad ATPasa de la membrana plasmática (Astolfi *et al*., 2005), alteraciones en la funcionalidad de la membrana plasmática (Fodor *et al*., 1995; Sandalio *et al*., 2001) y desequilibrios en el metabolismo del cloroplasto, inhibiendo la síntesis de clorofila y reduciendo la actividad de enzimas implicadas en la fijación de CO_2 (Ali *et al.,* 2000; Maksymiec *et al*., 2007). Las células del mesófilo

muestran concentraciones de Mg^{2+} más altas de lo normal, lo que se interpreta, según Küpper *et al.* (2002) como un mecanismo de defensa frente a la sustitución del Mg^{2+} por Cd^{2+} en las moléculas de clorofila.

Como ocurre con el Zn^{2+}, el Cd^{2+} no experimenta cambios redox y por lo tanto, a diferencia del Fe^{2+} o Cu^{2+}, no actúa directamente en la generación de ROS. Sin embargo, cierto estrés oxidativo producido por Cd se manifiesta con daños a membranas tales como peroxidación lipídica (Lozano-Rodríguez *et al*., 1997; Sandalio *et al*., 2001; Wua *et al*., 2003; Balestrasse *et al*., 2004), y también se han descrito daños oxidativos a proteínas por formación de grupos carbonilo (Romero-Puertas *et al*., 2002). Las actividades de las enzimas antioxidantes superóxido dismutasa (SOD), glutatión reductasa (GR), ascorbato peroxidasa (APX), peroxidasas (POD) y la catalasa (CAT), encargadas de la defensa celular frente a las ROS, experimentan reducciones o incremento de su actividad en función de la concentración de Cd incluida en el medio de crecimiento, la duración del tratamiento, el tipo de tejido y la especie vegetal de que se trate (Sandalio *et al*., 2001; Benavides *et al*., 2005). Diversos trabajos sobre los mecanismos implicados en la producción de radicales en respuesta al Cd han demostrado la producción indirecta de ROS en distintos compartimentos celulares (membrana plasmática, mitocondrias y peroxisomas) siendo la NADPH oxidasa una de las principales fuentes de generación de estos radicales (Olmos *et al.,* 2003; Romero-Puertas *et al*., 2004; Garnier *et al*., 2006).

Estrategias de tolerancia al Cd

La tolerancia a metales pesados no esenciales viene determinada por la reducción del transporte del mismo al interior de la célula y/o una mayor capacidad para secuestrar estos metales. En el caso del Cd la raíz constituye una de las principales barreras de defensa mediante diferentes mecanismos:

- Reducción del transporte o aumento de la extrusión del Cd por transportadores de cationes de la membrana plasmática (Thomine *et al*., 2000).
- Inmovilización del metal en el apoplasto por unión a los componentes de las paredes celulares, por ejemplo pectinas y carbohidratos extracelulares (Benavides *et al.,* 2005).
- Almacenamiento en vacuolas. Una vez dentro de la célula, el Cd puede

ser secuestrado por ácidos orgánicos, aminoácidos, fitoquelatinas y metalotioneínas, y posteriormente introducido en la vacuola para prevenir su toxicidad.

Las plantas que sobreexpresan la enzima fitoquelatina sintasa muestran una mayor tolerancia frente al Cd (Pomponi *et al.,* 2006). Otras posibles moléculas responsables de la acumulación del Cd son las metalotioneínas (MTs), pequeñas proteínas ricas en cisteína, si bien en las plantas no son las principales responsables de la detoxificación del Cd, como ocurre en células animales (Hamer, 1986).

En resumen, el mayor conocimiento sobre la homeostasis de metales en plantas lo tenemos sobre su absorción en la raíz y su posterior acumulación en vacuolas. Sin embargo, todavía se desconocen los mecanismos que rigen el flujo de metales entre los distintos tejidos, la carga y descarga del xilema y la localización subcelular en orgánulos como cloroplasto y mitocondria. Se han identificado y caracterizado numerosas proteínas transportadoras de micronutrientes (y/o metales pesados) y se sabe que un aspecto importante en el almacenamiento de los metales es la quelación de éstos con ligandos orgánicos, aunque hasta ahora los esfuerzos por identificar claramente ligandos de unión a Zn y Cd no han dado resultados (Ebbs *et al.*, 2002, Hernández-Allica *et al.*, 2006). Actualmente se están realizando estudios de alto rendimiento, de tipo ionómico, transcriptómico y proteómico, tanto en casos de deficiencia como de toxicidad, que permitirán arrojar luz sobre los procesos de homeostasis y tolerancia de metales en plantas (Baxter *et al.*, 2007; Winter *et al*., 2007; Weckwerth *et al*., 2008). En un futuro se espera tener éxito, tanto en las estrategias de biofortificación de especies de interés agronómico en metales esenciales como en las de fitorremediación de suelos contaminados por metales tóxicos que se intentan desarrollar en los últimos años.

Aunque es evidente que las plantas hiperacumuladoras son unos modelos excelentes para el estudio de sistemas extremos en almacenamiento de metales y para una mejor comprensión de la nutrición y homeostasis de micronutrientes y metales pesados no esenciales, la necesidad de trasladar ese conocimiento a especies de interés comercial es la que ha llevado al desarrollo de esta tesis.

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Capítulo 2

Objetivos

El trabajo de investigación desarrollado en esta tesis se ha basado en el estudio de dos especies de interés comercial: el tomate y la remolacha. Las plantas se han cultivado en cámaras climáticas de ambiente controlado en medio hidropónico. Se han estudiado los efectos del exceso de Cd y Zn, añadidos en forma de sales a la solución de crecimiento.

El objetivo general consisitió en un estudio descriptivo y detallado de los cambios fisiológicos inducidos por las toxicidades de Cd y Zn, con el fin de determinar las causas de los mismos, así como los mecanismos de transporte y tolerancia de especies de interés agronómico. Los objetivos concretos se detallan a continuación:

1. Caracterizar los cambios en el crecimiento y composición mineral a consecuencia de la toxicidad de Cd (10 y 100 μM) en plantas de tomate.

2. Caracterizar los cambios en el crecimiento y composición mineral a consecuencia del exceso de Zn (50, 100 y 300 μM) en plantas de remolacha.

3. Estudiar los efectos de la toxicidad de Cd en tomate y el exceso de Zn en remolacha sobre la fotosíntesis, composición pigmentaria y fluorescencia de clorofila.

4. Estudiar las limitaciones en el intercambio gaseoso, fotoquímica y bioquímica del proceso fotosintético a consecuencia del exceso de Zn en plantas de remolacha.

5. Estudiar los cambios en el metabolismo de ácidos orgánicos y la concentración de los mismos en diferentes tejidos de remolacha y tomate crecidos en exceso de Zn y Cd, respectivamente.

6. Caracterizar las concentraciones de Fe, Zn y Cd en el xilema de plantas de remolacha y tomate crecidas en exceso de Zn y Cd, respectivamente.

Capítulo 3

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

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ABSTRACT

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

Keywords: Citrate synthase, cadmium, Krebs cycle, phospho*enol*pyruvate carboxylase, photosynthesis, tomato.

INTRODUCTION

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

Most of the information available about Cd physiology in plants comes from studies with the Cd-hyperaccumulator *Thlapsi caerulescens* (Lombi *et al*., 2002) and Cd-tolerant plants such as *Arabidopsis halleri* (Weber *et al*., 2006; Zhao *et al*., 2006), whereas less information is available in commercial crops such as tomato. It is commonly assumed that Cd, as well as other heavy metals, are taken up by transporters of essential elements, because of the lack of specificity of these proteins. There is evidence that metal transporters from different families such as ZIP and Nramp are able to transport several divalent cations, including Cd (Korshunova *et al*., 1999; Pence *et al*., 2000; Thomine *et al*, 2000). Also, it has been described that a Ca transport pathway could be involved in Cd uptake (Clemens *et al*., 1998; Perfus-Barbeoch *et al*., 2002). Cadmium tolerance in plants is thought to involve internal metal detoxification processes, which may be achieved through both cellular and subcellular compartmentation (Vázquez *et al*., 1992; Küpper *et al*., 2000; Ma *et al*., 2005) and/or complexation with cellular ligands such as phytochelatins, organic

acids, cisteine and other low molecular weight thiols (Cobbett & Goldsbrough, 2002; Schat *et al*., 2002; Küpper *et al*., 2004; Ueno *et al*., 2005; Hernández-Allica *et al*., 2006). Although long distance Cd transport also contributes to Cd distribution and accumulation throughout the plant (Petit & van de Geijn, 1978; Herren & Feller, 1997; Cakmak *et al*., 2000; Chen *et al*., 2007), little is known about the chemical form(s) in which this heavy metal is present in xylem and phloem saps. Data available suggest that Cd may be associated in the xylem sap with small molecules such as organic acids (Cataldo *et al*., 1988; Senden & Wolterbeek, 1990).

Physiological effects of Cd toxicity in plants include inhibition of seed germination, major reductions in growth rates (Huang *et al*, 1974; Lozano-Rodríguez *et al*., 1997; Larbi *et al*., 2002), changes in photosynthetic efficiency, respiration and transpiration (Greger & Ögren, 1991; Krupa *et al*., 1993; Ciscato *et al*., 1999; Larbi *et al*., 2002) and alterations in nutrient homeostasis, including a Cd-induced, Fe deficiency (Wallace *et al*., 1992; Larbi *et al*., 2002) and changes in Mn, K, Mg and Ca uptake rates (Greger *et al*., 1991; Larbi *et al*., 2002; Dong *et al*., 2006). At the cellular level, Cd toxicity is known to cause alterations such as membrane damage, disruption of electron transport, inhibition/activation of enzymes and interaction with nucleic acids (Chaoui *et al*., 1997; Geuns *et al*., 1997; Clijsters *et al*., 1999; Leon *et al*., 2002; Chen *et al*., 2003a). Possible mechanisms by which these disorders are generated are induction of oxidative stress and replacement of elements such as Zn, Fe, and Mn, which are essential cofactors of many enzymes. Accordingly, there are several reports documenting oxidative stress following exposure to high concentrations of Cd (Smeets *et al*., 2005; Dong *et al*., 2006; Lin *et al*., 2007).

The relevance of tomato (*Lycopersicon esculentum*) in human nutrition is increasing, since it is generally considered as a healthy food because of the high content of lycopene and other health promoting natural compounds. Tomato is a constituent of the Mediterranean diet, and for instance in Spain (the first tomato producer in the UE and the 7th in the world; FAOSTAT database, http://faostat.fao.org/) this species ranks as one of the most important vegetables in terms of planting area and production (122.000 ha and 4,500,000 t/year). A large part of this crop is grown in greenhouses, using special substrates and fertilization techniques involving

reutilization of water, therefore implying an increased risk of heavy metal concentration increases (Gil *et al*., 2004). Therefore, there is a need to study the responses of food crops such as tomato to Cd toxicity. Thus, the aim of this work was to investigate the effects of two Cd concentrations, 10 and 100 μM, on the photosynthetic characteristics, growth, nutrient composition and several enzymatic activities involved in citric acid synthesis in this crop species.

MATERIALS AND METHODS

Plant material

Tomato (*L. esculentum* Mill cv. Tres Cantos) plants were grown in a growth chamber with a photosynthetic photon flux density (PPFD) at leaf height of 350 μmol $m^{-2}s^{-2}$ PAR, 80% relative humidity and at a 16 h-23 ºC/8 h-18 ºC, day/night regime. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for an additional two-week period in half-strength Hoagland nutrient solution (Terry, 1980) with 45 μM Fe(III)-EDTA, and then transplanted to 10 L plastic buckets (18 plants per bucket) containing half-strength Hoagland nutrient solution with 45 μM Fe(III)-EDTA and different concentrations of Cd. Treatments used were 0, 10 μM and 100 μM $CdCl_2$. Solutions were changed weekly. Plants were used for measurements 10-12 d after imposing Cd treatments (Picture 1). Young, completely expanded leaves were used for photosynthetic measurements and leaves and root tips were used for enzymatic measurements, except for Fe reductase activity that was measured in whole roots.

Chemical speciation of the nutrient solution

In silico estimations of the concentrations of Cd ionic species in the different nutrient solutions were carried out with MINTEQA2 from Windows (Version 1.50, Allison Geosience Consultants, Flowery Branch, GA, USA and HydroGeoLogic, Inc., Hermdon, VA, USA).

Growth parameters

Plants were divided into three fractions, leaves, stems and roots. Fresh (FW) and dry (DW) weights of each fraction, root to shoot ratios and water content per unit DW were also determined. The experiment was run with 3 different batches of plants, and

Picture 1. *Growth chamber with tomato plants grown in absence (control) and in presence of Cd for 10 days. From left to right: 0, 10 and 100 µM Cd.*

10 samples per treatment were taken for analysis in each experiment.

Analysis of Cd and mineral nutrient concentrations

All plant tissues were washed with distilled water. Samples were dried in an oven at 60 ºC for 72 h until constant weight. For Cd analysis, samples were digested with nitric acid and hydrogen peroxide (0.1 g in 8 mL HNO_3 and 2 mL H_2O_2) in a microwave system (Ethos Plus, Milestone, Bergamo, Italy). The digested material was diluted to 25 mL in MilliQ-water and mineral elemental analysis was carried out by optical inductively coupled plasma (ICP) spectrometry (ICP-OES spectrometer, IRIS Intrepid II XDL, Thermo Electron Corp., Franklin, MA, USA), equipped with a 2000 W RF generator and full wavelength coverage. For analysis of nutrients, samples were dry-ashed and dissolved in HNO_3 and HCl following the A.O.A.C. procedure (Association of Official Analytical Chemists, Washington DC, USA). Calcium (after La addition), Mg, Fe, Mn, Cu and Zn were determined by FAAS, K by FES and P spectrophotometrically by the molybdate-vanadate method (Igartua *et al*., 2000). Nitrogen was determined with an NA2100 Nitrogen Analyser (ThermoQuest, Milan, Italy). The experiment was run with 3 different batches of plants and 5 samples per treatment were taken for analysis in each experiment.

Enzyme activities

Extracts for measuring enzyme activities were made by grinding approximately 100 mg FW of root tip material (or 3 leaf disks, 0.96 cm^2 each) in a mortar with 1 mL of extraction buffer containing 30 mM sorbitol, 1% BSA and 1% PVP in 100 mM HEPES-KOH, pH 8.0. The slurry was centrifuged for 15 min at 10,000*g* and 4 ºC, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 1 mL (final volume) of the corresponding reaction buffer. Malate dehydrogenase (MDH, l-malate: NAD-oxidoreductase; EC 1.1.1.37) activity was determined as described in Dannel *et al*. (1995). Citrate synthase (CS, citrate (Si)-synthase; EC 2.3.3.1) was assayed spectrophotometrically according to Srere (1967) by monitoring the reduction of acetyl CoA to CoA with 5-5´-dithio-bis-2-nitrobenzoic (DTNB) acid at 412 nm. Isocitrate dehydrogenase (ICDH, isocitrate dehydrogenase ($NADP^+$); EC 1.1.1.42) and fumarase (fumarate hydratase; EC 4.2.1.2) activities were determined as described in Bergmeyer *et al*. (1974). Phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) activity was measured in a coupled enzymatic assay with MDH with 75 µL of extract in 2 mM phospho*enol*pyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH, and 100 mM Bicine-HCl, pH 8.5 (Vance *et al*., 1983). The experiment was run with 4 different batches of plants, and 3 plants per treatment were taken for activity measurements.

Root iron reductase activity measurements

Iron reductase activity was measured in whole root systems by following the formation of the Fe(II)-$BPDS_3$ complex from Fe(III)-EDTA (Zouari *et al*., 2001). Root Fe reductase activity was determined in intact plants after 2-3 h of light onset, 10 days after the Cd treatments were imposed. Plants were placed in 200 mL of a solution containing 1 mM MES, pH 5.5, 100 mM BPDS and 100 mM Fe(III)-EDTA in MilliQ water. The reaction was stopped at 30 min by removing the plant from the container, and absorbance readings of the assay solution at 535 nm were taken after centrifugation. Controls were also carried out in the absence of plants to correct for non-enzymatic Fe reduction. The experiment was run with 3 different batches of plants and 3 plants per treatment were taken for activity measurements.

Photosynthetic pigment analysis

Photosynthetic pigments were extracted with 100% acetone in the presence of Na ascorbate, and extracts were analysed spectrophotometrically. Photosynthetic pigments were also quantified by HPLC (Larbi *et al*., 2004). The experiment was replicated 3 times and 5 plants per treatment, illuminated for 3-4 h, were taken for pigment quantifications.

Gas exchange measurements

Measurements were made on attached leaves in the growth chamber with a portable gas exchange system (CIRAS-1; PP Systems, Hertfordshire, UK), using a PLC broad leaf cuvette in closed circuit mode. Transpiration rate (E), stomatal conductance (g_s), net photosynthetic rate (P_N) and sub-stomatal CO_2 concentration (C_i) were recorded during measurements. Experiments were made at ambient CO_2 concentration, 130-170 μmol $m^{-2}s^{-1}$ PPFD, and at the temperature and relative humidity prevailing in the growth chamber. All measurements were taken in leaves illuminated for 3-4 h. The experiment was run with 3 different batches of plants, and 7 plants per treatment were used for the measurements.

Modulated chlorophyll fluorescence analysis

Modulated Chl fluorescence measurements were made in attached leaves in the growth chamber with a PAM 2000 apparatus (H. Walz, Effeltrich, Germany). F_o (minimal Chl fluorescence yield in the dark) was measured by switching on the modulated light at 0.6 kHz; PPFD was below 0.1 μmol photon $m^{-2}s^{-1}$ at leaf surface. F_m (maximal Chl fluorescence yield in the dark) was measured at 20 kHz with a 1 s pulse of 6,000 μmol photons $m^{-2}s^{-1}$ of white light. The experimental protocol for the analysis of the Chl fluorescence quenching was as in Morales *et al*., (2000) and references therein. F_o and F'_o (minimal Chl fluorescence yield after light adaptation) were measured in presence of far red (FR) light (7 μmol photon $m^{-2}s^{-1}$) to fully oxidize the PSII acceptor side (Belkhodja *et al*., 1998; Morales *et al*., 1998; Logan *et al*., 2007). Dark-adapted, maximum potential PSII efficiency was calculated as F_v/F_m, where F_v is F_m -F_o (Morales *et al*., 1991; Abadía *et al*., 1999). Actual (Φ_{PSII}) and intrinsic (Φ_{exc}) PSII efficiency were calculated as $(F'_m- F_s)/F'_m$ and F'_v /F'_m, respectively. Photochemical quenching (qP) was calculated as $(F'_m- F_s)/F_v$. Non-

photochemical quenching (NPQ) was calculated as (F_m/F'_m) - 1. Experiments were carried out at ambient CO_2 concentration, 200-250 μmol $m^{-2}s^{-1}$ PPFD, and at the temperature and relative humidity prevailing in the growth chamber. All measurements were taken in leaves illuminated for 3-4 h. The experiment was replicated 3 times and 10 measurements were made per treatment.

RESULTS

In silico chemical speciation

In the Cd treatments, the major Cd chemical species predicted to occur in the nutrient solution was free Cd^{2+}, accounting for 87.8 and 89.8% of total Cd in the 10 and 100 μM treatments, respectively. Approximately 6.3% of total Cd was predicted to occur in the form of $CdSO_4^0$ in both treatments. Also, 4.8% and 1.7% of total Cd were predicted to occur as $Cd[EDTA]^{2-}$ in the 10 and 100 μM treatments, respectively. The species $CdCl^+$ was predicted to occur (1.1% of total Cd) only in the 10 μM Cd treatment.

Effects of Cd on growth

In the 10 μM Cd treatment the average root mass was larger than that found in the controls and the opposite occurred for leaves and stems (Picture 2, up), although changes were statistically significant only for leaves and stems (Fig. 1). In the 100 μM Cd treatment, however, both FW and DW in all fractions decreased significantly compared to the control values, and decreases were larger for shoot parts, leaf and stem, than for roots (Fig. 1). Also, both Cd concentrations increased the root/shoot ratio (data not shown). Water content was not changed with Cd treatments (Fig. 1).

Cd concentrations

Cadmium concentrations increased significantly in all plant parts when increasing Cd in the nutrient solution. Cadmium concentrations in control plants were 0.67 ± 0.46, 0.12 ± 0.04 and 0.28 ± 0.24 μg gDW^{-1} in roots, stems and leaves, respectively. With 10 μM Cd, Cd concentrations were 1607 ± 679, 152 ± 137 and 184 ± 54 μg gDW^{-1} in roots, stems and leaves, whereas with 100 μM Cd concentrations were much higher (4731 ± 1323, 1370 ± 338 and 1075 ± 358 μg gDW^{-1} in roots, stems and leaves, respectively). Total Cd amounts extracted per plant were 537 μg in the

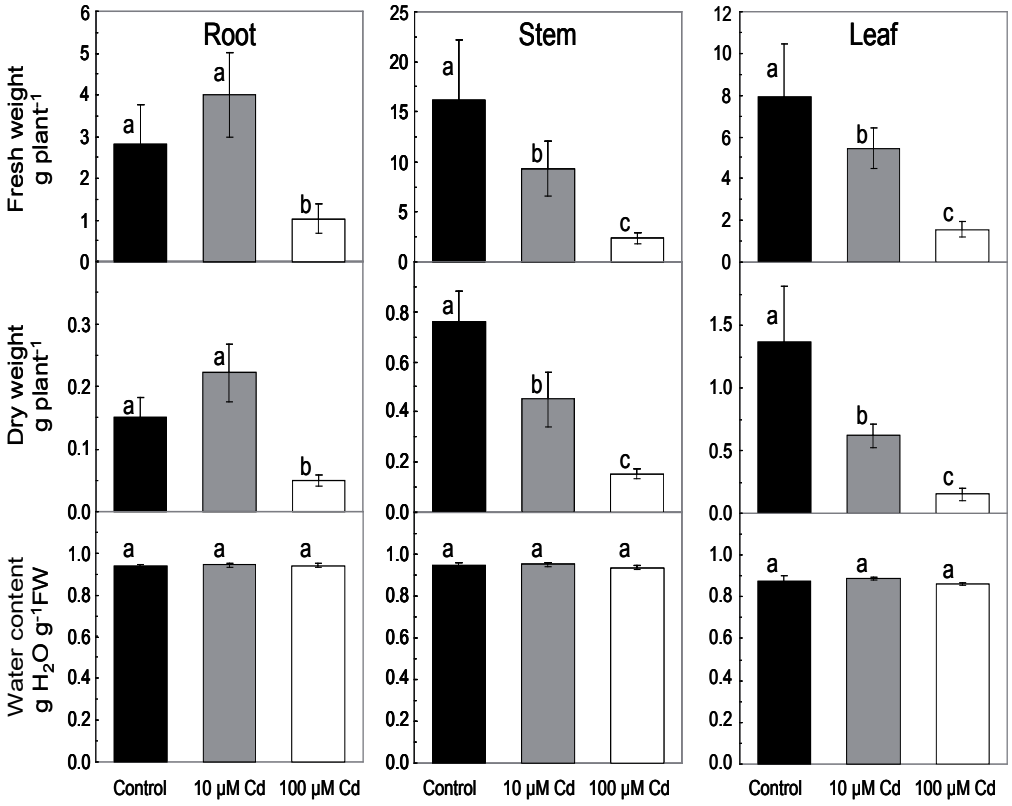

Fig. 1. *Fresh and dry masses (in g per plant) and water content (in g g^{-1} FW) of roots, stems and leaves of tomato plants grown in absence (control) and in presence of Cd (10 and 100 µM Cd). Data are means ± SD of 30 replications (3 batches of plants with 10 replicates per treatment). Columns marked with the same letter are not significantly different (Student's test) at the p < 0.05 level.*

10 µM Cd treatment (354, 69 and 114 µg in roots,stems and leaves, respectively) and 604 µg in the 100 µM Cd treatment (237, 205 and 161 µg in roots, stems and leaves, respectively). Therefore, Cd allocation (in percentage of total Cd, for root/stem/leaf) was 66/13/21 and 39/34/27 in the 10 and 100 µM Cd treatments, respectively.

Picture 2. *Reduction of leaf growth in plants grown with 0, 10 and 100 µM Cd for 10 days (upper panel, from left to right). Detail from leaves grown at 10 µM Cd; young leaves have chlorotic symptoms and older leaves presented necrotic parts (lower panel).*

Effects of Cd on plant mineral concentrations

Cd toxicity altered plant concentrations of several macroelements. Cd toxicity increased root concentrations of Mg only in plants grown with 10 μM Cd, whereas N concentrations decreased progressively with excess Cd in stems and leaves (12% and 25% in plants grown with 10 and 100 μM Cd, respectively, although changes were statistically significant only with 100 μM Cd) (Fig. 2). Potassium concentrations in roots decreased by 25% with both Cd treatments, whereas in leaves only the highest Cd treatment led to a significant 25% decrease (Fig. 2). Calcium and P concentrations did not show significant changes in any plant part with Cd treatments (Fig. 2).

Micronutrient concentrations were also affected by Cd in the nutrient solution (Fig. 3). Iron concentrations increased 2-fold in both roots and stems in plants grown with 100 μM Cd, whereas in leaves it did not change. No significant differences in Fe concentrations were measured in the 10 μM Cd treatment in roots and stems, but leaf Fe concentration decreased by 50% when compared to controls (Fig. 3). Zinc concentrations also increased (2-fold) in roots of plants grown with 100 μM Cd, but no significant differences were observed in stems and leaves in any of the treatments (Fig. 3). Copper concentrations in roots increased by 50% only with 100 μM Cd supply, whereas in leaves and stems it decreased by 40 and 50%, respectively (Fig. 3). In contrast, Mn concentrations decreased markedly in roots with both Cd treatments, whereas in stems and leaves it did not change significantly (Fig. 3), except for leaves at 10 μM Cd, where there was a slight but significant increase.

Effects of Cd on enzyme activities

The activities of five enzymes involved in organic acid metabolism in root tip and leaf extracts of tomato plants were measured. In root tip extracts, the activities of ICDH, CS and fumarase increased progressively as Cd concentration in the nutrient solution increased (Table 1). Activities of PEPC and MDH increased 2.7-fold and 1.4-fold respectively, in the 10 μM Cd treatment and no further increases were measured in the 100 μM Cd treatment when compared to controls (Table 1). Leaf extracts from plants grown with 100 μM Cd showed significant increases in the activities of ICDH (4.4-fold), CS (3.7-fold) and MDH (1.7-fold) when compared to activities measured in control leaves (Table 1).

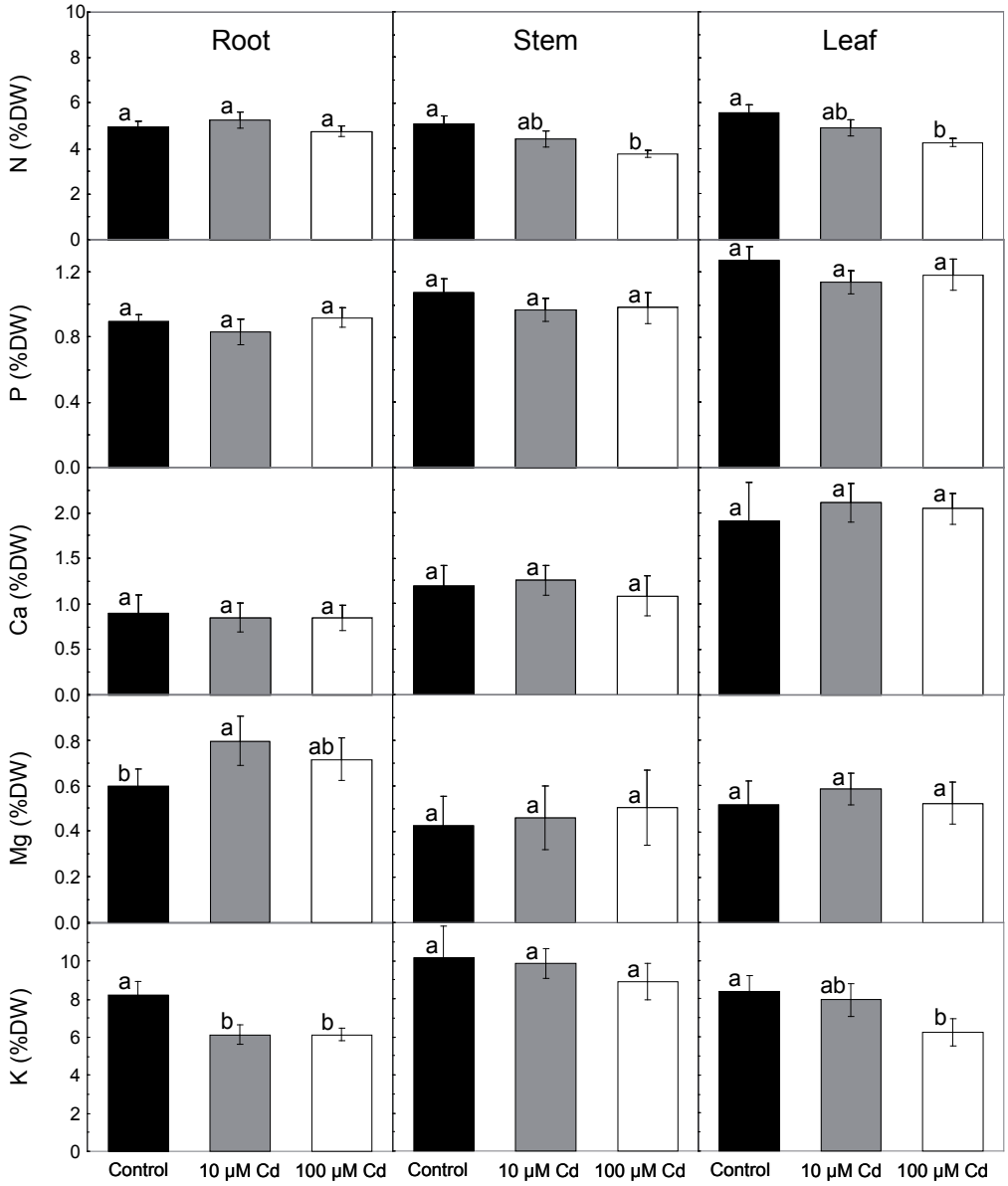

Fig. 2. *Macronutrient concentrations (in %DW) in roots, stems and leaves of tomato plants grown in absence (control) and in presence of Cd (10 and 100 µM Cd). Data are means ± SD of 15 replications (3 batches of plants with 5 replicates per treatment). Columns marked with the same letter are not significantly different (Student's test) at the $p < 0.05$ level.*

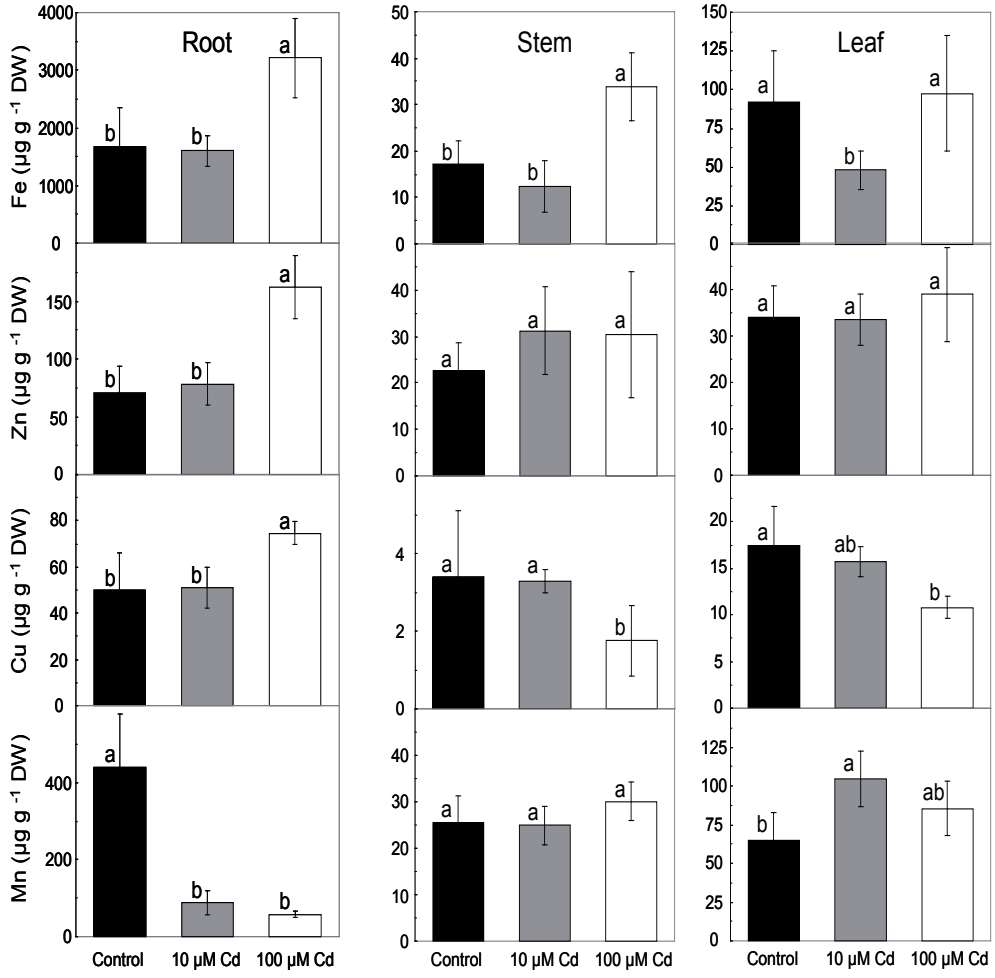

Fig. 3. *Micronutrient concentrations (in µg.g^{-1} DW) in roots, stems and leaves of tomato plants grown in absence (control) and in presence of Cd (10 and 100 µM Cd). Data are means ± SD of 15 replications (3 batches of plants with 5 replicates per treatment). Columns marked with the same letter are not significantly different (Student's test) at the p < 0.05 level.*

The activity of fumarase in extracts from the same leaves decreased by 50% when compared to controls, whereas the activity of PEPC did not change (Table 1). In the 10 µM Cd treatment, changes were not significant except for small increases in CS and MDH (Table 1).

Table 1. *Enzymatic activities in extracts of leaves and root tips (in µmol substrate g^{-1} FW min^{-1}) of control and Cd-treated (10 and 100 µM Cd) tomato plants. Data are means ± SE of 12 replications (4 batches of plants with 3 replicates per treatment). Data followed by the same letter within the same column are not significantly different (Student's test) at the $p < 0.05$ level.*

Leaves	ICDH	CS	Fumarase	MDH	PEPC
Control	0.20 ± 0.02 a	0.18 ± 0.01 a	315.8 ± 13.4 b	27.38 ± 0.81 a	0.95 ± 0.19 a
10 µM Cd	0.22 ± 0.08 a	0.25 ± 0.05 b	383.3 ± 61.5 b	36.24 ± 7.16 b	1.11 ± 0.26 a
100 µM Cd	0.88 ± 0.06 b	0.66 ± 0.21 c	142.5 ± 20.5 a	45.73 ± 0.81 c	1.15 ± 0.17 a

Roots	ICDH	CS	Fumarase	MDH	PEPC
Control	0.33 ± 0.03 a	0.05 ± 0.01 a	310 ± 85 a	13.07 ± 2.29 a	0.31 ± 0.10 a
10 µM Cd	0.59 ± 0.08 b	0.23 ± 0.04 b	763 ± 209 b	18.58 ± 1.33 b	0.85 ± 0.09 b
100 µM Cd	0.93 ± 0.11 c	0.42 ± 0.02 c	895 ± 375 b	19.61 ± 2.07 b	0.84 ± 0.09 b

Effects of Cd on root iron reductase activity

Roots of tomato became brownish when grown with Cd in the nutrient solution. Whole root Fe reductase activities decreased when Cd concentration increased in the nutrient solution. Iron reductase activity in control plants was 0.29 ± 0.06 µmol Fe reduced g^{-1} FW h^{-1}. Activities decreased by 38% (to 0.18 ± 0.03 µmol Fe reduced g^{-1} FW h^{-1}) and 62% (to 0.11 ± 0.03 µmol Fe reduced g^{-1} FW h^{-1}) in the 10 and 100 µM Cd treatments, respectively.

Effects of Cd on photosynthetic pigment composition

In the 10 μM Cd treatment leaf chlorosis was observed (Picture 2, down), and accordingly concentrations of all major photosynthetic pigments on a leaf area basis, except for β-carotene, were decreased when compared to those found in control plants (Fig. 4). In leaves of plants grown with 100 μM Cd, no marked chlorosis was observed but leaf size was reduced, moderate decreases in the concentrations of photosynthetic pigments (excepting β-carotene) were found and necrotic spots in the leaf blade were apparent. Leaf pigment decreases were (for the 10/100 μM Cd treatments) 50/44% for neoxanthin, 42/20% for lutein, 39/18% for violaxanthin, 40-35% for Chl a and 46-23% for Chl b. As a result of these changes, the average Chl a/b ratio increased in leaves of tomato plants treated with 10 μM Cd to 3.3 from the control values of 3.0, and decreased in those treated with 100 μM Cd to a value of 2.8, although these changes were not statistically significant at $p < 0.05$. The violaxanthin/Chl ratio increased in plants treated with 10 and 100 μM Cd (data not shown). Antheraxanthin and zeaxanthin were not detected in any of the treatments.

Effects of Cd on gas exchange parameters

Cd treatments reduced P_N and g_s when compared to control plants. Reductions in P_N were 31 and 73% in the 10 and 100 μM treatments, respectively, and reductions in g_s were 17 and 62%, respectively (Fig. 5). When compared to values found in control plants, transpiration rate increased in the 10 μM $CdCl_2$ treatment and decreased by 44% in the 100 μM $CdCl_2$ treatment (Fig. 5). C_i values did not change with Cd treatments (Fig. 5).

Effects of Cd on chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters including F_v/F_m ratios, actual PSII efficiency (Φ_{PSII}), intrinsic PSII efficiency (Φ_{exc}) and photochemical quenching (qP) did not change significantly in plants grown with 10 or 100 μM $CdCl_2$ when compared to control values (Table 2). Non-photochemical quenching increased with Cd in the nutrient solution, although this increase was only significant in the 100 μM Cd treatment (Table 2).

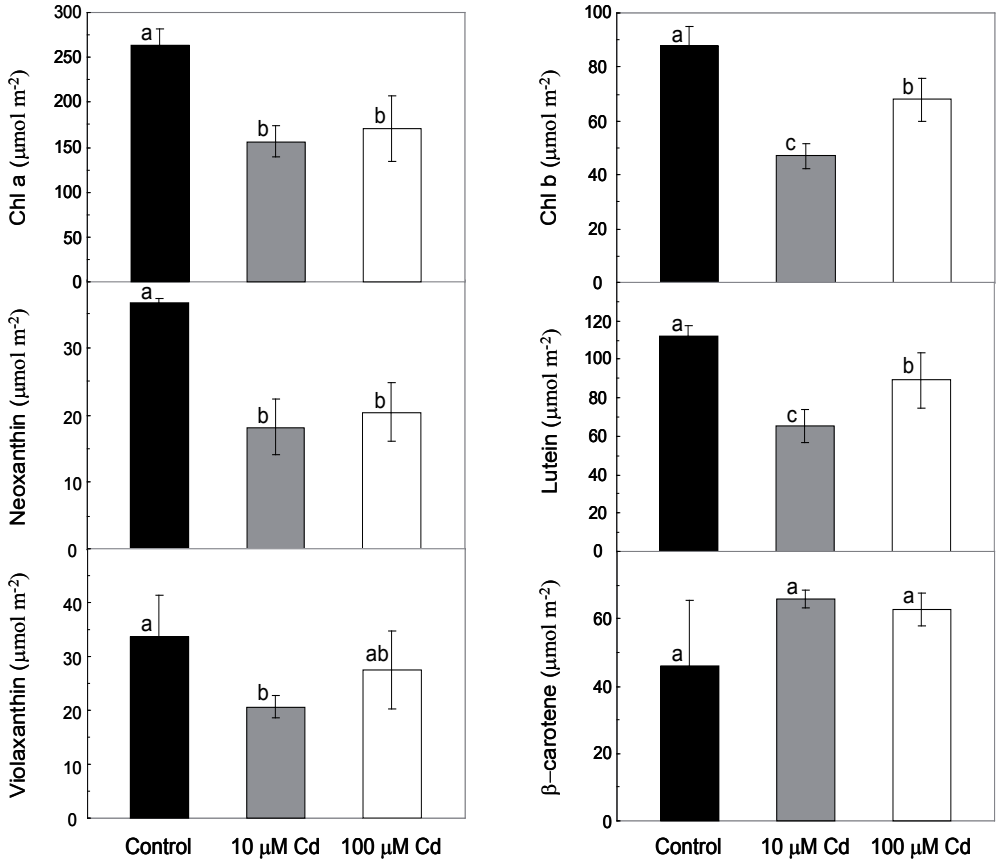

Fig. 4. *Leaf concentrations of photosynthetic pigments (carotenoids and chlorophylls, in µmol m^{-2}) in tomato plants grown in absence (control) and in presence of Cd (10 and 100 µM Cd). Data are means ± SD of 15 replications (3 batches of plants with 5 replicates per treatment). Columns marked with the same letter are not significantly different (Student's test) at the $p < 0.05$ level.*

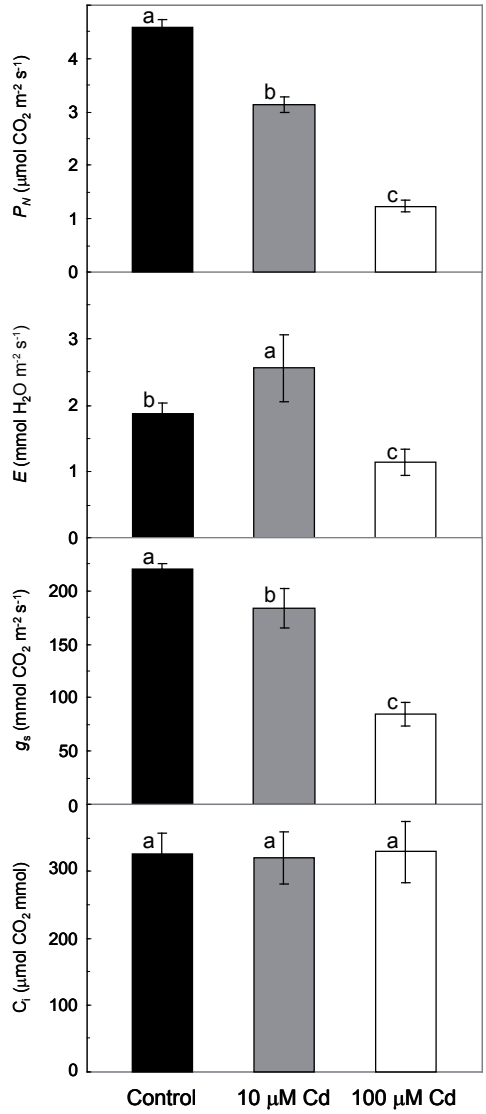

Fig. 5. *Gas exchange parameters in leaves of tomato plants grown in absence (control) and in presence of Cd (10 and 100 µM Cd). The incident PPFD was between 130 and 170 µmol $m^{-2}s^{-1}$. Data are means ± SD of 21 replications (3 batches of plants with 7 replicates per treatment). Columns marked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

Table 2. *Modulated chlorophyll fluorescence parameters in control and Cd-treated (10 and 100 μM Cd) tomato plants. The incident PPFD was between 130 and 170 μmol m^{-2} s^{-1}. Data are means ± SD of 30 replications (3 batches of plants with 10 replicates per treatment). Data followed by the same letter within the same column are not significantly different (Student's test) at the $p < 0.05$ level.*

	F_v/F_m	Φ_{PSII}	Φ_{exc}	qP	NPQ
Control	0.81 ± 0.01 a	0.66 ± 0.05 a	0.68 ± 0.04 a	0.98 ± 0.01 a	0.21 ± 0.01 a
10 μM Cd	0.81 ± 0.01 a	0.62 ± 0.05 a	0.65 ± 0.04 a	0.96 ± 0.02 a	0.25 ± 0.04 ab
100 μM Cd	0.79 ± 0.02 a	0.60 ± 0.08 a	0.62 ± 0.08 a	0.96 ± 0.02 a	0.34 ± 0.10 b

DISCUSSION

Cadmium treatment led to major effects in shoots and roots of tomato. Plant growth was reduced in both treatments, leaves showed chlorosis symptoms when grown at 10 μM Cd and necrotic spots when grown at 100 μM Cd, and root browning was also observed in both treatments. An increase in the activity of PEPC, involved in anaplerotic fixation of CO_2 into organic acids, was measured in roots of Cd exposed plants. Also, significant increases in the activities of several enzymes from the Krebs cycle were measured in roots of tomato plants grown with Cd. Cadmium excess caused several alterations on photosynthetic rates, photosynthetic pigments and chlorophyll fluorescence as well as in nutrient homeostasis.

Cadmium allocation in tomato plants depended on the Cd concentration in the nutrient solution. In the 10 μM Cd treatment, Cd^{2+} concentration in the nutrient solution was approximately 9 μM and the total amount of Cd extracted per plant was 537 μg. In the much stronger 100 μM Cd treatment, Cd^{2+} concentration in the nutrient solution was approximately 90 μM but the total amount of Cd extracted by plants was only 12% larger than that found with the 10 μM Cd treatment (604 μg per plant). However, Cd allocation was very different in the two treatments, and

a major increase in the metal allocated in shoots (from 34% to 61%) was found when Cd in the nutrient solution increased from 10 to 100 μM Cd. Conversely, the metal allocated in roots was 66% in the 10 μM Cd treatment and only 39% in the 100 μM treatment. In control plants less than 1 μg of Cd was taken up, most likely from Cd present in the salts used to build the nutrient solution, and the distribution of this metal was 17, 16 and 67% in roots, stems and leaves, respectively (results not shown). These data indicate that, as it occurs with other plant species, the Cd detoxification strategy in tomato plants grown with low Cd concentrations relies on accumulating Cd at the root level. At higher Cd concentrations, however, roots are overloaded with Cd and a significant mobilization to the shoot occurs.

The fact that total Cd uptake was only slightly larger in the 100 μM Cd treatment (10.8 mg Cd with 18 plants) when compared to that found in the 10 μM Cd (9.7 mg Cd with 18 plants) deserves further investigation. High Cd concentrations in the nutrient solution may lead to a large Cd uptake by the plant in the short-term subsequently impairing further metal uptake. Several explanations such as root Cd re-extrusion to the medium in the 100 μM Cd treatment are possible; however further studies including total Cd uptake data in the midst of the treatment period would be necessary to prove any possible hypotheses.

Cadmium supply increased root activities of PEPC and several enzymes from the Krebs cycle, including MDH, CS, fumarase and ICDH, with CS showing the largest increases (4.6- and 8.4-fold in the 10 and 100 μM Cd treatments, respectively). A coordinated increase in the root activities of PEPC, MDH and CS could cause carboxylation of PEP to OAA, and subsequently lead to malate and citrate, thus acting as an anaplerotic mechanism for CO_2 fixation. The ability to take up Cd in different species has been correlated to differences in the capacity of roots to secrete citric acid (Chen *et al*., 2003b; Duarte *et al*., 2007; Liu *et al*., 2007). Organic acid excretion by roots has also been reported in several nutritional stresses such as P and Fe deficiency, as well as in Al toxicity (Jones, 1998; Abadía *et al*., 2002; Liao *et al*., 2006; Kobayashi *et al*., 2007), and similar increases in the activities of PEPC, MDH and CS have been measured in P and Fe-deficient roots (Johnson *et al*., 1994; López-Millán *et al*., 2000b).

In tomato leaves PEPC activity did not increase with Cd supply in any of the treatments, suggesting that anaplerotic fixation of CO_2 is restricted to roots. Although significant increases in leaf activities of CS, MDH and ICDH were measured in the 100 μM Cd supply treatment, fumarase activity decreased. In the 10 μM Cd treatment however, leaf fumarase activity did not decrease and increases in CS, MDH and ICDH were less marked. Fumarase is unique to the mithochondrion and therefore is a convenient marker for the mitochondrial matrix (Siedow and Day, 2000). These results suggest that at high Cd supply leaf mitochondrial activity could be impaired and increases in CS, MDH and ICDH leaf activities may reflect cytoplasmic changes trying to cope with the reduction in citric acid cycle activities. An increase in ICDH has also been reported in leaves of *Phaseolus vulgaris* after Cd application (Smeets *et al.*, 2005). An increased activity of enzymes such as MDH and ICDH can also generate reducing power, which may play a role in redox mechanisms in plant cells. It has been widely described that Cd exposure causes oxidative stress, and accordingly several enzymes and metabolites involved in the plant defence mechanisms against oxidative stress are elicited by Cd (Lozano-Rodríguez *et al*., 1997; Smeets *et al*., 2005; Dong *et al*., 2006; Lin *et al*., 2007).

Cadmium treatments led to progressive decreases in net CO_2 uptake (P_N). Cadmium induced decreases in the leaf concentrations of chlorophyll and other photosynthetic pigment concentrations, and the effect was more marked in the 10 μM Cd treatment than in the 100 μM one. A similar effect was observed in sugar beet, and attributed to a Cd-induced Fe deficiency at low Cd treatments, where Cd effects on growth were still moderate (Larbi *et al*., 2002). At higher Cd levels, effects on growth were so strong that the requirement for Fe was much lower and Fe-deficiency symptoms were no longer evident (Larbi *et al*., 2002). No de-epoxidation of xanthophyll cycle pigments occurred even when mild leaf chlorosis occurred at 10 μM Cd. The same was observed in sugar beet grown at 50 μM Cd, but not at 10 μM Cd, since at low Cd zeaxanthin and antheraxantin were formed (Larbi *et al*., 2002). The lack of major effects of Cd on leaf electron transport rates, NPQ and xanthophyll cycle pigments de-epoxidation may support that Cd-treated tomato plants were not affected by photo-inhibitory processes. However, the possibility that the xanthophyll cycle pigments

photoprotection mechanism could be inactivated by Cd metal toxicity in tomato (and sugar beet) leaves cannot be ruled out at this stage.

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

Other alterations in micronutrient concentrations were observed in tomato plants grown with Cd. Major changes included increases in root Fe, Zn and Cu concentrations when plants were grown with 100 μM Cd and a progressive decrease in Mn concentration with increased Cd supply. Synergistic effects between high Cd concentrations and Fe, Cu and Zn root accumulation have been described before for other species (Larbi *et al.*, 2002; Liu *et al*., 2003) and in agreement with our data no such changes were observed in tomato roots with low Cd supply (Dong *et al*., 2006). It has been hypothesized that increases in root concentrations of divalent metals could be partially explained by Cd interference in nutrient uptake by affecting the permeability of plasma membranes (Dong *et al*., 2006). Also, the lack of specificity of members of several families of divalent metal transporters such as ZIP and Nramp could contribute to this fact. For instance, a member of the ZIP family, ZIP9, is induced in *Arabidopsis thaliana* roots in presence of Cd and is constitutively highly expressed in the roots of the accumulator *A. halleri* (Weber *et al*., 2006). Antagonistic effects between high Cd concentrations and Mn uptake and transport have been reported in many studies (Hernández *et al*., 1998; Larbi *et al*., 2002; Dong *et al*., 2006; Wu *et al*., 2007), and a reduction in Cd uptake in presence of Mn has been observed

(Cataldo *et al*., 1981). Reports are contradictory regarding the influence of Cd in shoot micronutrient concentrations; for instance $CdCl_2$ decreased Zn and Cu shoot concentrations in sugar beet (Larbi *et al*., 2002) whereas in tomato no differences were found (Dong *et al*., 2006, and this work). Differences among species may arise from the different micronutrient homeostasis and Cd detoxification mechanisms; for instance, at low Cd sugar beet is able to mobilize more Cd to shoots than tomato (up to 50 and 34% of total Cd, respectively).

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

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Capítulo 4

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

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ABSTRACT

The effects of high Zn concentration were investigated in sugar beet (*Beta vulgaris* L.) plants grown in a controlled environment in hydroponics. High concentrations of Zn sulphate in the nutrient solution (50, 100 and 300 μM) decreased root and shoot fresh and dry mass, and increased root/shoot ratios, when compared to control conditions (1.2 μM Zn). Plants grown with excess Zn had inward-rolled leaf edges and a damaged and brownish root system, with short lateral roots. High Zn decreased N, Mg, K and Mn concentrations in all plant parts, whereas P and Ca concentrations increased, but only in shoots. Leaves of plants treated with 50 and 100 μM Zn developed symptoms of Fe deficiency, including decreases in Fe, chlorophyll and carotenoid concentrations, increases in carotenoid/chlorophyll and chlorophyll a/b ratios and de-epoxidation of violaxanthin cycle pigments. Plants grown with 300 μM Zn had decreased photosystem II efficiency and further growth decreases but did not have leaf Fe deficiency symptoms. Leaf Zn concentrations of plants grown with excess Zn were high but fairly constant (230.260 $\mu g \cdot g^{-1}$ dry weight), whereas total Zn uptake per plant decreased markedly with high Zn supply. These data indicate that sugar beet could be a good model to investigate Zn homeostasis mechanisms in plants, but is not an efficient species for Zn phytoremediation.

Keywords: Heavy metal toxicity, iron deficiency, sugar beet, Zn toxicity.

INTRODUCTION

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

The mechanisms controlling Zn homeostasis in plants are still not fully known (Hacisalihoglu *et al*., 2004; Broadley *et al*., 2007; Krämer *et al*., 2007). Plant roots acquire Zn predominantly as the divalent ion, and the metal is then distributed throughout the whole plant in a complex series of processes. Several families of plant metal transporters have been identified in recent years (Krämer *et al*., 2007), with at least three being involved in Zn transport through membranes: ZIP (IRT-like proteins) (Grotz *et al*., 1998; Wintz *et al.,* 2003), CDF (Cation Diffusion Facilitator proteins) (Blaudez *et al*., 2003; Kim *et al*., 2004; Kobae *et al.,* 2004; Krämer, 2005) and P_{1B}-type ATP-ases (HMAs, metal transporting ATPases) (Hussain *et al*., 2004; Papoyan & Kochian, 2004; Verret *et al.,* 2004; Mills *et al*., 2005). The roles these transporters play in Zn uptake, efflux, compartmentalisation, storage and detoxification have been partially characterised (Krämer *et al*., 2007). After uptake, Zn can be transported in the xylem where it is chelated by different small molecules (Haydon & Cobbett, 2007), including organic acids such as malate and

citrate (White *et al*., 1981; Broadley *et al.,* 2007), His (Salt *et al.,* 1999; Kupper *et al.,*. 2004) and nicotianamine (von Wirén *et al.,* 1999; Callahan *et al*., 2006). Under high Zn supply, a large part of the Zn in the cell is also chelated by organic acids such as malate and citrate (Salt *et al.,* 1999; Sarret *et al*., 2002; Kupper *et al*., 2004), amino acids such as His and NA (Callahan *et al*., 2006), phytate (Rauser, 1999) and metallothioneins (Kawashima *et al*., 1992; Rauser, 1999; Papoyan & Kochian, 2004), and is most likely stored in vacuoles.

Zinc release to the environment may be associated with biotic or natural atmospheric processes, with the ratio of Zn emissions arising from human activities to those coming from natural causes being above 20 (Friedland, 1990). Human activities releasing Zn to the environment include fossil fuel combustion and the use of sewage sludge, manure and lime. In contaminated and acid soils some crops may suffer Zn toxicity, and species which have a high Zn uptake capacity, such as spinach and beet, could be more sensitive to its excess (Chaney, 1993; Broadley *et al.,* 2007). Bioaccumulation of trace metals in plant tissues may present a health risk to wildlife and potentially to humans (Singh & Agrawal, 2007).

The objective of the present study was to investigate the effects of high concentrations of Zn in the nutrient solution on growth, photosynthetic characteristics and nutrient composition of different parts of the model plant sugar beet (*Beta vulgaris* L.). The aim was to establish a basis for studies of the mechanisms of heavy metal transport in this model plant.

MATERIALS AND METHODS

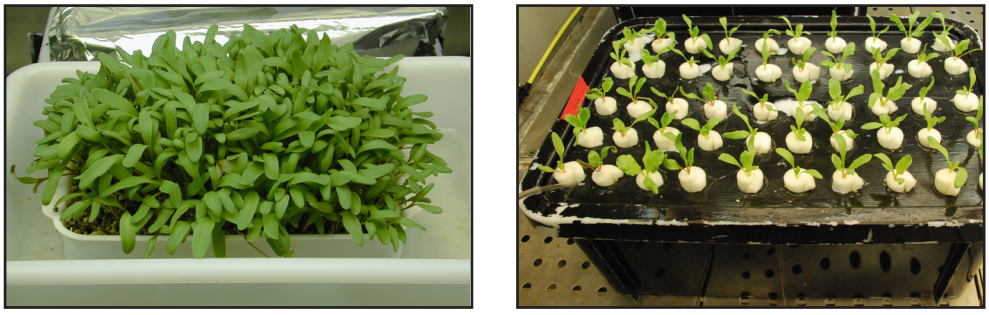
Plant material

Sugar beet (*Beta vulgaris* L. cv Orbis) was grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, measured with a LiCor sensor placed horizontally at maximum plant height, 80% relative humidity and a 16 h at 23 ºC /8 h at 18 ºC light/dark regime. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for an additional 2-week period in half-strength Hoagland nutrient solution (Terry, 1980) with 45 µm Fe(III)-EDTA (Picture 1), and then transplanted to 20-l plastic buckets (four plants

per bucket, Picture 2) containing half-strength Hoagland nutrient solution with 45 µM Fe(III)-EDTA and different concentrations of Zn. A concentration of 1.2 µM $ZnSO_4$ was used as a control, and the excess Zn treatments were 50, 100 and 300 µM $ZnSO_4$. Plants were used for measurements 9-10 days after imposing the high Zn treatments. Young, fully expanded leaves were chosen for all photosynthetic measurements.

Chemical speciation

In silico estimations of the concentrations of Zn ionic species in the different nutrient solutions were carried out with MINTEQA2 for Windows (Version 1.50, Allison Geoscience Consultants, Flowery Branch, GA and Hydro-GeoLogic, Inc., Hermdon, VA, USA).



Picture 1. *Two weeks- old sugar beet seedlings in vermiculite (left) and same seedlings transplanted to a 35-l bucket (right); seedlings were grown in this bucket for two more weeks before treatment onset.*

Picture 2. *Growth chamber with sugar beets in different stages of development.*

Growth parameters

Plants were divided in three fractions at day 10: shoots (leaf blades + petioles), main root and fine absorption roots. Fresh (FW) and dry weights (DW) of each fraction, root /shoot ratios and water content (WC) were also determined.

Mineral nutrient analysis

All plant tissues were washed with pure water. Samples were dried in an oven at 60 ºC for 76 h until constant weight. Samples were then dry-ashed and dissolved in HNO_3 and HCl following the AOAC procedure (Association of Official Analytical Chemists, Washington DC, USA). Calcium (after La addition), Mg, Fe, Mn, Cu and Zn were determined by FAAS, K by FES and P spectrophotometrically by the molybdate-vanadate method (Abadía *et al*., 1985; Igartua *et al.,* 2000). Nitrogen was determined with a NA2100 Nitrogen Analyzer (Thermo-Quest, Milan, Italy).

Photosynthetic pigment analysis

The leaf concentration of Chl was estimated on a leaf area basis in attached leaves using a SPAD portable apparatus (Konica Minolta Co., Osaka, Japan). For calibration, leaf disks were taken at day 10 after treatment, first analysed with the SPAD apparatus, then frozen in liquid N_2, pigments extracted with 100% acetone in the presence of Na ascorbate and the extracts analysed spectrophotometrically (Abadía & Abadía, 1993). Photosynthetic pigments were also quantified by HPLC (Larbi *et al*., 2004). In these experiments, leaf sampling was carried out in leaves illuminated for 3-4 h.

Gas exchange measurements

Nine days after treatments were imposed in the growth chamber, measurements were made on attached leaves using a portable gas exchange system (CIRAS-1, PP Systems, Herts, UK), using a PLC broadleaf cuvette in closed circuit mode. Transpiration rates (E), stomatal conductance (g_s), net photosynthetic rate (P_N) and substomatal CO_2 concentrations (C_i) were measured and calculated. Experiments were made at ambient CO_2 concentration, 130-170 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD, and at the temperature and relative humidity prevailing in the growth chamber. All measurements were taken in leaves illuminated for 3-4 h.

Modulated chlorophyll fluorescence analysis

Modulated Chl fluorescence measurements were made in attached leaves, 9 days after treatments were imposed in the growth chamber, using a PAM 2000 apparatus (H. Walz, Effeltrich, Germany). F_o was measured by switching on the modulated light at 0.6 kHz; PPFD was below 0.1 µmol photons $\cdot m^{-2} \cdot s^{-1}$ at the leaf surface. F_m was measured at 20 kHz with a 1 s pulse of 6000 µmol photons $\cdot m^{-2} \cdot s^{-1}$ of white light. The experimental protocol for the analysis of Chl fluorescence quenching followed Morales *et al*. (2000) and references therein. F_o and F'_o were measured in the presence of FR light (7 µmol photons $\cdot m^{-2} \cdot s^{-1}$) to fully oxidise the PSII acceptor side (Belkhodja *et al.,* 1998; Morales *et al.,* 1998; Logan et al., 2007). Dark-adapted, maximum potential PSII efficiency was calculated as F_v / F_m, where F_v is F_m / F_o (Morales *et al*., 1991; Abadía *et al.,* 1999). Actual (Φ_{PSII}) and intrinsic (Φ_{exc}) PSII efficiency were calculated as $(F'_m - F'_s)/F'_m$ and F'_v / F'_m, respectively. Photochemical quenching (qP) was calculated as $(F'_m - F_s) / F'_v$. Non-photochemical quenching (NPQ) was calculated as $(F_m / F'_m) - 1$. Experiments were carried out at ambient CO_2 concentration, 200-250 µmol $\cdot m^{-2} \cdot s^{-1}$ PPFD, and at the temperature and relative humidity prevailing in the growth chamber. All measurements were taken in leaves illuminated for 3-4 h.

Iron reductase activity measurements

Ferric chelate reductase (FC-R) activity was measured as described by Gogorcena *et al.* (2000) by following the formation of the Fe(II)-$BPDS_3$ complex from Fe(III)-EDTA. Root FC-R activity was determined in intact plants 2-3 h after light onset and at 4, 7 and 10 d after adding excess Zn to the nutrient solution. Plants were placed in 1-l buckets, in a solution containing 1 mM MES, pH 5.5, 100 µM BPDS and 100 µM Fe(III)-EDTA in MilliQ water. The reaction was stopped after 30 min by removing the plant from the bucket, and absorbance readings of the solution at 535 nm were taken using 1 ml aliquots after centrifugation at 10,000*g* for 1 min. Controls were also measured in the absence of plants to correct for non-enzymatic Fe reduction.

Statistical analysis

For statistical analysis, an LSD Bonferroni test was used for comparison of means on all data sets. The test used the SPSS software (v.14, SPSS Inc, Chicago, IL, USA).

RESULTS

Chemical speciation

In the 50, 100 and 300 μM $ZnSO_4$ treatments, the major Zn chemical species in solution was free Zn(II), accounting for 91-93% of total Zn (Table 1). Approximately 6% of total Zn was in the form $ZnSO_4$(aq) in the three high Zn treatments, whereas the concentration of the Zn(II)-EDTA complex was predicted to be very low, accounting for only 1.9, 1.4 and 0.2% of total Zn in the solutions containing 50, 100 and 300 μM Zn, respectively. In control solutions with 1.2 μM total Zn, approximately 86, 8 and 6% of total Zn was predicted to occur as Zn(II), Zn(II)-EDTA and $ZnSO_4$(aq), respectively (Table 1).

Table 1. *Major Zn chemical species in the nutrient solutions, in percentage of the total Zn present, estimated in silico with the software MINTEQA2.*

Total Zn concentration (μM)	Zn^{2+} (%)	Zn[EDTA] (%)	$ZnSO_4$(aq) (%)
1.2 (control)	86.0	7.5	5.8
50	91.2	1.9	6.0
100	91.7	1.4	6.0
300	92.9	0.2	6.0

Effects of excess Zn on growth

Sugar beet shoot and root fresh and dry mass decreased progressively when the Zn concentration in the nutrient solution increased (Fig. 1). Decreases were significant when considering whole plants (Picture 3), shoots, main roots and fine roots (Fig. 1). Also, plants treated with high Zn contained less water than control plants (Fig. 1). A significant decrease in the root/shoot ratio was only found with the 300 μM Zn treatment (data not shown). Zinc excess also decreased the number of leaves and leaf area, leaf margins were distorted or wrinkled and leaves were rolled inwards. Plants showed chlorosis symptoms in leaves and roots (Picture 3 and 4, see changes in leaf concentrations of photosynthetic pigments below).

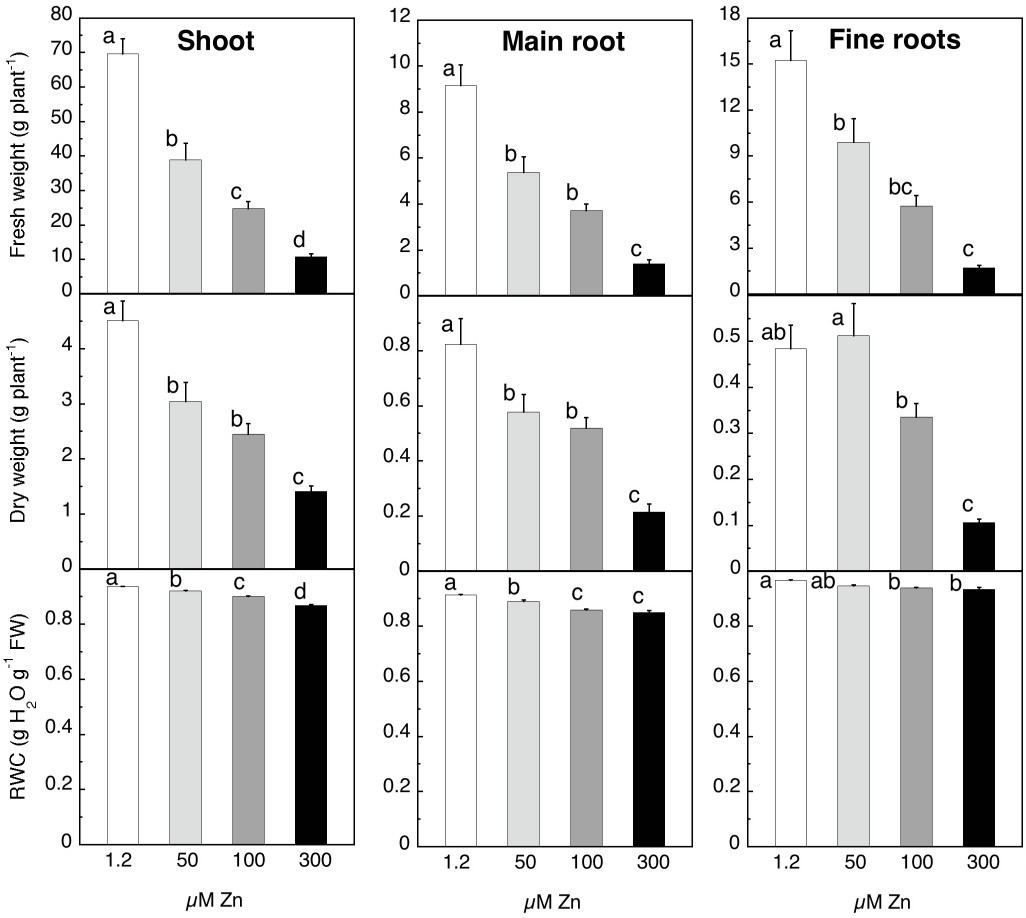


Fig.1.*Fresh and dry mass (in g per plant) and water content (in g g^{-1} FW) of shoots, main roots and fine roots of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (four different batches of plants, four to eight replicates in each batch). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤0.05 probability level.*

Picture 3. *Reduction of shoot and root growth in plants grown with 1.2, 50, 100 and 300 μM Zn for 10 days (upper panel- from left to right, respectively). Plants grown with 100 μM Zn showed chlorotic leaves and leaf margins were distorted and rolled inwards (lower panel).*

Picture 4. *Detail of the main root from a sugar beet plant grown at 50 µM Zn (left) and close up picture of fine roots from 100 µM Zn grown sugar beet plants showing yellow tips (right).*

Effects of excess Zn on plant nutrient concentrations

Zinc toxicity altered the plant concentrations of several nutrients (Fig. 2). Nitrogen and K concentrations decreased progressively with Zn excess in shoots, main and fine roots, whereas Mg concentrations decreased significantly only in shoots and main roots. Phosphorus concentrations increased in shoots and increased slightly in main roots, but did not change significantly in fine roots. Calcium concentrations were unaffected in roots and increased only in shoots of plants grown with 50 and 100 µM Zn. All of the macronutrient concentrations found, except for N, were in the normal ranges used for sugar beet (Jones *et al.,* 1991).

Micronutrient concentrations were also affected by high Zn in the nutrient solution (Fig. 3). Iron concentrations decreased by 40% in shoots (from approximately 110 to 170 µg g^{-1} DW) and increased by 25% in main roots in response to Zn excess. Manganese concentrations decreased progressively with Zn excess in shoots and fine roots, but shoot Mn concentrations always remained above 50 µg g^{-1} DW. Copper concentrations did not change significantly with Zn excess in shoots and main roots, although Cu concentrations in fine roots increased progressively, reaching approximately 120 µg g^{-1} DW in plants grown with 300 µM Zn, a value threefold higher than that found in control plants.

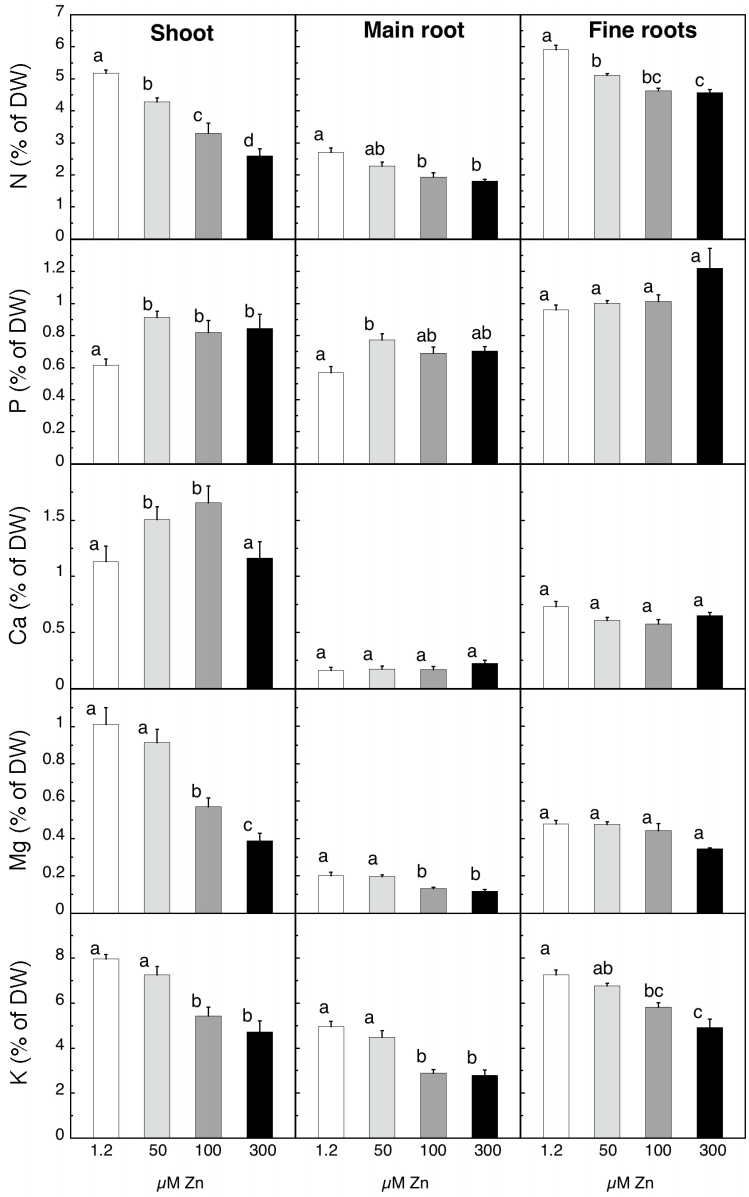


Fig. 2. *Macronutrient concentrations (in % DW) in shoots, main roots and fine roots of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (12-20, 8-16 and 4-8 samples for shoot, main and fine roots, respectively). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤0.05 probability level.*

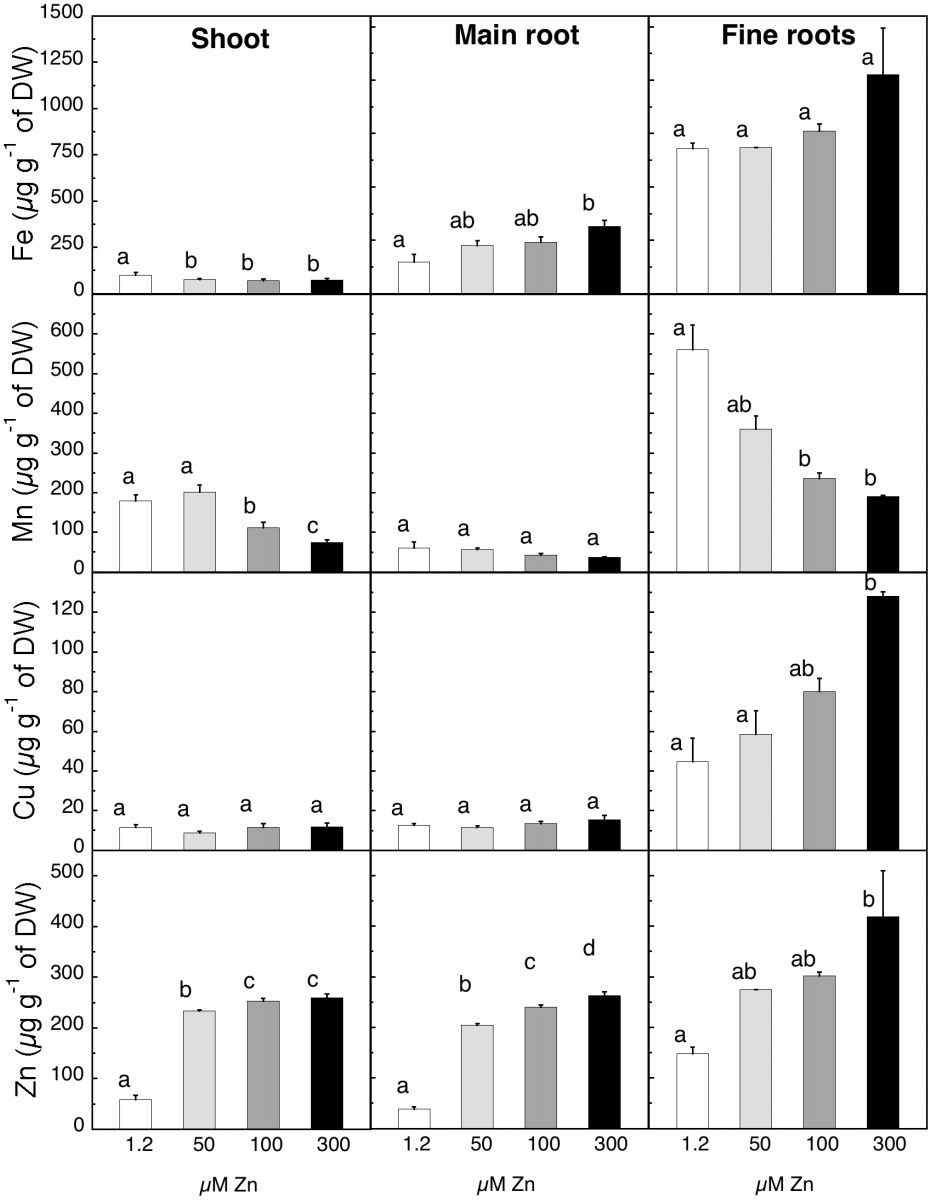


Fig. 3. *Micronutrient concentrations (in μg·g^{-1} DW) in shoots, main roots and fine roots of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (12-20, 8-16 and 4-8 samples for shoot, main and fine roots, respectively). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤0.05 probability level.*

Zinc concentrations increased significantly in all plant parts with high Zn in the nutrient solution. Increases were approximately fourfold in shoots, five- to sevenfold in main roots and two- to threefold in fine roots. Shoot Zn concentrations in plants grown with high Zn were 1233-259 $\mu g\, g^{-1}$ DW. Total Zn amounts extracted per plant were 367 μg in the 1.2 μM Zn treatment (263, 32 and 72 μg in shoot, main root and fine root, respectively), 968 μg in the 50 μM Zn treatment (709, 118 and 141 μg in shoot, main root and fine root, respectively), 844 μg in the 100 μM Zn treatment (618, 125 and 101 μg in shoot, main root and fine root, respectively), and 468 μg in the 300 μM Zn treatment (367, 57 and 44 μg in shoot, main root and fine root, respectively). Therefore, Zn allocation (as a percentage of total Zn) for shoot /main root /fine root was 72 /8 /20 in control plants, 73 /12-15 /12-15 in the 50 and 100 μM Zn treatments, and 78 /12 /9 in the 300 μM Zn treatment.

Effects of excess Zn on photosynthetic pigment composition

Leaf chlorosis was already seen in the 50 μM Zn treatment and, accordingly, concentrations of all major photosynthetic pigments on a leaf area basis were decreased when compared to those found in control plants (Fig. 4). In leaves of plants grown with 100 and 300 μM Zn, decreases in lutein, β-carotene, Chl*a* and Chl*b* were more marked than at 50 μM Zn, with reductions of approximately 40-50% when compared to the controls. However, total concentrations of violaxanthin cycle pigments (V + A + Z) were very similar, at 50 and 100 μM Zn, and decreased further only in the 300 μM Zn treatment (Fig. 4). In the case of neoxanthin, no further significant decreases were found at Zn concentrations above 50 μM in the nutrient solution.

In the 50 μM Zn treatment, the (V + A + Z) /Chl ratio did not change with respect to the value found in control plants, but the (A + Z) /(V + A + Z) ratio increased, indicating extensive de-epoxidation of violaxanthin cycle pigments (Fig. 5). In the 100 μM Zn treatment, however, both the (V+A+Z) /Chl ratio and the (A + Z) /(V + A + Z) ratio were markedly increased, while in the highest Zn treatment, 300 μM, neither the (V + A + Z) /Chl northe (A+Z) /(V + A + Z) ratios changed when compared to control ratios. Chl *a/b* ratios in leaves of sugar beet plants treated with 50 and 100 μM $ZnSO_4$ were higher than in leaves of plants grown with 300 μM $ZnSO_4$, with control plants having an intermediate value (Fig. 5).

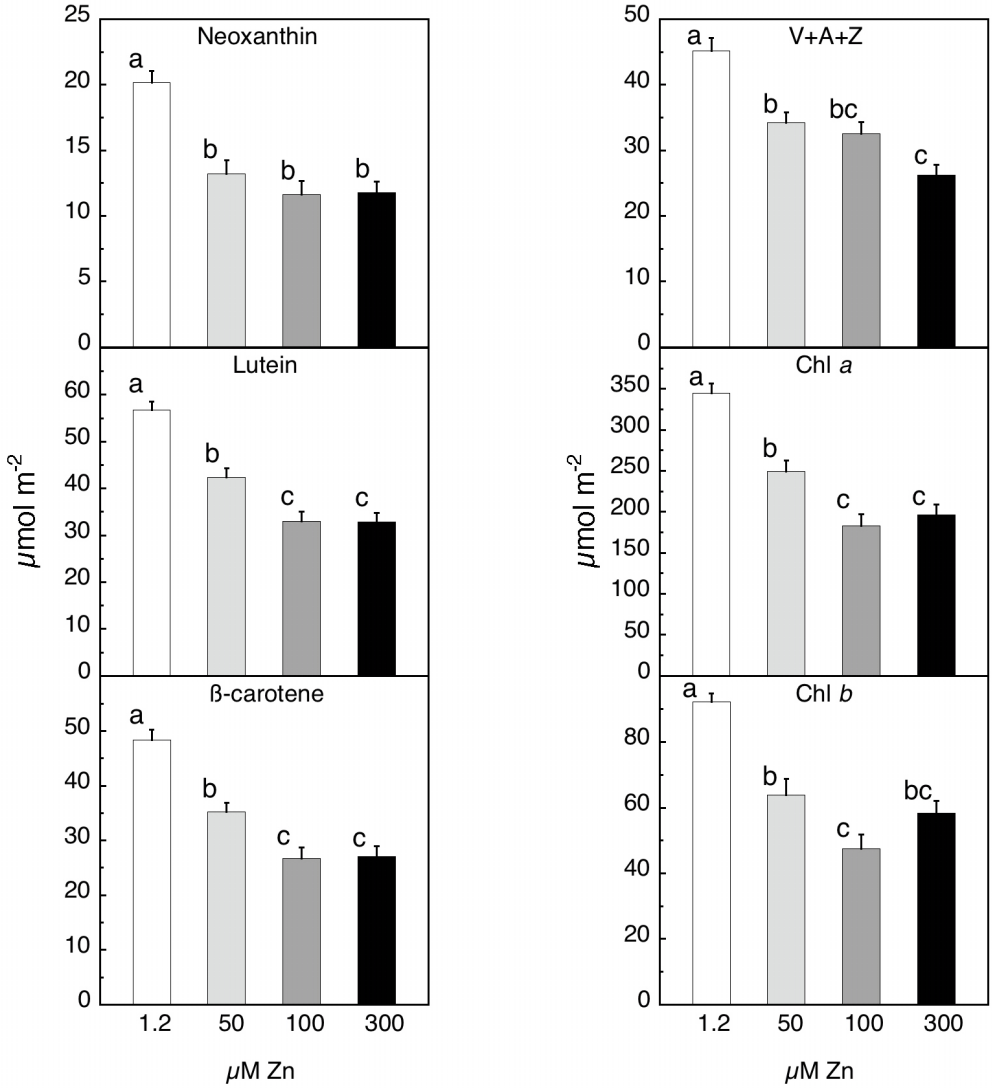


Fig. 4. *Leaf concentrations of photosynthetic pigments (carotenoids and chlorophylls, in µmol m^{-2}) in sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 µM Zn) for 10 days. Data are means ± SE (20 or more replications per treatment). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤0.05 probability level.*

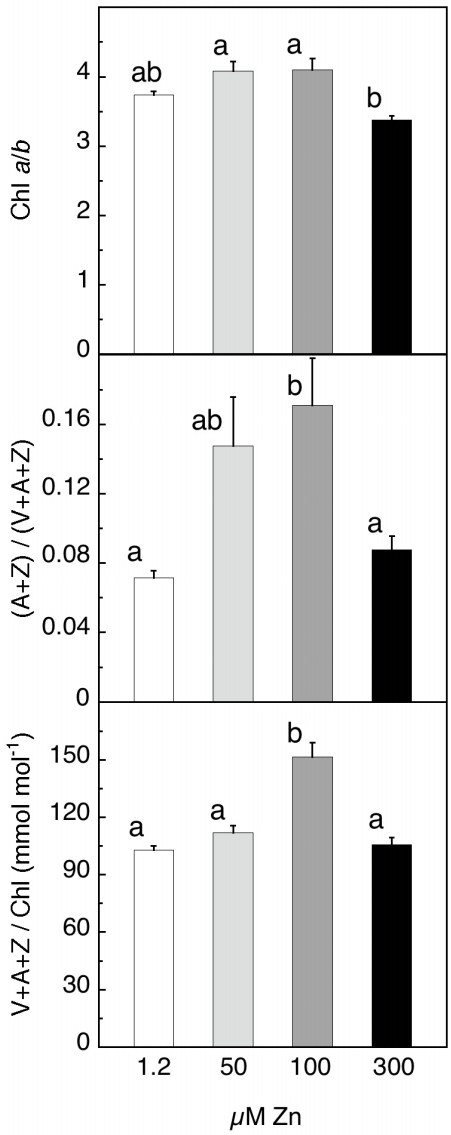


Fig. 5. *Ratios Chl* a/b *(in mol mol^{-1}), (A + Z) /(V+A+Z) (in mol mol^{-1}) and (V+A+Z) /Chl (in mmol mol^{-1}) in leaves of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 10 days. Data are means ± SE (20 or more replications per treatment). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤0.05 probability level.*

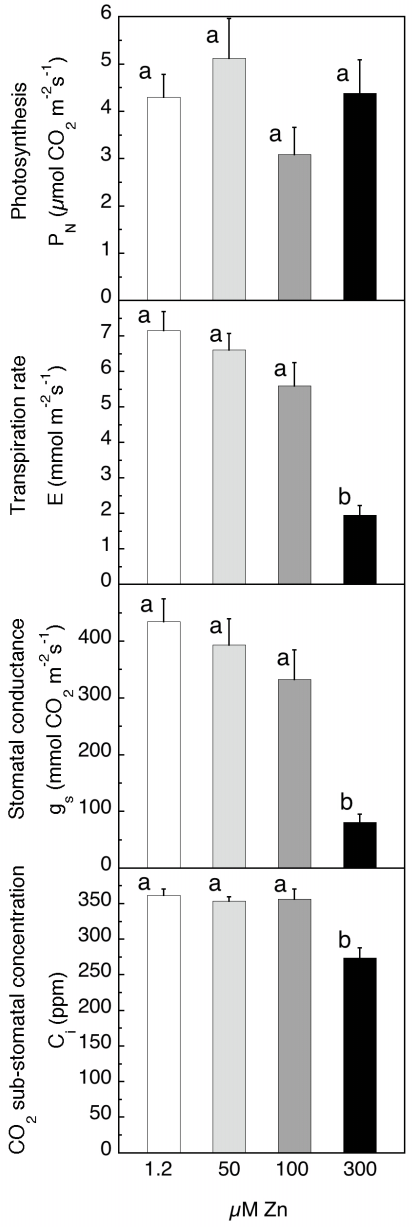


Fig. 6. *Gas exchange parameters.*

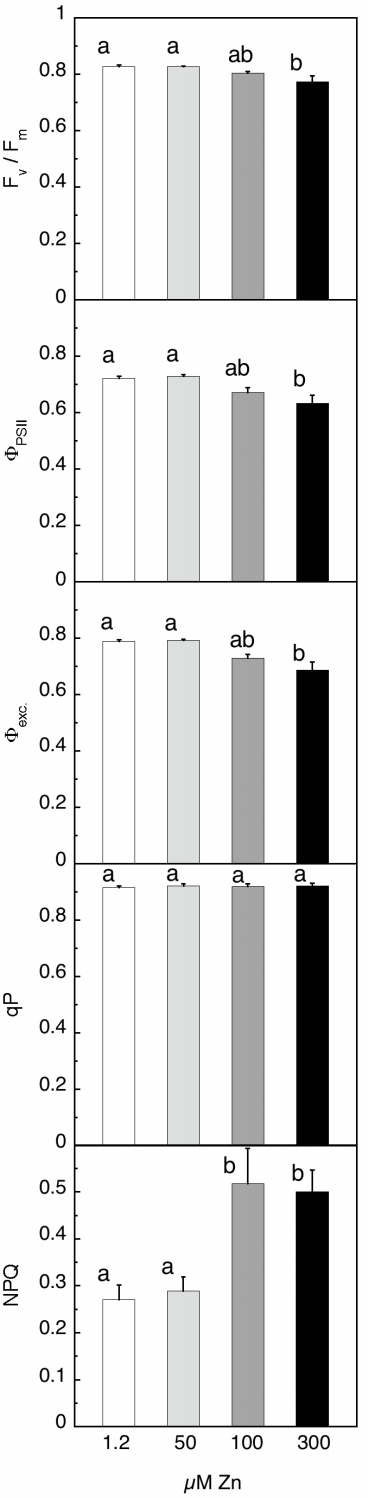


Fig. 7. *Modulated Chl fluorescence parameters.*

Fig. 6. *Gas exchange parameters in leaves of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 9 days. The incident PPFD was between 130 and 170 μmol m^{-2}. Data are means ± SE (two to three sets of measurements, 6-10 replications each). Columns marked with the same letter are not significantly different (LSD Bonferroni test) at the P ≤0.05 probability level.*

Fig. 7. *Modulated Chl fluorescence parameters in leaves of sugar beet plants grown with different Zn concentrations (1.2, 50, 100 and 300 μM Zn) for 9 days. The incident PPFD was between 200 and 250 μmol m^{-2}. Data are means ± SE (three sets of measurements, 13-17 replications each). Bars marked with the same letter were not significantly different (LSD Bonferroni test) at the P ≤0.05 probability level.*

Effects of excess Zn on gas exchange

No significant differences were measured in gas exchange parameters (P_N, E, g_s, or C_i) in plants grown with 50 and 100 μM Zn in the nutrient solution when compared to controls (Fig. 6). However, in the 300 μM Zn treatment E, g_s, and C_i decreased by 73, 82 and 24%, respectively, when compared to control plants, indicating marked stomatal closure. In these plants, P_N rates did not decrease significantly when compared to values found in control plants.

Effects of excess Zn on chlorophyll fluorescence parameters

Leaves of plants grown with the highest Zn concentration (300 μM) showed slight but significant decreases in F_v /F_m ratios (Fig. 7). Upon illumination, these leaves showed decreases in actual PSII efficiency (Φ_{PSII}) associated with decreases in intrinsic PSII efficiency (Φ_{exc}), since photochemical quenching (qP) did not change significantly when compared to control values (Fig. 7). No changes in chlorophyll fluorescence parameters were found in plants grown with 50 μM Zn, and only small, not significant, changes were found in plants grown with 100 μM Zn. Non-photochemical quenching, however, was markedly increased (twofold) both in the 100 and 300 μM Zn treatments (Fig. 7).

Effects of excess Zn on root Fe(III)-chelate reductase activity

Roots of sugar beet became brownish when grown with excess Zn, and in the 50 and 100 μM Zn treatments, some yellow root tips were observed 7 day after treatment onset. Whole root FC-R activity increased in plants grown with 50 μM Zn for 7 and 10 days when compared to control values (Table 2). However, in the time period studied, root FC-R activity of plants grown with 100 and 300 μM Zn did not show major changes when compared to controls.

Table 2. *Root Fe-reductase activity (in nmol Fe reduced g^{-1} FW min^{-1}) measured in intact plants grown in nutrient solution containing different concentrations of Zn. Data are means ± SE of two batches of plants (four replications each). Symbols * and **indicate significant differences from the control values (LSD Bonferroni test) at the P ≤0.05 and P ≤0.01 probability levels, respectively.*

Total Zn concentration (μM)	day 4	day 7	day 10
1.2 (control)	3.05 ± 0.31	1.81 ± 0.12	2.03 ± 0.39
50	2.68 ± 0.24	3.62 ± 0.42**	3.84 ± 0.22**
100	2.25 ± 0.13	1.52 ± 0.24	1.93 ± 0.19
300	1.89 ± 0.20*	1.67 ± 0.38	2.23 ± 0.26

DISCUSSION

Zn excess had different effects in sugar beet plants, and the type and extent of the effects were dependent on the Zn concentration in the nutrient solution. In general, excess Zn reduced plant growth, and leaves showed symptoms of chlorosis and signs of damage. Effects were also apparent in roots, with depressed growth and browning. Effects on photosynthetic rates, photosynthetic pigments and chlorophyll fluorescence were markedly different, depending on the Zn concentration in the nutrient solution.

A moderate Zn treatment (50 μM) led to Zn(II) concentrations in the nutrient solution of approximately 45 μM and to shoot concentrations of approximately 230 μg Zn g^{-1} DW. At this Zn concentration, shoot and root growth were already markedly affected when compared to controls. Shoots had less Fe and N, and more P and Ca

than control plants. Leaves had mild chlorosis and less Fe than control plants, with decreases in the concentrations of all photosynthetic pigments. Although no significant changes were seen in gas exchange or chlorophyll fluorescence parameters, de-epoxidation of the xanthophyll cycle (V + A + Z) pigments did occur, indicating slight thylakoid stress. In roots, 50 μM Zn led to some yellow tips, and to a slight increase in whole root FC-R activity. All these data indicate that, besides the marked decrease in growth, 50 μM Zn in the nutrient solution caused moderate Fe deficiency. In Fe-deficient sugar beet, moderate decreases in photosynthetic pigments do not affect photosynthetic rates (Larbi *et al*., 2006). This behaviour is similar to that observed in 50 μM Zn-treated sugar beet plants.

The intermediate Zn treatment (100 μM Zn) led to nutrient solution Zn(II) concentrations of approximately 90 μM, but shoots had only marginally higher concentrations of Zn (approximately 250 μg $Zn \cdot g^{-1}$ DW) than those of plants grown with 50 μM Zn. In these plants, shoot and root growth were further decreased. Shoot concentrations of N, Mg, K and Mn decreased when compared with values found at 50 μM Zn. Concentrations of P and Ca were still higher, and Fe was lower, than in the controls. Leaves had stronger chlorosis and a similar Fe concentration than that at 50 μM Zn, with the concentrations of photosynthetic pigments decreasing further, except for neoxanthin and V + A + Z pigments, thus leading to increases in the ratio (V + A + Z) /Chl. No significant changes were seen in gas exchange and in most chlorophyll fluorescence parameters, but both de-epoxidation of V + A + Z pigments and an increase in NPQ occurred, indicating marked thylakoid stress. In roots, the 100 μM Zn treatment led to some yellow tips, but no change in whole root FC-R activity was observed. This may be associated to a deleterious effect of high Zn on enzyme activity, as shown to occur with other heavy metals such as Cd and Pb (Larbi *et al.,* 2002; Chang *et al*., 2003). All these data indicate that 100 μM Zn in the nutrient solution caused strong decreases in growth and significant photosynthetic stress that was not necessarily related to Fe deficiency.

Using an even higher Zn treatment (300 μM), Zn(II) concentrations in the nutrient solution were estimated to be approximately 270 μM, but leaves still had approximately 250 μg $Zn\ g^{-1}$ DW. This treatment led to further decreases in shoot

and root growth. Leaves had intermediate chlorosis, between that found at 50 and 100 μM Zn, and the Fe concentration was similar to that found at 50 μM Zn, but the ratio (V+A+Z) /Chl, the de-epoxidation state of the V + A + Z pigments and photosynthetic rates were not changed when compared to control values. Stomatal conductance, transpiration, C_i, F_v/F_m, Φ_{PSII}, and Φ_{exc} decreased, whereas the NPQ was still quite high. Similar results were reported with four different *Datura* species grown in vermiculite with much higher Zn concentrations (5 mM) (Vaillant *et al*., 2005). In roots, the 300 μM Zn treatment led to the appearance of some yellow tips, but no change in whole root FC-R activity was observed. All these data indicate that 300 μM Zn in the nutrient solution causes a strong decrease in growth, stomatal closure and signs of photosynthetic stress, again not necessarily related to Fe deficiency. The lack of effect on violaxanthin cycle pigments suggests that formation of the thylakoid pH gradients may be strongly impaired by high Zn. The fact that Zn excess causes marked stomatal closure with little effect on C fixation rates points to the possibility that a futile cycle may exist in plants grown in high Zn, where the C respired may be re-fixed by photosynthesis. Also, carbonic anhydrase, a Zn-containing enzyme whose activity correlates with leaf Zn concentration (Hacisalihoglu & Kochian, 2003; López-Millán *et al.,* 2005), could contribute to the low C_i values found. This possibility should be explored in further studies.

Our results indicate that Zn homeostasis is tightly controlled in sugar beet, since when the Zn concentration in the nutrient solution increased (from 50 to 300 μM Zn), Zn shoot concentrations only increased marginally, from 236 to 259 $\mu g\, g^{-1}$ DW, and Zn allocation to the shoot was little changed. Therefore, sugar beet can be used as a good model plant to study Zn homeostasis in non-hyper-accumulator plant species. Treatment with two doses of sewage sludge containing Zn and other metals led to sugar beet leaf Zn concentrations of approximately 75 $\mu g\, g^{-1}$ DW (Singh & Agrawal, 2007). In four *Datura* species grown in vermiculite with high concentrations of Zn in the nutrient solution, leaf Zn concentrations were higher than 300-500 $\mu g\, g^{-1}$ DW (Vaillant *et al*., 2005). Different mechanisms have been implicated in the regulation of Zn homeostasis, including downregulation of Zn root uptake systems, Zn chelation by low-molecular weight compounds, and /or subcellular compartmentalisation of

excess Zn in the apoplast or vacuoles (Hall, 2002). Most of the knowledge to date comes from the study of Zn hyperaccumulator plants, such as *Arabidopsis halleri* and *Thlapsi caerulescens*, in which the major strategy for Zn detoxification consists of metal sequestration in vacuoles from mesophyll cells (Lasat *et al*., 1998; Kobae *et al.,* 2004; Kupper *et al*., 2004). However, in non-hyperaccumulators other mechanisms might make a higher contribution to cope with excess Zn. Further studies should be directed to analyse, in sugar beet plants treated with excess Zn, the underlying mechanisms that contribute in this species to control Zn homeostasis, with special emphasis on chemical speciation in xylem sap, subcellular Zn distribution and the storage forms in vacuoles and /or apoplastic compartments.

Data presented here indicate that sugar beet is not a Zn accumulator and is unlikely to have potential for Zn bioremediation. This contrasts with data obtained for Cd and Pb with the same plant species (Larbi *et al*., 2002). This results from the fact that high Zn causes a very strong growth decrease, whereas the concentration of Zn in tissues does not increase greatly (i.e., 260 μg Zn g^{-1} DW in plants grown with 300 μM Zn in the nutrient solution). In consequence, the amount of Zn removed per plant was larger with 50 than with 100 or 300 μM Zn in the nutrient solution. This is in contrast to Cd and Pb, since sugar beet shoots may contain up to 500 μg Cd or Pb g^{-1} DW in plants grown with 50 μM Cd or 2 mM Pb in the nutrient solution (Larbi *et al*., 2002). Sugar beet plants took up approximately 4, 2 and 1% of the total nutrient solution Zn in the treatments containing 50, 100 and 300 μm Zn, respectively. Considering a possible shoot dry mass production of 1.5 ton ha^{-1} (usual values in a sugar beet commercial crop are approximately 13 ton ha^{-1}), and a possible leaf Zn concentration of 200 μg g^{-1} DW, metal removal would be approximately 300-600 g Zn ha^{-1}, an amount clearly insufficient for Zn phytoremediation.

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

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Capítulo 5

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

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SUMMARY

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

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

INTRODUCTION

Zinc (Zn) availability is generally high in low-pH soils (Foy *et al*., 1978; Chaney, 1993), and the Zn concentration in plants can reach toxic levels (Broadley *et al*., 2007). Zn is a major industrial pollutant of terrestrial and aquatic environments (Barak & Helmke, 1993) in most regions of the world, including North and South America, Europe, Africa and Asia (Forstner, 1995). Zn toxicity occurs when leaf concentrations reach 400-500 μg g^{-1} dry weight (Marschner, 1995). Zn toxicity includes symptoms similar to those of cadmium (Cd) or lead (Pb) toxicity (Foy *et al*., 1978; Larbi *et al*., 2002; Fodor *et al*., 2005). Excess Zn reduces growth, leading to stunted plants (Horler *et al*., 1980; Sagardoy *et al*., 2009) and produces changes in root growth and morphology (Vaillant *et al*., 2005; Broadley *et al*., 2007). Young leaf chlorosis in plants grown in high Zn has been suggested to be caused by deficiencies in iron (Fe) or magnesium (Mg) (Marschner, 1995; Sagardoy *et al*., 2009). The ratio Chl*a* : Chl*b* has been found to decrease with Zn toxicity (Monnet *et al*., 2001; Schuerger *et al*., 2003; Vaillant *et al*., 2005), suggesting that Chl*a* is more affected than Chl*b*. However, in other species, leaves of Zn-treated plants may remain green when Zn concentrations are high, suggesting that inhibition of leaf expansion could have a greater effect than Chl breakdown or reduced biosynthesis (Horler *et al*., 1980; Sagardoy *et al*., 2009). Therefore, the effects of Zn excess in plants are highly variable, depending on the Zn concentration and the species in question.

Zn toxicity may inhibit photosynthesis at various steps and through different mechanisms. Generally, Zn toxicity decreases net photosynthesis (A_N) (Van Assche *et al*., 1980; Vaillant *et al*., 2005; Dhir *et al*., 2008; Mateos-Naranjo *et al*., 2008), but it is a subject of debate whether this decrease is limited by stomatal conductance (g_s), mesophyll conductance (g_m), increased respiration, decreased photochemistry, impaired biochemistry, or a combination of several of these factors (Sharma *et al*., 1995; Di Baccio *et al*., 2009; Shi & Cai, 2009). It has been observed that high Zn concentrations could cause blockage of xylem elements (Robb *et al*., 1980), large decreases in root biomass (Vaillant *et al*., 2005) and decreases in leaf water content (Bonnet *et al*., 2000), all of which may lead to impaired g_s, in turn decreasing transpiration rates and photosynthesis (Schuerger *et al*., 2003; Vaillant *et al*., 2005;

Sagardoy *et al*., 2009). Increased mesophyll resistance to CO_2 diffusion has also been suggested to occur with Zn excess (Van Assche *et al*., 1980; Prasad & Strzalka, 1999). However, the effects of Zn toxicity on mesophyll diffusion conductance to CO_2 (g_m) have only been assessed using a parameter that combines g_m and carboxylation activity. To date, no study has analyzed the effects of Zn toxicity on g_m, in spite of the fact that Zn is a cofactor of carbonic anhydrases (CAs), which facilitate g_m by catalyzing the interconversion between CO_2 and HCO^-_3 in the weakly alkaline chloroplast stroma (Evans *et al*., 2009).

Respiratory metabolism also interacts with photosynthesis, and respiratory mutants (especially those with altered alternative oxidase (AOX) activity) show decreased photosynthesis, as well as decreased g_s and g_m (reviewed by Flexas *et al*., 2008). The effects of Zn excess on respiration are a matter of controversy. On the one hand, in vitro studies have suggested that Zn inhibits the activity of the mitochondrial cytochrome bc1 complex, probably by interfering with the enzyme's reaction with ubiquinol at the UQH_2 binding niche that oxidizes UQH_2, the quinol oxidase (Q_o) site (Link & von Jagow, 1995), as well as the activity of AOX (Affourtit & Moore, 2004). On the other hand, in vivo studies have shown that respiration is enhanced progressively with increasing Zn doses (Ismail & Azooz, 2005), and use of inhibitors of cytochrome bc1 and AOX has suggested that there is a specific increase in AOX activity (Webster & Gadd, 1999), although, to the best of our knowledge, no report on the in vivo activity of AOX under Zn excess has been published. Regardless of the mechanisms involved, Zn-induced alterations in respiration would result from metabolic effects that could potentially reduce net photosynthesis.

The effects of Zn excess on the photochemistry and biochemistry of photosynthesis are also a matter of controversy. In some studies, the quantum yield of photosystem II (PSII)-related electron transport, estimated at low photosynthetic photon flux density (PPFD), was not affected in Zn-treated plants (Van Assche & Clijsters, 1986a), in agreement with studies showing only small decreases (from 0.82 to 0.70) of the maximum potential PSII efficiency (F_v/F_m) under Zn excess (Schuerger *et al*., 2003; Dhir *et al*., 2008; Sagardoy *et al*., 2009). However, other studies have shown an inhibition of thylakoid electron transport rates (ETRs) by high Zn concentrations

(Kim & Jung, 1993). Specific effects on PSII photochemistry, related to competitive substitution of manganese (Mn) by Zn at the site of water photolysis inhibiting photosynthetic electron transport and oxygen evolution, have also been reported (Van Assche & Clijsters, 1986a; Ralph & Burchett, 1998). Consistent with this, Zn excess was found to decrease PSII efficiency (Φ_{PSII}) and noncyclic photophosphorylation (Van Assche & Clijsters, 1986a; Bonnet *et al*., 2000; Schuerger *et al*., 2003; Sagardoy *et al*., 2009), although, alternatively, Φ_{PSII} could be limited by excess Zn-mediated reductions in dark photosynthetic processes. Other studies have shown no changes in the ADP : ATP and $NADP^+$: NADPH ratios with changes in Zn concentration (Dhir *et al*., 2008). In some studies, the decrease in F_v/F_m was associated with increased levels of minimal Chl fluorescence in the dark (F_o) (Vaillant *et al*., 2005), suggesting PSII photoinactivation or photooxidation, consistent with the observation that lipid peroxidation was found to be enhanced in plants treated with Zn excess (Chaoui *et al*., 1997). The activity of antioxidant enzymes such as Mn-superoxide dismutase (Mn-SOD) and ascorbate peroxidase (APX) increased in plants exposed to high concentrations of Zn (del Río *et al*., 1985; Chaoui *et al*., 1997; Bonnet *et al.*, 2000). Catalase (CAT) activity was decreased in high-Zn plants (Chaoui *et al.*, 1997; and references therein), which may suggest decreased photorespiratory activity. However, photosynthetic ETR : A_N ratios increased under Zn excess (from *c*. 9 in controls to 31-77; recalculated from Monnet *et al*., 2001), which is probably indicative of increased electron consumption diverted to photorespiration or to alternative processes.

Regarding other photoprotection mechanisms, leaf reflectance increases under Zn toxicity (Horler *et al*., 1980). Sagardoy *et al*. (2009) reported low xanthophyll cycle activity, with low de-epoxidation values, under Zn excess. While the latter may suggest that the formation of the thylakoid pH gradient may be impaired under excess Zn, Dhir *et al*. (2008) showed an increase in trans-thylakoid ΔpH under Zn toxicity as a consequence of increased activity of photosystem I (PSI). Finally, specific effects on Rubisco carboxylase capacity (Van Assche & Clijsters, 1986b; Monnet *et al*., 2001; Mateos-Naranjo *et al*., 2008) and the Calvin cycle (Chaney, 1993) have been reported, although other studies have reported only slight Zn effects on Rubisco (Van Assche *et al*., 1980; Dhir *et al*., 2008).

The aim of this work was to investigate the effects of Zn toxicity on photosynthesis and respiration rates in the model plant sugar beet (*Beta vulgaris*). A complete analysis of photosynthetic activity has been used to separate stomatal, mesophyll and biochemical contributions to the reductions in photosynthesis (Grassi & Magnani, 2005), similar to those used for water stress and recovery (Grassi & Magnani, 2005; Galmés *et al*., 2007a,b; Flexas *et al*., 2009; Gallé *et al*., 2009) and leaf ageing (Grassi & Magnani, 2005; Flexas *et al*., 2007, 2009).

MATERIALS AND METHODS

Plant material and growth conditions

Sugar beet (*Beta vulgaris* L. cv Orbis) seeds were germinated and grown in vermiculite for 3 wk, in a growth chamber at a constant temperature (25 ºC) and a PPFD of 600 µmol m^{-2} s^{-1} with a 16 h :8 h light/dark regime. Plants were then moved to a glasshouse, and transplanted into 20-l plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution (Terry, 1980) with 45 µm Fe(III)-EDTA. Once plants were established, experiments with different concentrations of Zn were initiated. Zn ($ZnSO_4$) concentrations were 1.20 µM (control), 100 µM and 300 µM. Treatments used were based on a previous study (Sagardoy *et al*., 2009). Half-strength Hoagland's solution was added, when necessary, several times throughout the experiment. Plants were used for measurements 10-14 d after the treatments were imposed. Measurements were performed in young, fully expanded leaves, 3 to 4 h into the light period.

Growth parameters

At the end of the experiment, 14 d after the start of the treatments, plants were collected and separated into roots and shoots. Fresh weights (FWs) and dry weights (DWs) were determined for each fraction, and water content (WC) was calculated as (FW - DW) /FW. Leaf areas were measured with an AM-100 leaf area meter (ADC, Herts, UK). Eight plants per treatment were used.

Mineral analysis

For nutrient analysis, plant tissues were washed with pure water and dried in an oven at 60 ºC for 76 h to constant weight. Samples were then dry-ashed and dissolved

in HNO_3 and HCl following the Association of Official Analytical Chemists (AOAC) procedure (Association of Official Analytical Chemists, Washington, DC, USA); Zn was determined by flame atomic absorption spectroscopy (FAAS) (Igartua *et al*., 2000).

Gas exchange and chlorophyll fluorescence measurements

Stomatal conductance (g_s) was estimated nondestructively with a portable leaf porometer (SC-1; Decagon Devices, Pullman, WA, USA). Porometry was used for rapid assessment of stomatal status throughout the experimental period, in order to ensure all experiments were performed under similar initial experimental conditions.

Leaf gas exchange and Chl fluorescence were measured simultaneously using an open gas exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated Chl fluorescence chamber head (Li-6400-40 leaf chamber fluorometer; Li-Cor, Inc.) according to Flexas *et al*. (2007). These measurements were typically carried out on days 10-13 after the start of the treatments. All measurements were performed at 25 ºC and 1500 µmol photons m^{-2} s^{-1} (10% blue light). The reference CO_2 concentration (C_a) was set at 400 µmol CO_2 mol^{-1} air, and vapor pressure deficit (VPD) was kept at 2.0 ± 0.2 kPa.

In addition to net photosynthesis (A_N) and g_s, the sub-stomatal CO_2 concentration (C_i) was calculated. The possible occurrence of C_i overestimation was evaluated in preliminary experiments in all treatments as follows. Average g_s values were found to be much higher (data not shown) than those typically causing cuticular-associated C_i overestimations (Boyer *et al*., 1997; Flexas *et al*., 2009). Also, leaf Chl fluorescence images (obtained using a Fluor-CAM; PSI Instruments, Brno, Czech Republic; data not shown) demonstrated that patchiness did not occur, indicating that heterogeneous stomata closure did not cause errors in the calculation of C_i (Terashima, 1992).

The incorporated fluorometer allowed determination of the actual PSII efficiency ($\Phi_{PSII} = (F'_m - F') / F'_m$, where F' and F'_m are the steady-state and maximum Chl fluorescences, respectively) (Genty *et al*., 1989). F'_m was determined with a light-saturating pulse of *c*. 8000 µmol photons m^{-2} s^{-1}.

The ETR was then calculated as Φ_{PSII} • PPFD • α. In this equation, α (a term

that includes the product of leaf absorbance and the partitioning of absorbed quanta between PSI and PSII) was determined for each treatment as the slope of the relationship between Φ_{PSII} and Φ_{CO2} (i.e. the quantum efficiency of gross CO_2 fixation), obtained by varying the light intensity under nonphotorespiratory conditions in an atmosphere containing < 1% O_2 (Valentini *et al*., 1995). Three light curves per Zn treatment were recorded to determine α.

Mesophyll conductance (g_m) was estimated from combined gas exchange and Chl fluorescence measurements (Harley *et al*., 1992) as A_N /(C_i - (Γ*(J_{flu} + 8(A_N+ R_d)) / (J_{flu} - 4(A_N+ R_d))) by the variable J method. In this equation, A_N and C_i were obtained from gas exchange measurements at saturating light, whereas Γ* was taken after Bernacchi *et al*. (2002), and dark respiration was used as a proxy for R_d (Pinelli & Loreto, 2003). J_{flu} is the electron transport rate calculated from fluorescence measurements, R_d is the mitochondrial respiration in the light, and Γ* is the CO_2 compensation concentration in the absence of mitochondrial respiration.

Calculated g_m values were used to convert A_N- C_i into A_N- C_C curves (where C_C is the chloroplastic CO_2 concentration) using the equation C_C= C_i - (A_N /g_m). The maximum carboxylation and ETR capacities ($V_{c,max}$ and J_{max}, respectively) were calculated from the A_N-C_C curves, using Rubisco constants and the temperature dependence of Rubisco kinetic parameters described on a C_C basis by Bernacchi *et al*. (2002). The Farquhar model was fitted to the data by applying iterative curve-fitting (minimum least-square difference) using Microsoft Excel's Solver tool.

Photosynthesis limitation analysis

Relative photosynthetic limitations were partitioned into their functional components, following the method of Wilson *et al*. (2000), modified by Grassi & Magnani (2005) to take into account g_m. This approach requires measuring A_N, g_s, g_m and $V_{c,max}$, and it allows for the partitioning of photosynthesis limitations into different components related to stomatal (SL) and mesophyll conductance (MCL) and leaf biochemical characteristics (BL), compared with a hypothetical reference state when g_s, g_m and $V_{c,max}$ are at their maximum. This approach makes it possible to compare absolute or relative limitations to assimilation over any period of time, assuming that a reference maximum assimilation rate can be defined as a standard

(see Grassi & Magnani (2005) for further details and formulae underlying this rationale). In our case, control plants were fixed as reference maximum values, and their limitations were set to 0 (see Table 3). Diffusional (DL) and nonstomatal (NSL) limitations are taken as the sum of SL and MCL, and of MCL and BL, respectively.

Respiratory measurements

An isotope ratio mass spectrometer (Delta Plus XP; Thermo LCC, Bremen, Germany) with a dual-inlet system from the Serveis Científico Tècnis of the Universitat de les Illes Balears (UIB) was used for respiratory measurements. Analysis of respiration and oxygen-isotope fractionation was performed in 10 cm^2 leaf discs at a controlled temperature of 25 ºC as in Florez-Sarasa *et al*. (2007). The electron partitioning through the alternative oxidase pathway (τ_a) was calculated as $(\Delta n - \Delta c) / (\Delta a - \Delta c)$, where Δn, Δc, Δa are the oxygen isotope fractionation in the absence of inhibitors, in the presence of SHAM (hydroxy-salicylic acid) and in the presence of KCN, respectively. For inhibitor treatments, leaf discs were incubated in the presence of 10 mM KCN for 30 min and the Δa value obtained was 32‰. As cytochrome oxidase pathway discrimination has been shown to be remarkably constant in several species (Ribas-Carbó *et al*., 2005), a Δc value of 20.0‰ was used. The individual activities of the cytochrome oxidase (v_{cyt}) and alternative oxidase (v_{alt}) pathways were obtained from the total oxygen uptake rate (V_t) and τ_a as $V_t (1 - \tau_a)$ and $V_t \tau_a$, respectively. Four discs per treatment were collected for respiratory measurements 10-12 d after Zn treatments were imposed.

Experiments on stomatal conductance responses and application of the Ball-Woodrow-Berry model

Stomatal conductance responses were studied with the Li-6400 gas exchange system using three different treatments: leaf desiccation (DES treatment); exogenous application of abscisic acid (ABA treatment); and changes in relative humidity (RH treatment). The DES treatment was carried out by cutting the leaf petiole in air and letting the leaf desiccate for ≥90 min under ambient conditions. Measurements were taken every 1 min, using four leaves per treatment. Exogenous application of ABA consisted of a single application of 100 μM ABA (ABA was previously dissolved in MeOH) to the plant nutrient solution. Measurements were taken in attached leaves

for 4 d after the start of the treatment, using four replicates per treatment. In the RH treatment, 70%, 50% and 30% RH values were used. Measurements were carried out once g_s had reached a steady-state level (typically 20-40 min after changing the RH value), using four leaves per treatment. DES and RH experiments were carried out in plants not treated with ABA.

To assess the effects of Zn stress on the stomatal conductance response to environmental conditions and its coupling to photosynthesis, the model proposed by Ball *et al*. (1987) was used, plotting g_s vs $(A_N H_S)/C_s$, where A_N is the net photosynthesis rate (in μmol CO_2 $m^{-2}s^{-1}$), C_s is the CO_2 concentration at the leaf surface (μmol mol^{-1}) and H_S is the RH value (%). Data obtained from the A_N- C_i curves and RH and ABA measurements (9-25 data points depending on the treatment) were used.

Xylem sap collection

In order to analyze ABA concentration, sugar beet xylem sap was obtained using leaf petioles as described elsewhere (López-Millán *et al*., 2000a). Malate dehydrogenase (MDH, EC 1.1.1.37) was used as a cytosolic contamination marker by checking the activity in xylem sap against the corresponding activities in petiole total homogenates.

Xylem from six plants per treatment was analyzed for ABA following the procedures described in Albacete *et al*. (2008) on a high-performance liquid chromatography (HPLC) /mass spectrometry (MS) system (CEBAS-CSIC, Murcia, Spain).

Enzyme assays

Extracts used for the measurement of carbonic anhydrase (CA, EC 4.2.11) activity were prepared by grinding fresh leaf discs (0.95 cm^2) in 1 ml of extraction buffer (50 mM HEPES-NaOH, pH 8.3, 0.5 mM EDTA, 10 mM dithiothreitol (DTT), 10% (v /v) glycerol and 1% (v /v) Triton X-100) at 4 ºC. Extracts were centrifuged at 2,400*g* for 10 min at 4 ºC. Supernatants were put in Eppendorf tubes and frozen at -20 ºC until assayed. CA activity was measured using a method adapted from that of Gillon & Yakir (2000). Assays were carried out in 7-ml flat-bottom glass vials placed on ice with continuous stirring. A volume of 4.5 ml of reaction buffer (Na-Barbital 20 mM, pH 8.3) was supplemented with 75 μl of extract, and 1.5 ml of CO_2-saturated water

(at 0 ºC) was added to start the reaction. CA activity was obtained from the reaction time of pH change from 8.3 to 7.3.

Scanning electron microscopy

Leaf samples were taken from sugar beet plants grown according to Sagardoy *et al*. (2009), and electron microscopy was used to study leaf surfaces (scanning electron microscopy (SEM); ICB-CSIC, Zaragoza, Spain) and cryo-fractured leaf pieces (low temperature-SEM (LT-SEM); Cryotrans CT-1500, Oxford, UK). A Hitachi S-3400 N microscope (Krefeld, Germany) was used to visualize hydrated leaf surfaces. Fresh pieces were trimmed to an adequate size, mounted on stubs and observed directly (uncoated) with an accelerating voltage of 1 kV. Also, fresh leaf sections were mounted on aluminum stubs with adhesive (Gurrs, O.C.T. B.D.H, UK; Gurr®, OCT compound, BDH, Poole, UK), cryofixed in slush nitrogen (-196 ºC), cryotransferred to a vacuum chamber at -180 ºC, and fractured using a stainless steel spike. Once inside the microscope, samples underwent superficial etching in a vacuum (at -90 ºC and 2 kV for 120 s), and were overlaid with gold (Au) for observation. Fractured samples were observed at low temperature using a Zeiss digital scanning microscope (DSM 960), employing secondary and back-scattered electrons (SEM-BSE).

Hydrated leaf surfaces were analyzed by measuring stomatal density and pore size on the adaxial and abaxial sides of the leaf. Stomatal density was calculated from SEM images by measuring the number of stomata in a given leaf surface. Stomatal density data are the mean ± SE of ten (adaxial) and three (abaxial) images in each treatment. Pore size was measured manually using the Adobe Photoshop CS3 image analysis software. Pore size data are the mean ± SE of 50 (adaxial) and 25 (abaxial) stomata from the same images used in stomatal density measurements.

The spaces occupied by intercellular air spaces and substomatal cavities were quantified using the SEM images (nine images from control leaves and eight from 300 μM Zn-grown plants) and the ImageJ Image Processing and Analysis software from the Wright Cell Imaging Facility (http://www.uhnresearch.ca/facilities/wcif). Chloroplast size was measured manually in each SEM image by counting the number of pixels occupied by the axis of the chloroplast relative to the scale, using the Adobe

Photoshop CS3 image analysis software (for 18 chloroplasts each in the control and high-Zn treatments, using nine and eight images, respectively).

Protoplast and chloroplast isolation and size measurements

Intact protoplasts and chloroplasts were isolated as described in González-Vallejo *et al*. (2000) and using the chloroplast isolation kit (Sigma), respectively, and measured using the Adobe Photoshop CS3 image analysis software. Data shown are for 40 protoplasts and 30 chloroplasts in each treatment (obtained using eight different images in each case).

RESULTS

Growth parameters

High Zn concentrations in the nutrient solution reduced whole-plant DW and leaf area (Table 1), and also shoot and root DW (data not shown) in sugar beet plants grown in a glasshouse. Plants treated with high Zn contained less water than control plants (Table 1). These results agree with previously reported data for Zn-stressed sugar beet plants grown at lower PPFD in a growth chamber (Sagardoy *et al.*, 2009).

Table 1 *Growth parameters, water content (WC) and zinc (Zn) concentrations in sugar beet plants grown in a glasshouse in hydroponics with different Zn concentrations for 14 d. Data are the mean ± SE of eight replicates. Different letters indicate significant differences (Duncan's test) at $P < 0.05$.*

	Zn treatment		
	1.2 μM	100 μM	300 μM
DW (g per plant)	2.8 ± 0.4 a	1.3 ± 0.2 b	0.8 ± 0.2 b
Area per leaf (cm^2)	136.7 ± 5.5 a	48.3 ± 5.2 b	29.8 ± 3.0 b
WC (%)	96.8 ± 0.2 a	91.1 ± 0.1 b	87.6 ± 0.3 c
Zn in roots (μg g^{-1} DW)	136.1 ± 15.0 a	218.4 ± 2.9 b	202.9 ± 0.1 c
Zn in shoots (μg g^{-1} DW)	129.7 ± 11.9 a	1223.7 ± 64.5 b	1184.3 ± 102.7 b

Zinc concentration increased significantly in tissues of plants grown with high Zn in the nutrient solution; 1.5-to 1.6-fold in roots and almost 10-fold in shoots (Table 1). Leaf Zn concentrations were markedly higher than those found in a previous growth chamber study (Sagardoy *et al*., 2009). This was probably a result of the reduced plant growth rates in the glasshouse (this study) as compared with growth chamber conditions (Sagardoy *et a*l., 2009). However, total amounts of Zn extracted per plant (362, 1139 and 742 µg in the 1.2, 100 and 300 µM Zn treatments, respectively) were comparable to those found in Sagardoy *et al*. (2009). Zn allocation (as a percentage of total Zn, for shoot : roots) was 78 : 22%, 92 : 8% and 94 : 6% in the 1.2, 100 and 300 µM Zn treatments, respectively.

Photosynthetic parameters and respiratory measurements

Leaves of plants grown in high Zn showed 50% decreases in photosynthetic rate (A_N), with no significant differences between the 100 and 300 µM Zn treatments (Table 2). However, although there was a tendency for some parameters to decrease with excess Zn, Duncan analysis showed no significant differences at $P < 0.05$ in F_v/F_m ($P < 0.061$), ETR ($P < 0.056$) and Φ_{PSII} ($P < 0.056$) (Table 2). There were significant

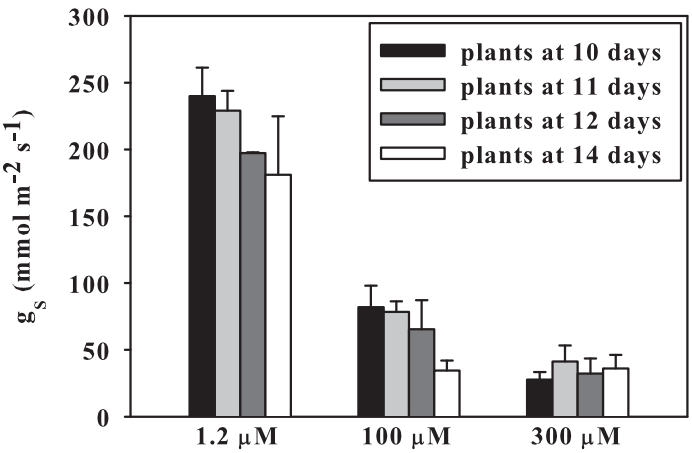

Fig. 1. *Stomatal conductance (g_s) of sugar beet plants grown in a glasshouse in hydroponics with different zinc (Zn) concentrations, measured 30 s after clipping of leaves with a portable leaf porometer. Measurements were carried out 14 d (first batch of plants) or 10 to 12 d (second batch of plants) after the beginning of treatments. Data are the mean ± SE of four replicates.*

differences in F_v/F_m only when Tukey's analysis ($P < 0.027$), a softer statistical test, was used. Stomatal conductance (g_s) was reduced by 70% in excess Zn, whereas mesophyll conductance was reduced by 44%, and in both cases differences between the 100 and 300 μM Zn treatments were not significant. Similar g_s changes were observed when using a leaf porometer (Fig. 1).

The slope of the relationship between Φ_{PSII} and Φ_{CO2} (α) was found to be 0.44 in all Zn treatments (not shown). The intercept of the relationship, which indicates the amount of electrons channeled to alternative sinks (Laisk & Loreto, 1996; Long & Bernacchi, 2003), was very close to zero, being slightly negative in the controls (-0.024) and in plants grown at intermediate Zn concentrations (-0.031) and positive but of similar magnitude (0.025) in the 300 μM Zn-grown plants (not shown). As a negative Φ_{PSII} is not possible, these differences are mere statistically non-significant deviations from the origin. At the measuring CO_2 concentration (400 μmol CO_2 mol^{-1} air), C_i and C_C were lower (30-38% and 35-48%, respectively) in plants grown in an excess of Zn, although no differences were found between the two high Zn concentrations (Table 2). The A_N vs C_i and A_N vs C_C curves also showed that, in excess Zn, C_i reached values of 700-800 μmol CO_2 mol^{-1} air, whereas C_C never reached values of 400 μmol CO_2 mol^{-1} air (Fig. 2). Analysis of the data shown in Fig. 2 indicated that there were no significant differences between treatments in slope ($V_{c,max}$); $P < 0.198$) and (J_{max}), although the latter parameter could not be calculated in the 300 μM Zn treatment (Table 2).

The A_N vs C_i curve for Zn-treated plants saturated at values below the saturation values for control plants, which would appear to reflect a biochemical limitation that is not associated with CO_2 availability. However, when A_N was expressed on a C_C basis, CO_2 availability was found to be clearly responsible for the decreased photosynthesis. Data from the limitation analysis also showed that biochemical limitations were negligible in excess Zn-grown plants (Table 3). In other words, the 'apparent' biochemical limitation observed in the A_N vs C_i curves can be fully explained by the decreased mesophyll conductance in excess Zn-treated plants.

Similar to Grassi & Magnani, (2005) the control treatment can be defined as the actual reference where all three parameters (g_s, g_m and $V_{c,max}$) were at their

Table 2. *Photosynthetic parameters measured with a Li-6400 gas exchange system at 400 µmol CO_2 mol^{-1} air in sugar beet plants grown in hydroponics with different zinc (Zn) concentrations. Data are the mean ± SE of five replicates. Different letters indicate significant differences (Duncan's test at P < 0.05).*

	Zn treatment		
	1.2 µM	100 µM	300 µM
A_N (µmol CO_2 m^{-2} s^{-1})	21.4 ± 1.2 a	12.4 ± 1.4 b	11.1 ± 1.8 b
F_v/F_m	0.821 ± 0.001 a	0.807 ± 0.005 a	0.802 ± 0.008 a
ETR (µmol e^- m^{-2} s^{-1})	143.8 ± 3.1 a	114.0 ± 5.8 a	120.0 ± 17.3 a
Φ_{PSII}	0.218 ± 0.005 a	0.173 ± 0.009 a	0.182 ± 0.026 a
g_s (mol CO_2 m^{-2} s^{-1})	0.231 ± 0.033 a	0.070 ± 0.014 b	0.055 ± 0.010 b
g_m (mol CO_2 m^{-2} s^{-1})	0.389 ± 0.091 a	0.243 ± 0.055 ab	0.204 ± 0.048 b
C_i (µmol CO_2 mol^{-1} air)	286 ± 8 a	200 ± 11 b	176 ± 17 b
C_C (µmol CO_2 mol^{-1} air)	221 ± 19 a	143 ± 14 b	115 ± 9 b
$V_{c,max}$ (µmol CO_2 m^{-2} s^{-1})	104.4 ± 4.5 a	102.8 ± 5.0 a	126.5 ± 19.1 a
J_{max} (µmol CO_2 m^{-2} s^{-1})	128.7 ± 6.7 a	122.6 ± 1.3 a	

Table 3. *Photosynthesis limitation parameters (%) in sugar beet plants grown in hydroponics with different zinc (Zn) concentrations. The control, 1.2 µM Zn treatment was taken as a reference, for which all limitations were set to 0 (see text for a full explanation). TL, total conductance; SL, stomatal conductance; MCL, mesophyll conductance; BL, biochemical limitation. Data are the mean ± SE of five replicates*

	Zn treatment	
	100 µM	300 µM
TL	42 ± 6	48 ± 8
SL	38 ± 5	42 ± 7
MCL	4 ± 2	5 ± 2
BL	0	0

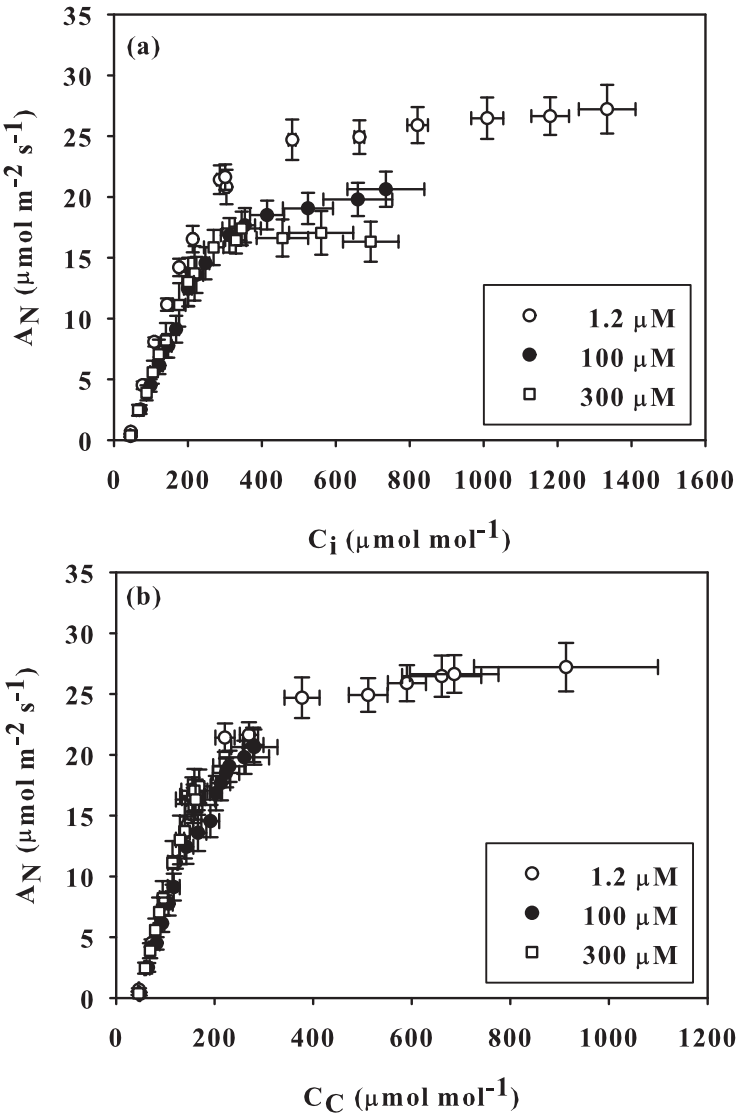

Fig. 2. *Relationships between net photosynthesis (A_N) and (a) substomatal (C_i) and (b) chloroplastic (C_C) CO_2 concentrations in sugar beet plants grown in hydroponics with different zinc (Zn) concentrations. Data are the mean ± SE of five replicates.*

maximum: g_s and g_m declined in response to Zn treatment, whereas $V_{c,max}$ did not change significantly (although it tended to increase; Table 2). Therefore, the same $V_{c,max}$ value was used for all treatments, so that all biochemical limitations (BL) were 0 (Table 3). Using the A_N, g_s, g_m and $V_{c,max}$ values found, stomatal conductance limitation (SL) was estimated to account for 79% -86% of the total limitation (TL), whereas nonstomatal limitations (NSL = MCL + BL) were only 14% and 21% in the 100 and 300 µM Zn-treated plants, respectively (Table 3). The implications of the fact that $V_{c,max}$ values tended to increase with excess Zn merit some consideration. Although mathematically one could calculate a negative limitation, such a value would imply the alleviation of a pre-existing limitation in the control plants. But, if control plants had some limitation, then the net photosynthesis displayed by them would not be really maximal and therefore control plants could not be used as a reference. Establishing a hypothetical optimal state may be uncertain, as maximum values in all three parameters (g_s, g_m and $V_{c,max}$) would result in net photosynthesis values higher than any of those measured but still unknown; that is, the total limitation could not be properly stated and hence a limitation analysis would not be possible. Because of this, even when $V_{c,max}$ values were (non-significantly) higher in treated than control plants, BL was considered null and not negative, as explained by Flexas *et al*. (2009). Nevertheless, we also performed the analysis allowing negative limitations to occur. The results were identical in 100 µM plants, whereas in 300 µM plants SL, MCL and BL become 49, 6 and -7, respectively, as compared with 42, 5 and 0 when BL was forced to 0. In other words, the main conclusion remains the same, that is, that SL was by far the most important limitation and BL did not occur in response to high Zn.

Stomatal conductance experiments

The response of stomatal conductance was studied by inducing stomatal closure, in a short-term (DES) and a long-term (ABA) treatment, and also by using different vapor pressure deficits (RH treatment). After detachment, g_s increased transiently for a few minutes in control plants. This transient gs increase was smaller at 100 µM Zn and absent at 300 µM Zn (Fig. 3a). These data show the progressive inability of stomata in Zn-treated plants to respond to hydraulic stimuli. In the long-term

experiments with ABA, g_s decreased markedly in the controls whereas in plants grown in 300 μM Zn it was not affected (Fig. 3b). Also, when relative humidity was reduced, g_s decreased in the controls but not in the two high-Zn treatments (Fig. 3c).

The Ball–Berry model (g_s vs (A_N.H_s) /C_s) distinguished clearly between control and 300 μM Zn-treated plants (Fig. 4a). However, when ABA was used, stomata closed in the controls and the data for controls and 300 μM Zn-treated plants had similar slopes (Fig. 4b).

Biochemical parameters and respiratory measurements

CA activity in leaves was similar in control and 100 μM Zn-treated plants, whereas in plants grown at 300 μM Zn it increased markedly (by 80%; Table 4). Xylem ABA concentrations were reduced by 70% at 100 μM Zn compared with the controls, whereas at 300 μM Zn changes were not statistically significant (Table 4).

Leaf respiration increased 2-fold in both high-Zn treatments when compared with the control. The increase was more marked for the cytochrome oxidase pathway (COP; 2.3-fold) than for the alternative oxidase pathway (AOP; 1.8-fold) respiration pathway (Table 5).

Scanning electron microscopy images

Leaf surface samples were scanned at x300 and x3000. Both the adaxial (Fig. 5c) and abaxial (Fig. 5d) epidermis of high-Zn plants had a smoother appearance than those of control plants (Fig. 5a,b). Moreover, excess Zn induced a decrease in both stomatal density and size (Table 6, Fig. 5). A closer look at stomata showed structural differences, and plants grown at 300 μM Zn (Fig. 6c,d; adaxial and abaxial sides, respectively) had rounder stomata and smaller stomatal slits than the control plants (Fig. 6a,b; adaxial and abaxial sides, respectively). In some cases, the stomatal slit of high-Zn leaves appeared to be completely sealed.

Mesophyll anatomical features and the arrangement of chloroplasts around the mesophyll cell plasma membrane were different in plants grown under high Zn concentrations than in the controls. LT-SEM showed a more compact mesophyll tissue in Zn-treated plants (Fig. 7c) than in the controls (Fig. 7a), with smaller cells.

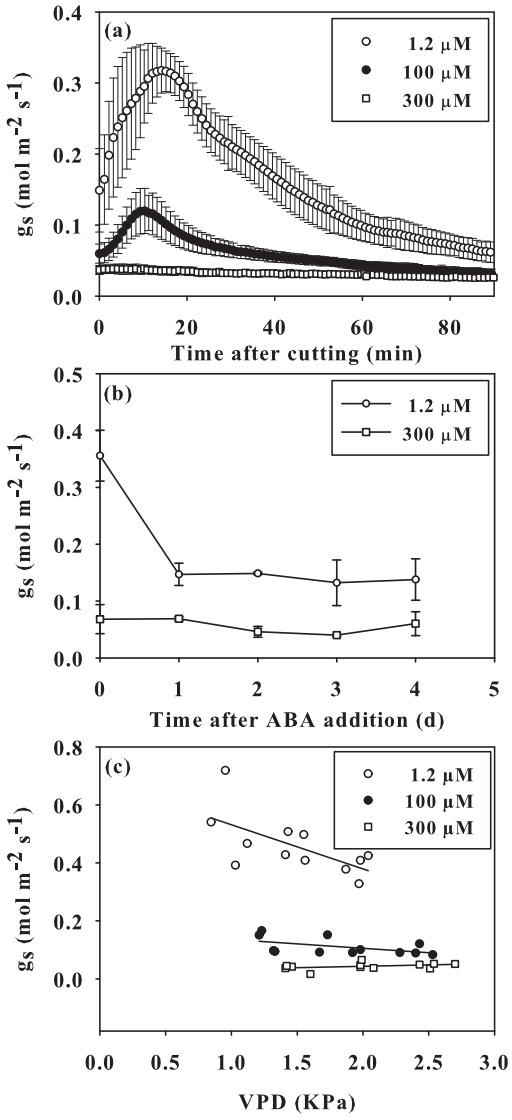

Fig. 3. *Variations in stomatal conductance (g_s), measured with a Li-6400 gas exchange system, in sugar beet plants grown in hydroponics with different zinc (Zn) concentrations in response to three different treatments: (a) leaf desiccation by cutting the leaf petiole and taking measurements every 1 min (DES; mean ± SE of four replicates); (b) exogenous addition of 100 µM ABA (ABA; mean ± SE of three replicates); and (c) relative humidity (RH) treatment, where measurements were taken at 70%, 50% and 30% RH in four leaves per treatment. VPD, vapor pressure deficit.*

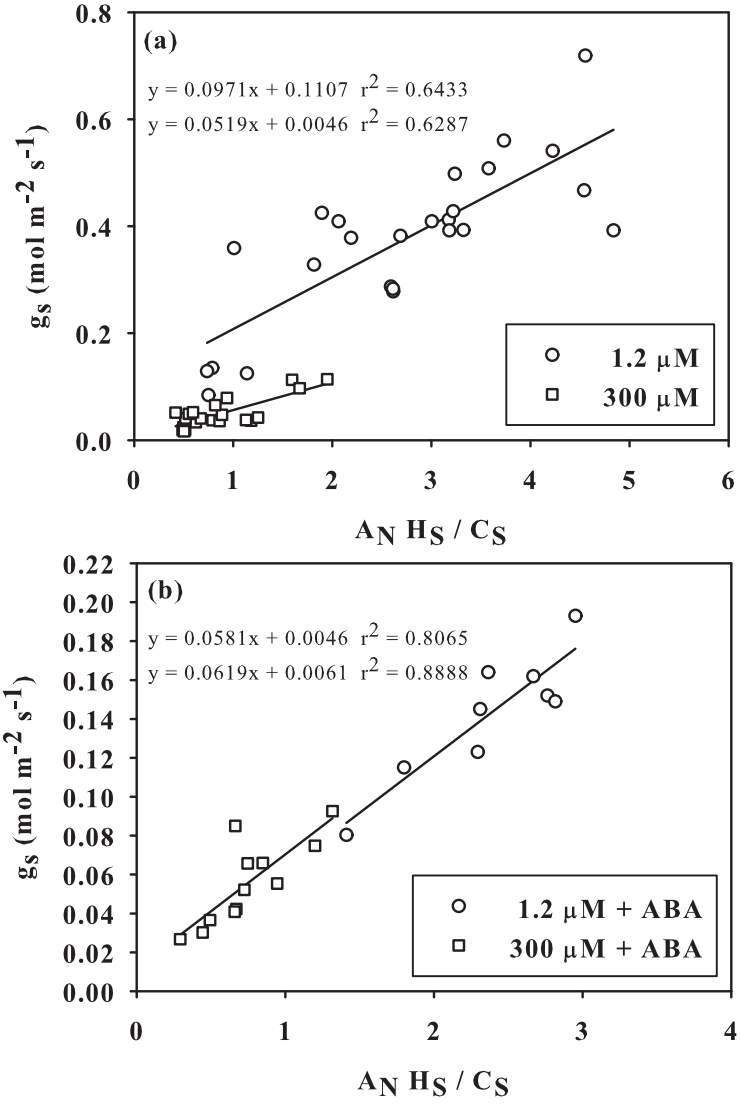

Fig. 4. *Ball-Berry model equations, calculated in sugar beet plants grown in hydroponics with different zinc (Zn) concentrations before (a) and after (b) ABA treatment application. A total of 9-25 data points obtained from control and Zn-stressed plants were used.* g_s*, stomatal conductance.*

Table 4. *Carbonic anhydrase (CA) activity in leaf extracts and abscisic acid (ABA) concentration in xylem sap from sugar beet plants grown in hydroponics with different zinc (Zn) concentrations. Data are the mean ± SE of five and six replicates, respectively. Different letters indicate significant differences (Duncan's test) at P < 0.05.*

	Zn treatment		
	1.2 µM	100 µM	300 µM
CA (µmol CO_2 m^{-2} s^{-1})	305.5 ± 12.3 a	328.8 ± 29.6 a	550.5 ± 44.7 b
ABA (ng ml^{-1})	111.4 ± 22.7 a	33.5 ± 5.0 b	63.6 ± 7.1 ab

Table 5. *Total leaf respiration (V_t) and the contribution of the cytochrome oxidasepathway (COP; v_{cyt}) and alternative oxidase pathway (AOP; v_{alt}) in sugar beet plants grown in hydroponics with different zinc (Zn) concentrations. Data are the mean ± SE of four replicates. Different letters indicate significant differences (Duncan's test) at P < 0.05.*

	Zn treatment		
	1.2 µM	100 µM	300 µM
V_t (µmol O_2 m^{-2} s^{-1})	0.37 ± 0.04 a	0.81 ± 0.02 b	0.80 ± 0.05 b
v_{cyt} (µmol O_2 m^{-2} s^{-1})	0.25 ± 0.03 a	0.59 ± 0.02 b	0.58 ± 0.04 b
v_{alt} (µmol O_2 m^{-2} s^{-1})	0.12 ± 0.02 a	0.22 ± 0.01 b	0.23 ± 0.01 b

Table 6. *Stomatal density and pore size in the abaxial and adaxial epidermis of hydroponically grown control and 300 μM zinc (Zn)-grown sugar beet plants. Stomatal density data are the mean ± SE of ten (adaxial) and three (abaxial) images, and pore size data are the mean ± SE of 50 (adaxial) and 25 (abaxial) stomata from the same images used in stomatal density measurements (Fig. 5). Different letters indicate significant differences (Duncan's test) at P < 0.05.*

	Zn treatment			
	1.2 μM adaxial	1.2 μM abaxial	300 μM adaxial	300 μM abaxial
Density (stomata mm^{-2})	218 ± 7 a	223 ± 3 a	172 ± 5 b	156 ± 12 b
Pore size (range in μm)	9–22	10–18	7–14	6–15
Pore size (mean in μm)	14.5 ± 0.3 a	14.0 ± 0.5 a	10.8 ± 0.2 b	10.1 ± 0.4 b

Isolated protoplasts were smaller in 300 μM high-Zn plants than in controls (33 ± 1 vs 46 ±3 μm, respectively; n = 40). Also, intercellular spaces and abaxial and adaxial substomatal cavities were smaller in the high-Zn leaves. The space occupied by intercellular air spaces decreased by 31% in the highest Zn treatment, from 9.8% (controls) to 6.8% (300 μM Zn-grown plants) of the whole mesophyll, whereas that occupied by the substomatal cavities decreased by 27%, from 5.2% (controls) to 3.8% (300 μM Zn-grown plants) of the mesophyll.

Chloroplast length was reduced in high-Zn leaves 4 (Fig. 7d) compared with controls (Fig. 7b); data obtained from the SEM images were 2.8 ± 0.2 vs 3.9 ± 0.1 μm (n = 18) in 300 μM high-Zn and control plants, respectively. Isolated chloroplasts were also smaller in high-Zn plants (3.0 ± 0.2 μm) than in the controls (4.5 ± 0.3 μm) (images not shown; n = 30). High Zn concentrations reduced the apparent adherence of chloroplasts to the mesophyll cell plasma membrane (Fig. 7d) compared with control plants (Fig. 7b).

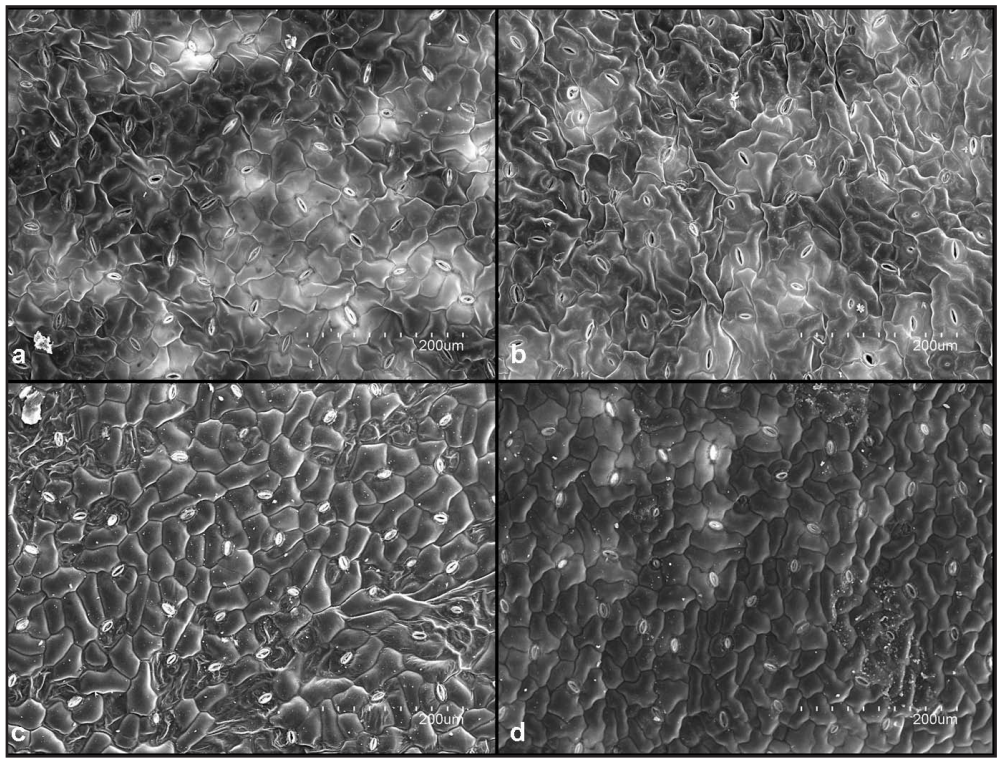

Fig. 5 *Images of the leaf surface (magnification x300) of sugar beet plants grown in hydroponics from control (a, adaxial; b, abaxial) and 300 μM zinc (Zn) (c, adaxial; d, abaxial) treatments, taken with scanning electron microscopy (SEM), showing alterations in guard cell development caused by excess Zn (lower stomatal density on the leaf surface; numbers are presented in Table 6). Stomatal density data were obtained from ten (adaxial) and three (abaxial) images for each treatment.*

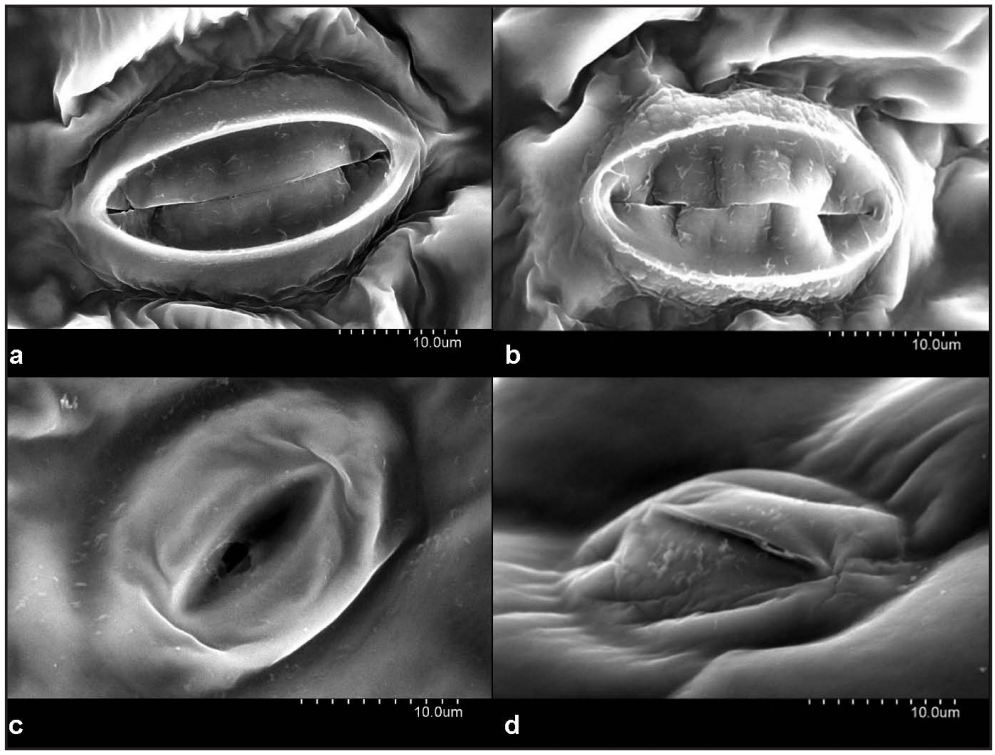

Fig. 6 *Images of stomata (magnification (c) (d) x3000) of sugar beet plants grown in hydroponics from control (a, adaxial; b, abaxial) and 300 μM zinc (Zn) (c, adaxial; d, abaxial) treatments, taken with scanning electron microscopy (SEM), showing reductions in pore size and the sealing of some stomata in response to Zn toxicity. Pore size data were obtained from 50 (adaxial) and 25 (abaxial) stomata from the same images used in stomatal density measurements.*

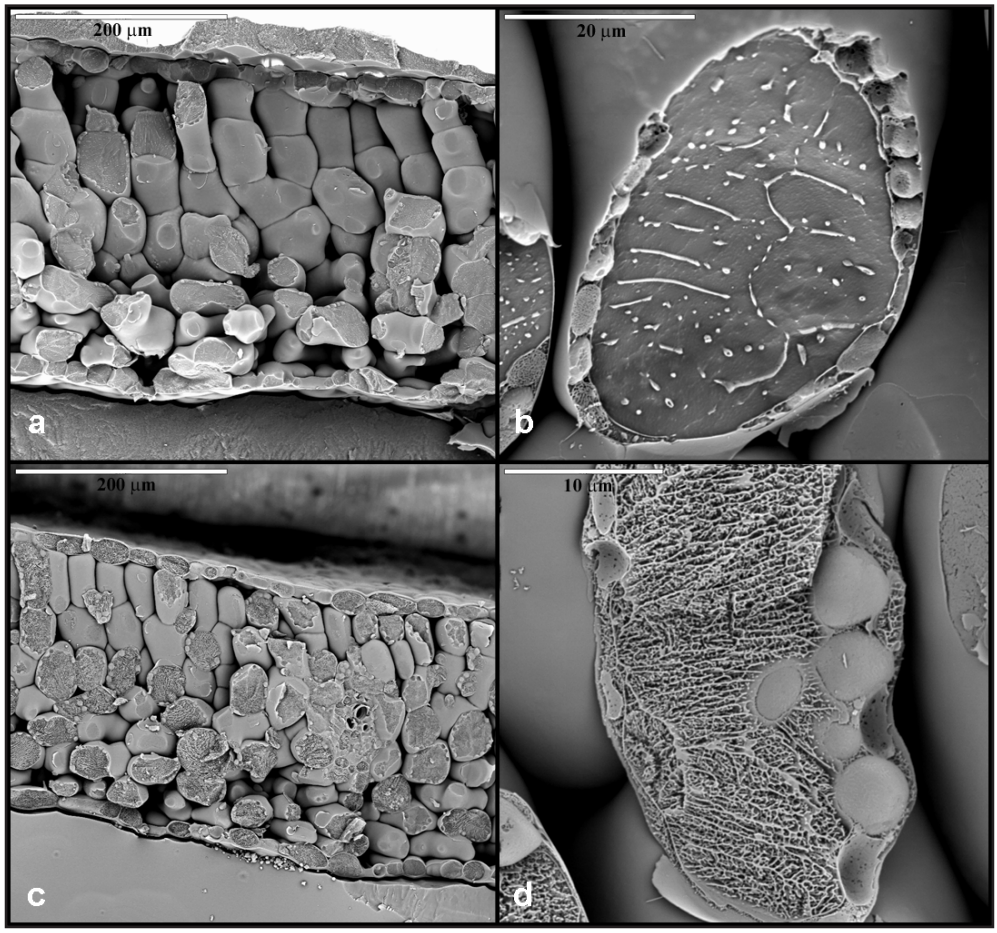

Fig. 7 *Images of leaf cross-sections (a, c) and mesophyll cell details (b, d) of sugar beet plants grown in hydroponics from control (a, b) and 300 µM zinc (Zn) (c, d) treatments, taken with low-temperature scanning electron microscopy (LT-SEM), showing the excess Zn-mediated reduction in the intercellular spaces in the mesophyll and in the substomatal cavities. We used nine images from control leaves and eight from 300 µM Zn-grown plants, and the images shown are representative of each treatment. Note the different scales in (b) and (d). Light-colored objects on the right in (d) are (c) (d) chloroplasts.*

DISCUSSION

The aim of the present study was to investigate the causes of the decrease in photosynthesis that occurs under Zn stress. Both 100 and 300 μM $ZnSO_4$ resulted in high Zn concentrations in roots and shoots, far above the leaf critical toxicity concentrations (400-500 μg g^{-1} DW; Marschner, 1995), with shoot Zn concentrations being similar in the 100 and 300 μM Zn treatments. Both biomass and photosynthetic rates decreased markedly in Zn-treated plants compared with the controls. Decreased growth and photosynthesis under excess Zn have already been described for other species (Ismail & Azooz, 2005; Vaillant *et al*., 2005; Mateos-Naranjo *et al*., 2008). In a previous study, we described changes in gas exchange properties and photosynthetic pigments in sugar beet grown under high Zn at lower PPFDs than those used in the present study (Sagardoy *et al*., 2009). The most important effect of high Zn on photosynthetic parameters was a 70% decrease in stomatal conductance, with mesophyll conductance decreasing by 44%, whereas other possible causes for photosynthetic rate decreases such as PSII photochemistry were not significantly affected. A photosynthesis limitation analysis (Grassi & Magnani, 2005) revealed that, of a total photosynthesis limitation of 42-48% under excess Zn, up to 38-42% could be accounted for by SLs and only 4-5% could be accounted for by MCLs, whereas significant BLs did not occur. Furthermore, the decrease in stomatal conductance was caused by physical and/or structural stomatal changes, whereas hydraulic and chemical signaling, which usually control stomatal closure (Christmann *et al*., 2005, 2007), were not involved. Stomata of high Zn-treated plants did not respond at all to either chemical or hydraulical signals, and the concentration of ABA in the xylem was decreased rather than increased under excess Zn, indicating that stomatal closure was not mediated by ABA signals. Also, g_s in Zn-treated plants was unaffected by exogenous ABA and changes in VPD. In summary, Zn-treated plants showed a stomatal closure similar to that of control plants supplied with exogenous ABA, according to a Ball-Woodrow-Berry analysis.

Zn-stressed plants had a lower stomatal frequency and a smaller stomatal size than control plants, and similar characteristics were found in *Phaseolus vulgaris* grown in high Zn (Van Assche *et al*., 1980). Scanning electron microscopy showed

that stomata of 300 μM Zn-grown plants were round in shape and had a shorter slit than the stomata of. control plants, and in many cases the slit was apparently sealed with unidentified, wax-like substances. Preliminary experiments (using a 10 s wash with chloroform:MeOH 2:1, a 10 s wash with hexane or a sequential combination of the two procedures) confirmed the waxy nature of these substances. Further studies are needed to elucidate the nature of this stomatal seal, and the mechanism for its accumulation under excess Zn.

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

The photochemistry-related parameters F_v/F_m, ETR and Φ_{PSII} were not decreased significantly under high Zn, consistent with previous observations in sugar beet grown in relatively low PPFDs (Sagardoy *et al*., 2009) as well as in other species (Van Assche & Clijsters, 1986a; Schuerger *et al*., 2003; Dhir *et al*., 2008). This indicates that decreased photosynthesis was not caused by impaired leaf photochemistry. Zn treatments did not induce the operation of alternative sinks for electrons. On the one hand, this evidence comes from the close to 0 intercept of the relationship between Φ_{PSII} and Φ_{CO2} under nonphotorespiratory conditions (Laisk & Loreto, 1996; Long & Bernacchi, 2003). On the other hand, the ETR:A_N ratios increased under Zn excess, from 6.7 in the controls to 10.8 in the 300 μM Zn treatment (calculated from Table 2); values for ETR:A_N + respiration (values taken from Table 5) ratios were lower, at 6.6 (controls) and 10.1 (300 μM Zn), whereas those for the ETR:A_N + respiration + photorespiration (not shown) ratios were lower still, at 5.2 (controls) and 6.9 (300 μM Zn), indicating photorespiration as the cause of the increased ETR:A_N ratios in excess Zn-grown plants and ruling out the existence of alternative sinks for

electrons under Zn excess. The slope of the relationship between Φ_{PSII} and Φ_{CO2} was not affected by the Zn treatments, which suggests that there were no changes either in leaf absorbance or in energy partitioning between PSI and PSII. Photosynthetic biochemistry was also unaffected by Zn, based on the in vivo estimates of $V_{c,max}$ and J_{max}. Nevertheless, Zn excess increased the activity of CA, a metalloprotein with Zn in its active center, although only at 300 μM. Zn was previously reported to inhibit CA in vitro at high concentrations (Ivanov *et al*., 2007). Although it has been suggested that CA is involved in the regulation of mesophyll conductance to CO_2 (Gillon & Yakir, 2000), g_m did not increase under excess Zn, but instead decreased by 44% compared with the control. This decrease, moderate when compared with the much greater decrease in stomatal conductance, may be related to the increase in CA activity.

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

(Priault *et al*., 2006; Juszczuk *et al*., 2007).

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

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Capítulo 6

Carboxylate metabolism in sugar beet plants grown with excess Zn

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ABSTRACT

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

Keywords: Heavy metal toxicity, carboxylate metabolism, sugar beet, Zn toxicity.

INTRODUCTION

Zinc is an essential element for plant cell physiological processes, but can also be toxic when present in excess (Broadley *et al*., 2007). Agricultural soils are often contaminated with heavy metals due to anthropogenic sources, and in these soils, some crops may take up large amounts of Zn that can be stored in edible parts (Broadley *et al*., 2007). High concentrations of Zn in fruits and vegetables pose a threat to food quality and safety, and a risk to animal and human health (White & Broadley, 2005).

Plant roots acquire Zn as Zn(II), and then the metal is distributed throughout the whole plant in a complex series of processes not yet fully elucidated, involving several families of metal transporters (Krämer *et al*., 2007; Pilon *et al*., 2009; Puig & Peñarrubia, 2009; White & Broadley, 2009). In the xylem, Zn could be transported chelated by different small molecules, including organic acids, histidine and nicotianamine (NA) (Broadley *et al*., 2007; Trampczynska *et al*., 2010). With Zn excess, a large part of the Zn in the cell can be chelated by organic acids, amino acids such as histidine and NA, phytate and metallothioneins (Callahan *et al*., 2006; Broadley *et al*., 2007), and most likely stored in vacuoles.

In the model plant sugar beet (*Beta vulgaris* L.), which has a great capacity to accumulate heavy metals (Larbi *et al.*, 2002), Zn toxicity symptoms include Fe deficiency-induced chlorosis in young leaves, altered plant mineral composition, and growth decreases (Sagardoy *et al*., 2009). Zinc excess in sugar beet increases leaf respiration rates and decreases photosynthetic rates due to reductions in stomatal and mesophyll conductance to CO_2 associated with changes in stomatal frequency, morphology and functioning, and also to changes in mesophyll ultrastructure, leading to a CO_2 depletion in the sub-stomatal chamber and at the Rubisco carboxylation site (Sagardoy *et al*., 2010).

Our aim was to investigate the effects of high Zn concentrations in the carboxylate metabolism of *B. vulgaris*, measuring the activities of enzymes involved in these processes in roots and leaves and carboxylate concentrations in roots, xylem sap and leaves.

MATERIALS AND METHODS

Plant material

Sugar beet (*B. vulgaris* L. cv. Orbis) was grown hydroponically in a growth chamber in controlled conditions (Sagardoy *et al*., 2009). Seeds were germinated, pre-cultured for 2 weeks in control conditions and then treatments were imposed (Sagardoy *et al.*, 2009). A concentration of 1.2 µM $ZnSO_4$ was used as the Zn-sufficient control, and Zn excess treatments were 50, 100 and 300 µM $ZnSO_4$. Samples (roots, leaves and xylem sap) were taken 10 days after imposing treatments, frozen in liquid N_2 and stored at -80 ºC.

Enzyme assays

Extracts were made by grinding frozen roots or leaves (100 mg of fresh weight) in a mortar with 1 mL of extraction buffer (López-Millán *et al*., 2001). Activities of five enzymes involved in carboxylate metabolism: malate dehydrogenase (MDH, EC 1.1.1.37), citrate synthase (CS, EC 2.3.3.1), isocitrate dehydrogenase (ICDH, EC 1.1.1.42), fumarase (EC 4.2.1.2) and phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), were measured in 1 mL final volume of the corresponding reaction buffer (López-Millán *et al*., 2001). The experiment was repeated with four different batches of plants, and two plants per treatment were used as replicates within each batch.

Xylem sap collection

Xylem sap was collected from sugar beet petioles by centrifugation as described in López-Millán *et al*. (2000a), using malate dehydrogenase activity as cytosolic contamination marker. Samples were collected from four plants per treatment in three different batches of plants.

Carboxylate analysis

Leaf and root material (ca. 100 mg fresh weight) were extracted in a Restch MM301 mill (Restch, Düsseldorf, Germany) with 1 mL of cold 4% (w/v) meta-phosphoric acid (MPA), supplemented with 200 nmol of isotopically-labeled malic (^{13}C4) and succinic (1,4-^{13}C2) acids (Cambridge Isotope Labs, Andover MA). Homogenates were centrifuged at 15,000*g* for 20 min at 4 ºC, supernatants were collected and the pellets were re-extracted by vortexing with extraction solution for 3 min. Xylem sap was diluted 20-fold with 1 mL of extraction solution, vortexed

for 3 min and centrifuged at 15,000*g* for 5 min at 4 ºC. All samples were filtered (0.22 m, PVDF), taken to a final volume of 2 mL with 0.1% (v/v) formic acid and immediately analyzed by HPLC-TOFMS (Jiménez *et al*., 2010). Four plants of each treatment from the same batch were analyzed. Citric, malic, oxalic and succinic acids were detected and quantified in all tissues analyzed, whereas other carboxylates were below the limits of detection.

RESULTS AND DISCUSSION

The type and extent of the effects of Zn excess on sugar beet carboxylate metabolism were dependent on the Zn concentration in the nutrient solution. Two of the Zn concentrations, 50 and 100 µM, had similar effects, whereas the highest Zn concentration, 300 µM, had markedly different effects.

Effects of 50-100 µM Zn

The treatments with 50 and 100 µM Zn led to nutrient solution Zn(II) concentrations of 46 and 92 µM (as calculated with MINTEQA software), respectively, and to shoot concentrations of approximately 230-250 µg Zn g^{-1} DW (Sagardoy *et al*., 2009). These values are above optimal levels for sugar beet (Benton-Jones *et al*., 1991), and plants show toxicity symptoms (Sagardoy *et al*., 2009).

Root extracts from plants grown with 50 µM Zn showed significant increases in PEPC and CS activities (1.8- and 1.6-fold, respectively), when compared to controls (Table 1). Root fumarase activity decreased by 34%, whereas the activities of ICDH and MDH did not change significantly (Table 1). In the 100 µM Zn treatment, significant increases in root extract activities of CS and PEPC (2.1- and 1.7-fold, respectively) were also observed when compared to controls. The ICDH root activity decreased by 34%, whereas changes in fumarase and MDH were not significant (Table 1)

The total root carboxylate pool did not change with 50 µM Zn. Malic and succinic acid concentrations decreased in this treatment (by 32 and 35%, respectively), whereas citric and oxalic acid concentrations did not change. In the 100 µM Zn treatment, the total root carboxylate pool increased significantly (1.3-fold; Table 2), with citric and malic acid concentrations increasing (4.5- and 1.4-fold, respectively)

and oxalic and succinic acid concentrations not changing significantly compared to the controls (Table 2).

In xylem sap, the total carboxylate pool increased in plants grown with 50 and 100 µM Zn (3.4- and 2.3-fold, respectively) compared to controls. Increases occurred for citric (5.1- and 5.8-fold) and malic acid (4.2- and 3.5-fold) in plants grown with 50 and 100 µM Zn, respectively (Table 2). On the other hand, oxalic and succinic acid concentrations increased 2.5- and 2.7-fold in 50 µM Zn-grown plants, whereas no significant changes were observed in 100 µM Zn-grown plants (Table 2). The transpiration rates of 50-100 µM Zn-grown plants diminished slightly (10-20%) compared to controls (Sagardoy *et al*., 2009).

Table 1. *Enzymatic activities in extracts of roots and leaves (in µmol substrate g^{-1} FW min^{-1}) from 38 d old sugar beet plants grown with different Zn concentrations for 10 days. Data are mean ± SE of four batches (two plants per treatment in each batch). Different letters indicate significant differences (Duncan's test) at $p < 0.05$.*

	Zn treatment			
	1.2 µM	50 µM	100 µM	300 µM
Roots				
ICDH	0.382 ± 0.069 c	0.333 ± 0.090 bc	0.253 ± 0.063 b	0.076 ± 0.018 a
CS	0.072 ± 0.012 a	0.118 ± 0.028 b	0.148 ± 0.022 b	0.141 ± 0.026 b
Fumarase	0.631 ± 0.106 b	0.419 ± 0.055 a	0.526 ± 0.124 ab	0.410 ± 0.044 a
MDH	1.211 ± 0.318 a	1.747 ± 0.471 a	1.178 ± 0.335 a	b.d.l.
PEPC	0.079 ± 0.018 b	0.145 ± 0.044 c	0.131 ± 0.037 bc	0.016 ± 0.013 a
Leaves				
ICDH	0.567 ± 0.085 b	0.743 ± 0.093 c	0.582 ± 0.085 bc	0.295 ± 0.041 a
CS	0.126 ± 0.022 a	0.283 ± 0.025 c	0.253 ± 0.027 bc	0.198 ± 0.032 b
Fumarase	0.420 ± 0.060 a	0.937 ± 0.253 b	1.616 ± 0.391 c	2.403 ± 0.486 d
MDH	9.444 ± 1.347 c	5.838 ± 0.401 b	2.025 ± 1.185 a	2.060 ± 0.271 a
PEPC	0.641 ± 0.117 b	0.542 ± 0.044 b	0.513 ± 0.056 b	0.294 ± 0.027 a

b.d.l.: below detection limit.-

Table 2. *Organic acid concentrations in roots, leaves (in µmol g^{-1} FW) and xylem sap (in µM) of 38 d old sugar beet plants grown with different Zn concentrations for 10 days. For leaves and roots, data are mean ± SE of four plants per treatment (from one batch). For xylem sap, data are mean ± SE of three batches of plants (four plants per treatment). Different letters indicate significant differences (Duncan's test) at $p < 0.05$.*

	Zn treatment			
	1.2 µM	50 µM	100 µM	300 µM
Roots				
Oxalic acid	11.82 ± 0.54 a	11.95 ± 0.22 a	13.31 ± 1.64 a	21.44 ± 0.57 b
Citric acid	0.52 ± 0.10 a	0.48 ± 0.09 a	2.36 ± 0.13 b	2.08 ± 0.77 b
Malic acid	1.98 ± 0.28 b	1.34 ± 0.16 a	2.86 ± 0.10 c	1.47 ± 0.61 ab
Succinic acid	0.17 ± 0.01 b	0.11 ± 0.02 a	0.15 ± 0.01 b	0.07 ± 0.03 a
Total	14.48 ± 0.54 a	13.88 ± 0.37 a	18.68 ± 1.72 b	25.06 ± 1.92 c
Xylem sap				
Oxalic acid	584 ± 131 ab	1483 ± 403 c	690 ± 30 b	553 ± 63 a
Citric acid	56 ± 22 a	286 ± 31 b	324 ± 90 b	212 ± 47 b
Malic acid	462 ± 112 a	1957 ± 333 c	1619 ± 293 bc	1255 ± 331 b
Succinic acid	36 ± 10 b	97 ± 29 c	23 ± 4 b	8 ± 3a
Total	1138 ± 220 a	3823 ± 657 d	2657 ± 279 c	2027 ± 337 b
Leaves				
Oxalic acid	58.09 ± 7.21 b	88.73 ± 6.41 c	63.73 ± 2.43 b	31.78 ± 4.40 a
Citric acid	1.75 ± 0.52 a	4.17 ± 0.72 b	4.63 ± 0.72 b	2.99 ± 1.12 ab
Malic acid	15.66 ± 3.83 a	27.15 ± 5.65 bc	31.62 ± 2.60 c	17.04 ± 4.29 ab
Succinic acid	0.47 ± 0.09 b	0.52 ± 0.13 b	0.51 ± 0.05 b	0.26 ± 0.03 a
Total	75.97 ± 11.37 b	120.57 ± 8.59 d	100.49 ± 5.36 c	52.00 ± 9.12 a

Leaf extracts from plants grown with 50 μM Zn showed significant increases in the activities of CS, fumarase and ICDH (2.2-, 2.2- and 1.3-fold, respectively), compared to controls (Table 1). In these plants, leaf PEPC activity did not change significantly, whereas MDH activity decreased by 38%. In plants grown with 100 μM Zn, a similar pattern was observed; significant increases were measured in the activities of fumarase and CS (3.8- and 2.0-fold), changes were not significant for ICDH and PEPC, and MDH activity decreased by 79% (Table 1).

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

Based on our data, a metabolic model of the carboxylate metabolism in sugar beet plants grown under Zn excess is proposed (Fig. 1). Our results indicate that several changes in the root carboxylate metabolism occur upon treatment with 50-100 μM Zn (Fig. 1A): (i) an increase of anaplerotic C fixation associated to increases in the root activities of CS and PEPC; (ii) an alteration in TCA activity, based on the decreases in ICDH (at 100 μM Zn) and fumarase (at 50 μM Zn) activities; and (iii) an increased flow of carboxylates from roots to leaves via xylem, supported by the several-fold increase in the total carboxylate pool in the xylem sap and the slight (at 100 μM Zn) or no (at 50 μM Zn) change of the same pool in roots (Table 2). Moreover, calculations of C flow in xylem sap based on transpiration rates (Sagardoy *et al*., 2009) and carboxylate concentrations indicate that in 50-100 μM Zn-grown plants, C flow was higher (1.5 and 1 mol C $m^{-2}s^{-1}$, respectively) than in control plants (0.4 mol C $m^{-2}s^{-1}$). In leaves, in contrast to what happens in roots, PEPC activity did not change, TCA activity was enhanced and leaf citric and malic acid concentrations increased 2-3-fold in the 50-100 μM Zn-grown plants (Tables 1 and 2). These results suggest that carboxylates transported from roots are used as respiratory substrates to support metabolism in leaves with low photosynthetic rates. This conclusion is also supported by the increases in leaf respiration observed under Zn excess (Sagardoy

et al., 2010). Increases in xylem carboxylate concentrations and root PEPC activity have also been described in other stresses causing photosynthetic damage, such as Cd toxicity, Fe deficiency and P stress (Johnson & Allan, 1994; López-Millán *et al*., 2000b; Wei *et al.*, 2007). Based on these observations, we hypothesize that anaplerotic C fixation in roots and subsequent transport of carboxylates to shoots may constitute a general mechanism to cope with situations causing reduced photosynthetic activity.

Effects of 300 µM Zn

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

Root extracts from plants grown with 300 µM Zn showed a significant increase in the activity of CS (2-fold) when compared to the activity measured in controls, whereas the activities of ICDH, fumarase and PEPC decreased by 80, 35 and 80%, respectively, and MDH activity was no longer detected (Table 1). In these roots, the total carboxylate pool increased 1.7-fold (Table 2); citric and oxalic acid concentrations increased (4- and 1.8-fold, respectively), whereas succinic acid concentration decreased by 59%, and no significant changes were found for malic acid when compared to controls (Table 2). In xylem sap from 300 µM Zn-grown plants, the total carboxylate pool increased 1.8-fold when compared to controls. Citric and malic acid concentrations increased (3.8- and 2.7-fold, respectively), whereas succinic acid concentration decreased by 78% and no significant changes were observed for oxalic acid (Table 2). The transpiration rates of the 300 µM Zn-grown plants were markedly diminished (by 75%) when compared to controls (Sagardoy *et al.*, 2009).

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

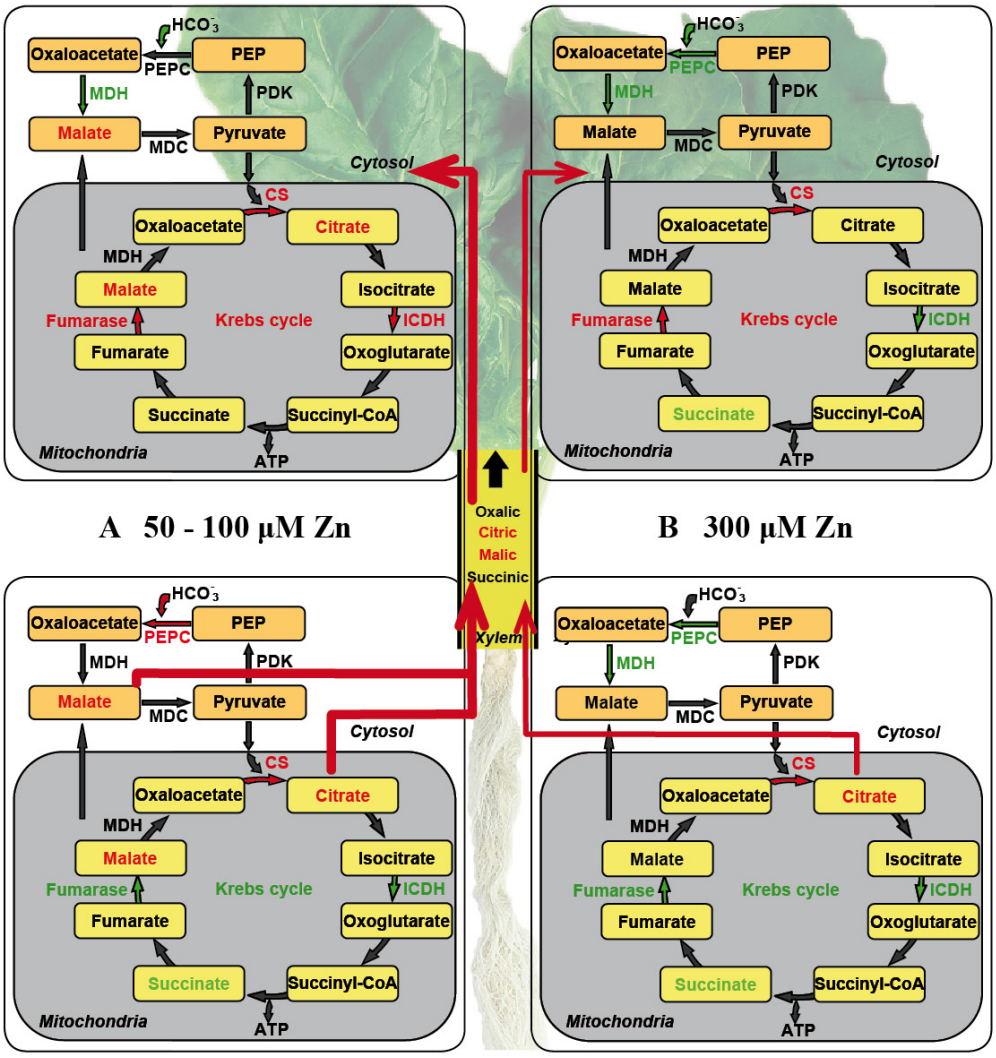

Fig. 1. *Metabolic model for carboxylate metabolism in sugar beet plants grown with Zn excess. Colors indicate either increases (red) or decreases (green). The treatments of 50 and 100 µM Zn cause an enhancement in C assimilation in the root cytosol via increases in PEPC activity. The carboxylates would be exported via xylem providing respiratory substrates to the shoot. This metabolic pathway would not be fully operative at 300 µM Zn, where only CS (in roots and leaves) and fumarase (in leaves) had higher activities than the controls. Phosphoenolpyruvate, PEP; phosphoenolpyruvate carboxylase, PEPC; malate dehydrogenase, MDH; malate decarboxylase, MDC; citrate synthase, CS; isocitrate dehydrogenase, ICDH; pyruvate dehydrogenase kinase, PDK.*

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Capítulo 7

Effects of Zn and Cd toxicity on metal concentrations in the xylem sap of Beta vulgaris *and* Lycopersicon esculentum

ABSTRACT

Physiological effects of Zn and Cd toxicity in plants include alterations in nutrient homeostasis such as a Zn- and Cd-induced Fe-deficiency. The aim of this work was to study the changes in Fe, Zn and Cd concentrations in the xylem sap of two model plant species, sugar beet and tomato, grown with different Zn and Cd concentrations. Plants were grown in hydroponics and treatments were 10 and 50 µM Zn for Zn toxicity in sugar beet, and 10 and 50 µM Cd for Cd toxicity in sugar beet and tomato. Xylem sap was collected 4 and 8 days after the onset of the treatments. Samples were analyzed by ICP/OES and ICP/MS. Xylem sap Zn concentrations of plants treated with Zn excess were between 2- and 8-fold (in 10 µM Zn and 50 µM Zn treatments, respectively) higher than those found in controls, whereas Fe concentrations were not affected. Cadmium treatments significantly increased xylem Cd concentrations but the time of exposure affected differently both species, with sugar beet showing a linear trend, and tomato a rapid initial uptake at 4 days and a decrease thereafter. Iron concentrations in the xylem sap increased in sugar beet plants grown with Cd and decreased in tomato, while Zn concentrations changed only in tomato plants, increasing with the two Cd treatments. Cadmium toxicity resulted in large species-specific alterations of the Fe and Zn xylem sap concentrations, but Zn toxicity in sugar beet did not. These data provide the framework for further studies on the identity of the Zn and Cd chemical forms in the xylem sap.

Keywords: Zn toxicity, Cd toxicity, xylem, sugar beet, tomato.

INTRODUCTION

The effect of heavy metals on plant physiology, including the essential micronutrients Zn and Cu and toxic metals such as Pb, Hg and Cd -present in the environment mostly via anthropogenic sources- has been widely studied in the last decade (Clemens, 2006). One of the major goals of these studies is to understand the interactions between exogenous toxic metals and essential mineral nutrients (Clemens, 2001). Heavy metal toxicity in plants is determined by soil-plant interaction factors, which determine heavy metal mobility to the plant, and by plant specific factors including root absorption, translocation and detoxification mechanisms. Cadmium has many physical and chemical similarities with Zn, as they belong to group II of the periodic table, and therefore may cause competition effects altering mineral balances. Accordingly, different studies report that Cd ions compete with Zn ions for accumulation in shoots (Green *et al*., 2003; Clemens, 2006). In sugar beet, excess of Zn in the nutrient medium decreases Fe translocation from roots to leaves, resulting in Fe deficiency in shoots (Sagardoy *et al.*, 2009). In wheat grown with Cd excess, Zn addition reduces the Cd shoot:root ratio whereas in other crops addition of Zn may have a synergistic effect in Cd uptake (Green *et al*., 2003). Zinc is an essential heavy metal but can also be toxic when present in high amounts in the soil environment (Broadley *et al*., 2007). Zinc excess interferes at the loading site of the roots and decreases the rate of absorption or translocation of essential nutrients to plants, causing mineral imbalances (Rout & Das, 2003). For instance, Cayton *et al.* (1985) reported that the absorption and translocation of plant nutrients such as Fe, Mg, K, P and Ca varied with Zn concentration in the soil. Also, it has been reported that Zn inhibits root Fe translocation in soybean plants (Ambler *et al*., 1970).

Effects on sugar beet (*Beta vulgaris* L.) and tomato (*Lycopersicon esculentum* L.) grown in hydroponics under Zn (Sagardoy *et al.*, 2009, 2010) and Cd excess (Picture 1; Larbi *et al.*, 2002; López-Millán *et al*., 2009b) have been reported previously. These two model plants accumulate large amounts of heavy metals in roots and are able to translocate high amounts of the metals to the shoots *via* xylem sap. Physiological effects of these toxicities in sugar beet and tomato include reductions in plant growth, photosynthetic pigments, photosynthesis and alterations in micronutrient

homeostasis such as a Zn- and Cd-induced Fe deficiency, which could be partially due to a failure in Fe long-distance transport (Larbi *et al*., 2002; López-Millán *et al.*, 2009b; Sagardoy *et al.*, 2009). Interestingly, increases in organic acid concentrations were found in xylem sap of sugar beet plants grown with 50 and 100 μM $ZnSO_4$ (Sagardoy *et al*., 2011) similarly to what occurs in Fe-deficient plants.

Little is known about the chemical form(s) in which these heavy metals are present in xylem sap (Salt *et al.*, 1999; Küpper *et al.*, 2004; Ma *et al*., 2005). Analytical data evidences are available for Ni ligands in xylem sap -His- (Krämer *et al*., 1996) but not for other metals such as Zn and Cd. It has been speculated that Zn can be associated with sulfur ligands such as those in cysteine, glutathione or phytochelatins (Milner & Kochian, 2008). Also, using X-ray absorption, Salt *et al.* (1999) suggested that Zn was mostly found as free hydrated Zn^{2+} ion with a small part portion bound to organic acids in xylem sap from *Thlaspi caerulescens,* although formation of Zn(II)-citrate in xylem sap has been predicted with different speciation software packages (White *et al*., 1981; Mullins *et al*., 1986). On the other hand, studies with the NA-free mutant from tomato chloronerva indicate that NA plays a major role in long distance transport of Zn. With regard to Cd, X-ray data showed that Cd is chelated by oxygen or nitrogen ligands in the xylem (Salt *et al*., 1995), and indirect evidence for an involvement of phytochelatins in translocation of Cd has also been reported (Gong *et al*., 2003). Because this lack of data, the aim of this work was to study the changes induced by Zn and Cd toxicities in xylem sap Fe, Zn and Cd concentrations in sugar beet and tomato plants, thus providing the framework for further studies of heavy metal speciation in the plant xylem sap.

MATERIALS AND METHODS

Plant material

Sugar beet (*Beta vulgaris* L. cv. 'Orbis') and tomato (*Lycopersicon esculentum* L. cv. 'Tres Cantos') seeds were germinated and grown in a growth chamber in hydroponics with half-strength Hoagland nutrient solution as described in Sagardoy *et al*. (2009) and López-Millán *et al*. (2009b), respectively. Four weeks old sugar beet plants were treated with 1.2 μM $ZnSO_4$ (control), 10 and 50 μM $ZnSO_4$ and 10

and 50 μM $CdCl_2$. Three weeks old tomato plants were treated with 0 (control), 10 and 50 μM $CdCl_2$. Plants were grown in these conditions for 4 or 8 days, and then plants were sampled for xylem sap.

Xylem sap sampling

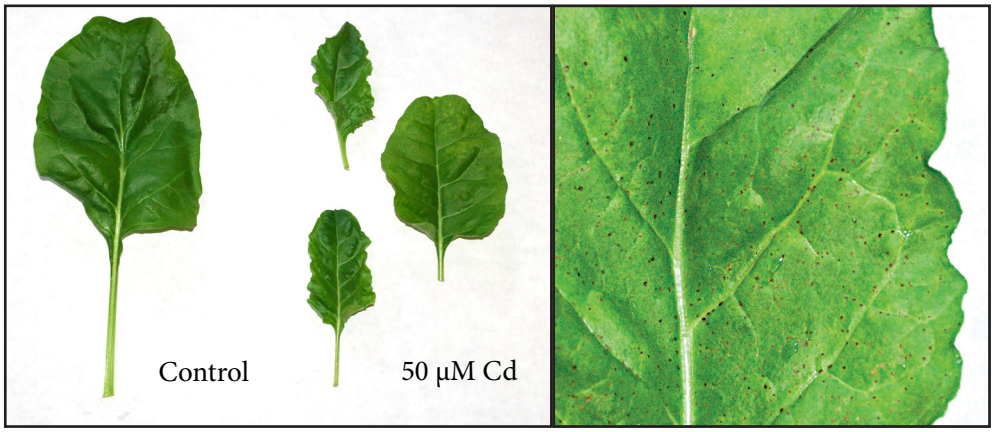
Sugar beet xylem sap was isolated from petioles by excision under water with a razor blade as in Sagardoy *et al*. (2011). Tomato xylem sap was obtained by pooling the fluid bled for 30 min after stem decapitation (Picture 2; López-Millán *et al*., 2009a). Malate dehydrogenase (EC 1.1.1.37) was used as cytosolic contamination marker of xylem sap samples (López-Millán *et al*., 2000). Enzymatic activity was determined with oxalacetate as substrate (Dannel *et al*., 1995) by measuring the decrease in A_{340} due to the enzymatic oxidation of NADH. The reaction was carried out with 5 μL of extract in 0.15 mM NADH, 0.6 mM oxalacetate, and 70 μM Tris-HCl, pH 9.5. Xylem sap was collected from 5 plants per treatment (in 3 different batches of plants) 4 and 8 days after imposing the high Zn or Cd treatments, frozen in liquid N_2 and stored at -80 ºC until analyzed.

Fe, Zn and Cd concentration analyses

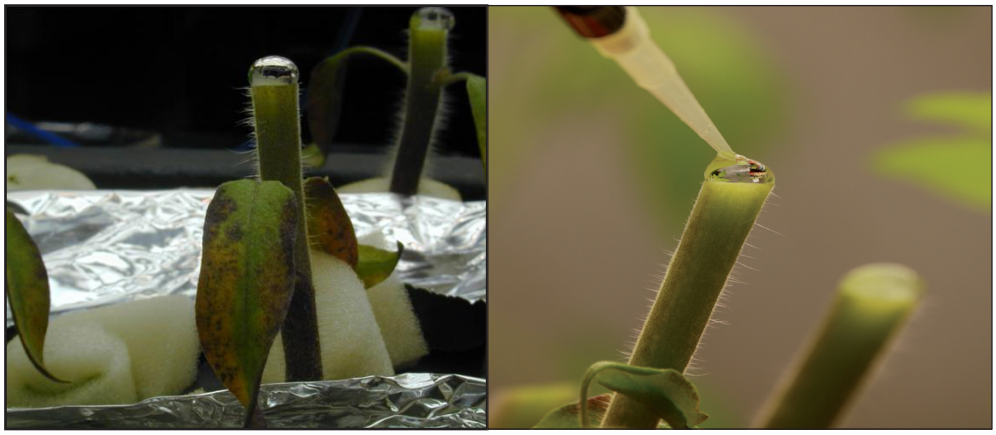
Xylem sap was diluted 20 and 50-fold (for tomato and sugar beet, respectively) and taken to a final volume of 5 mL with 1% HNO_3. Samples were analyzed for Fe, Zn and Cd concentrations by Inductively Coupled Plasma (ICP) using two different detection methods, Optical Emission Spectroscopy (OES) and Mass Spectrometry (MS) for high and low metal concentrations, respectively, at the SCT-SSR (UB, Barcelona).

Organic acid concentration analyses

Tomato xylem sap was diluted 5-fold with 1 mL of cold 4% (w/v) metaphosphoric acid (MPA) in water, containing 200 nmol isotopically labeled malic and succinic acids. Samples were vortexed for 3 min and centrifuged at 15,000*g* for 5 min at 4 ºC. Supernatants were collected and filtered through a 0.22 μm PVDF filter. Samples were taken to a final volume of 2 mL with 0.1% (v/v) formic acid and immediately analyzed by HPLC-TOFMS. Analyses were carried out as in Sagardoy *et al*. (2011). Four replicates of each treatment were done.



Picture 1. *Reduction of leaf growth in sugar beet plants grown with 50 µM Cd for 8 days compared to control leaf (left). Close up detail of a leaf from a plant grown at 50 µM Cd showing necrotic spots (right).*



Picture 2. *Xylem bleeding from tomato stems after shoot decapitation (left) and sampling procedure by pipetting (right). Pictures taken by R. Rellán.*

RESULTS

Zn, Fe and Cd concentrations in sugar beet

In sugar beet, Zn concentration in xylem sap of control plants (1.2 μM Zn) was approximately 19 μM and did not change with sampling time. Plants grown at 10 μM Zn had Zn concentrations approximately 3-fold higher than controls and did not change with sampling time; however, at higher Zn supply (50 μM), Zn concentration increased 4.5- and 7.5-fold when sampled at 4 and 8 days, respectively (Table 1). Iron concentration in xylem sap of control plants was 4.5 μM. In plants grown with excess Zn, xylem Fe concentrations at both sampling dates ranged from 5 to 9 μM (Table 1); these concentrations were always higher than those found in the controls, although differences were not statistically significant. Cadmium concentrations were very low, and below the detection limit in some xylem sap samples.

Table 1. *Metal concentrations (in μM) in the xylem sap of sugar beet plants grown with different Zn and Cd concentrations for 4 and 8 days. Data are means ± SE of five replicates. Different letters in the same row indicate significant differences (Duncan´s test) at p < 0.05.*

S. beet	**Control**	**Zn treatments**			
		10 μM		50 μM	
		4 days	8 days	4 days	8 days
Fe	4.53 ± 0.61 a	8.62 ± 1.23 a	5.25 ± 0.43 a	6.41 ± 0.74 a	6.72 ± 1.73 a
Zn	19.18 ± 3.59 a	58.91 ± 2.07 b	48.67 ± 4.32 b	90.14 ± 7.95 c	148.05 ± 15.60 d
Cd	0.04 ± 0.02 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.02 ± 0.00 a	0.05 ± 0.00 a

S. beet	**Control**	**Cd treatments**			
		10 μM		50 μM	
		4 days	8 days	4 days	8 days
Fe	4.53 ± 0.61 a	4.50 ± 0.72 a	10.69 ± 1.90 c	3.55 ± 0.59 a	7.16 ± 0.64 b
Zn	19.18 ± 3.59 a	16.31 ± 1.56 a	18.81 ± 3.02 a	13.97 ± 1.29 a	25.58 ± 6.42 a
Cd	0.04 ± 0.02 a	3.91 ± 0.81 b	6.47 ± 1.45 bc	5.02 ± 0.75 b	9.67 ± 1.66 c

In the Cd toxicity experiment, Cd concentration was 0.04 µM in xylem sap of sugar beet control plants, whereas Cd concentrations were 4 and 5 µM in the 10 and 50 µM Cd treatments, respectively, when sampled at 4 days, and increased to 6.5 and 10 µM, respectively, when sampled at 8 days (Table 1). Xylem sap Zn concentrations in plants grown with excess Cd ranged from 14 to 26 µM and no differences were found between treatments or sampling dates (Table 1). Iron concentration in xylem sap of plants sampled at 4 days did not change with Cd supply, whereas when plants were sampled at 8 days Fe increased 2.4- and 1.6-fold in the 10 and 50 µM Cd treatments, respectively (Table 1).

Zn, Fe and Cd concentrations in tomato

In tomato plants, Cd concentration in control xylem sap samples was 0.01 µM, whereas in plants grown with excess Cd reached higher levels than those of sugar beet grown with the same Cd concentrations. Also, and conversely to what occurs in sugar beet, Cd decreased at 8 days. Cadmium concentrations in xylem sap were 25 and 95 µM in the 10 and 50 µM Cd treatments, respectively, when sampled at 4 days, and decreased to 9 and 42 µM, respectively, when sampled at 8 days (Table 2). In contrast to sugar beet, high Cd levels were associated with increased Zn and decreased Fe concentrations. Zinc concentration in xylem sap of control tomato plants was 3 µM, significantly lower than in sugar beet control plants, and increased 4.6- and 3.6-fold in xylem sap from 10 and 50 µM Cd treatments sampled at 4 days, and approximately 2.6-fold in xylem sap from both treatments when sampled at 8 days (Table 2).

Table 2. *Metal concentrations (in µM) in xylem sap of tomato plants grown with different Cd concentrations for 4 and 8 days. Data are means ± SE of five replicates. Different letters in the same row indicate significant differences (Duncan´s test) at p < 0.05.*

Tomato	**Control**	**Cd treatments**			
		10 µM		50 µM	
		4 days	8 days	4 days	8 days
Fe	9.76 ± 1.41 b	6.07 ± 2.29 b	7.58 ± 1.15 b	1.18 ± 0.20 a	2.50 ± 0.59 a
Zn	2.67 ± 0.29 a	12.17 ± 0.67 c	7.35 ± 0.51 b	9.55 ± 1.08 bc	7.10 ± 0.30 b
Cd	0.01 ± 0.00 a	25.09 ± 1.61 c	8.66 ± 0.54 b	94.63 ± 7.39 e	41.86 ± 10.95 d

In these plants, Fe concentration in xylem sap did not change significantly in the 10 μM Cd treatment (although it showed a tendency to decrease) and decreased approximately 80% in the 50 μM Cd treatment at both sampling dates (Table 2).

Organic acid concentrations

Concentrations of organic acids were determined in xylem sap of tomato plants grown at different Cd levels, and only citric and malic acid concentrations were found to be above the detection limit. In tomato xylem sap from plants grown with 10 μM Cd, citric acid concentration decreased by 54% at 4 days and did not change at 8 days when compared to controls. In the 50 μM Cd treatment no significant changes were found at 4 days, but increased 2-fold at 8 days (Table 3). Malic acid concentration increased 1.8- and 1.6-fold at 4 days and 3.7- and 3.1-fold at 8 days in plants treated with 10 and 50 μM Cd, respectively (Table 3).

Table 3. *Organic acid concentrations (in μM) in xylem sap of tomato plants grown with different Cd concentrations for 4 and 8 days. Data are means ± SE of four replicates. Different letters in the same row indicate significant differences (Duncan´s test) at p < 0.05.*

Tomato	**Control**	**Cd treatments**			
		10 μM		50 μM	
		4 days	8 days	4 days	8 days
Citric acid	28.34 ± 3.77 b	13.02 ± 2.52 a	26.52 ± 4.98 b	24.72 ± 3.08 b	55.82 ± 8.39 c
Malic acid	90.45 ± 19.94 a	159.45 ± 26.26 b	332.42 ± 55.55 c	146.05 ± 33.56 ab	275.72 ± 43.79 c

DISCUSSION

Cadmium toxicity had different effects in Fe, Zn and Cd concentrations in xylem sap depending on the plant species, metal concentration in the nutrient solution and exposure time. In sugar beet, xylem sap Cd concentration increased progressively with time, whereas tomato xylem sap Cd concentrations reached maximum values at day 4 and then decreased (ca. 50%) at day 8. At this sampling time, the Cd concentration in tomato xylem sap was similar to that in the nutrient solution in both treatments, suggesting that Cd exclusion mechanisms were overruled or were not

active. These species-based differences of Cd concentrations in xylem sap with time of exposure might reflect different mechanisms of root Cd-uptake, sequestration or xylem loading rates, which could determine differences in tolerance to Cd toxicity. Data presented in Larbi *et al.* (2002) indicate that sugar beet is able to transport 50-74% of Cd from the solution to the shoots. Also, the metal allocated in shoots in tomato plants increased from 34% to 61% when Cd in nutrient solution increased from 10 to 100 µM (López-Millán *et al.*, 2009b).

Differences in Cd loading found in these two species may cause differences in metal balances. Accordingly, Zn and Fe concentrations in xylem sap were affected by Cd excess in both species in opposite ways. In sugar beet plants, the xylem sap Zn concentration did not change and the Fe concentration increased with Cd excess. However, in tomato plants the xylem sap Zn concentration increased and the Fe concentration decreased in response to Cd. In previous works with sugar beet shoots, Zn concentrations decreased and Fe concentrations remained constant in the presence of Cd (Larbi *et al.*, 2002) suggesting that, in sugar beet, Cd could compete with Zn and not with Fe, possibly at the xylem loading step and less likely in the leaf uptake step. In tomato, shoot Fe concentrations were reduced by 50% when plants were grown at 10 µM Cd (López-Millán *et al*., 2009b). These results and those presented here suggest a competition between Fe and Cd at the tomato root level that does not exist in sugar beet. This might be associated to the higher Cd concentrations found in tomato with respect to those found in sugar beet. Interestingly, no competition but a synergistic effect was observed in tomato between Cd and Zn, and this was not found in sugar beet. This could be related to the induction of the Fe transporter, due to the Cd-induced Fe deficiency in tomato (and not in sugar beet), which is known to transport also Cd. It should be noted that although Zn xylem sap concentrations were higher in the presence of Cd, Zn concentration did not change in shoots (Sagardoy *et al*., 2009), indicating a tight regulation of Zn shoot concentrations probably at the xylem unloading and/or leaf uptake levels.

Excess Zn in sugar beet caused only minor effects on xylem sap Fe concentration, which showed slight, not significant increases. However, excess Zn caused a 40% decrease in shoot Fe concentration (Sagardoy *et al*., 2009), suggesting that the

competition occurs at leaf uptake level.

Malic acid concentration in tomato xylem sap increased in both Cd treatments and at both sampling dates. Several Krebs cycle enzyme activities, including MDH and CS, are increased in roots of tomato plants grown with Cd toxicity (López-Millán *et al*., 2009), and their activities might account for malic acid concentration increase in xylem sap. Although Fe reductase activity does not change or even decreases in tomato plants exposed to Cd toxicity (López-Millán *et al*., 2009b), Fe deficiency symptoms are indeed observed in these plants. These observations, together with the previously commented competition between Cd and Fe in tomato, suggest that increased organic acid synthesis in roots and transport to the shoots may take place in tomato plants exposed to Cd, as it occurs in Fe deficiency. This response would act as an anaplerotic pathway for CO_2 fixation, that could help overcome the reduction in photosynthetic rates reported in these plants (López-Millán *et al*., 2009b).

In summary, this work shows that Cd toxicity alters the Fe-Zn balance differently in tomato and sugar beet, and the effects also depend on the time exposure to Cd. Sugar beet is able to take up Cd in a dose and time dependent manner whereas in tomato Cd concentration initially shows a sharp increase (10-fold higher than sugar beet). This may be the cause of the lower tolerance of this species to Cd toxicity when compared to sugar beet, and could be possibly related to the competition effect observed betwen Cd and Fe. Finally, these data provide the basis for future studies on putative Cd and Zn ion ligands by LC-MS.

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Capítulo 8

Discusión general

La mayoría de los estudios realizados sobre la toxicidad de metales pesados en plantas se han desarrollado con especies hiperacumuladoras, y aún es muy poco lo que se conoce de la fisiología de especies de interés agronómico sometidas a este estrés. En esta tesis he trabajado con dos especies comerciales, la remolacha y el tomate, cultivadas en condiciones controladas y con exceso de Zn y Cd. El objetivo general ha sido describir los cambios fisiológicos que se producen en estas plantas y buscar las causas de los mismos, así como comprender mejor sus mecanismos de transporte y tolerancia.

La remolacha y el exceso de Zn

La remolacha, *Beta vulgaris*, se ha utilizado habitualmente como planta modelo de Estrategia I en nuestro grupo, ya que es muy eficiente en la toma y utilización de Fe en condiciones de deficiencia del mismo, y existen numerosos datos sobre su fisiología y bioquímica (http://www.stressphysiology.com). Además, se sabe que es una planta capaz de acumular altas concentraciones de metales pesados (Introducción, Tablas 2 y 3), lo que fue confirmado por Larbi *et al*. (2002) al estudiar su crecimiento en presencia de Cd o Pb. La similitud entre Cd y Zn, así como la importancia de este último como elemento esencial pero peligroso si está en exceso, llevó a su estudio en profundidad.

Lo primero que observamos es que, al aumentar la concentración de Zn en la solución nutritiva, el tamaño de las plantas disminuye. Las hojas tienen los bordes enrollados y muestran clorosis en las hojas jóvenes, principalmente cuando las plantas se cultivan con 100 µM de Zn. De forma progresiva con el tratamiento, las raíces se acortan y oscurecen y se desarrollan raíces laterales. Los análisis minerales realizados indican que la concentración de Zn aumenta en toda la planta cuando se compara con la concentración existente en las plantas control; es decir, la remolacha absorbe Zn en exceso y lo moviliza desde la raíz a las hojas en todos los tratamientos. De todo el Zn que acumula la remolacha, entre el 72% (control) y el 78% (300 µM) se encuentra en las hojas (Sagardoy *et al.*, 2009). Por tanto, la remolacha se comporta como una especie tolerante al Zn y no como una especie sensible, que muchas veces acumulan la mayor parte del metal en la raíz. La remolacha es capaz

de mantener una concetración del metal en hoja alrededor de 250 $\mu g.g^{-1}$ (4 veces superior a la encontrada en controles) en todos los tratamientos de Zn. Por tanto, es una buena planta modelo para el estudio de la homeostasis de Zn en especies no hiperacumuladoras. Recopilando los datos obtenidos durante esta tesis se puede hacer un seguimiento del metal desde que es absorbido por la raíz hasta su almacenamiento en hoja.

El exceso de Zn en solución causa alteraciones en la absorción de otros nutrientes esenciales. El Zn puede impedir la entrada de ciertos elementos a las células por competición en transportadores no específicos, pero también puede tener un efecto sinérgico en otros elementos y favorecer su asimilación. En el caso de la remolacha, en las raíces disminuyen las concentraciones de N, Mg y K mientras que aumenta la absorción de P, Fe y Cu, aunque este último sólo en las raíces finas (Sagardoy *et al*., 2009). Parte del Zn absorbido se almacenará en las vacuolas de las células de raíz y parte se transportará *via* xilema a las hojas. Los análisis han mostrado que a medida que aumenta la concentración de Zn en la solución nutritiva y el tiempo de exposición al tratamiento, la concentración de Zn en xilema también aumenta, y además que el transporte de Fe no se ve afectado. Aún no se conoce la forma química en que es transportado el Zn. Se ha especulado con que el Zn podría unirse a moléculas que contienen ligandos de S, como cisteína, glutatión o fitoquelatinas (Milner & Kochian, 2008). Los estudios de Salt *et al*. (1999) con la especie hiperacumuladora *Thlaspi caerulescens* indican que se transporta como ión libre Zn^{2+} y, en menor proporción, unido a ácidos orgánicos. La formación del ligando Zn^{2+}-citrato también ha sido predicha con diferentes programas de especiación química (White *et al*., 1981; Mullins *et al.*, 1986). En nuestro caso, hemos encontrado una concentración de citrato en xilema entre 5 y 6 veces superior a la control, pero para confirmar que se forma un complejo con Zn serán necesarios estudios ulteriores con técnicas como LC-MS, capaces de detectar este tipo de compuestos.

En las hojas encontramos que el aumento de Zn reduce la concentración de otros elementos como N, Mg, K, Fe y Mn. Parece, por tanto, que el Zn interfiere en la descarga de Fe del xilema a hoja. El descenso en Fe y Mg explicaría la clorosis que observamos en las hojas de plantas crecidas con 50 y, principalmente, con 100 µM

Zn. La concentración de todos los pigmentos fotosintéticos disminuye, y el ciclo de las xantofilas (ciclo VAZ) se encuentra activado en estos tratamientos. Además, en el caso de las plantas crecidas con 50 μM de Zn encontramos un ligero incremento en la actividad de la reductasa férrica de raíz (FC-R) y el comportamiento se asemeja al que presenta la remolacha con una deficiencia moderada de Fe (Larbi *et al.*, 2006). Las plantas con el tratamiento de 100 μM Zn muestran una clorosis muy marcada, y a la activación del ciclo VAZ hay que sumarle un incremento en la disipación de energía (NPQ), que llega a ser doble del valor normal. Sin embargo, en estas plantas no hay un aumento en la actividad de la FC-R, lo que lleva a pensar que el estrés fotosintético que sufren no estaría relacionado directamente con una deficiencia inducida de Fe (Sagardoy *et al*., 2009).

Las plantas crecidas con 300 μM de Zn muestran mayores daños: la transpiración, conductancia estomática y concentración de CO_2 sub-estomática se encuentran muy reducidas, una tendencia que ya ocurría en los tratamiento intermedios. pero que en estas plantas se observa incluso a intensidades de luz bajas. El estudio del proceso de fotosíntesis confirma que aunque la tasa de fijación de C en hoja se reduce en un 50%, ni los parámetros que se asocian a la fotoquímica del proceso (eficiencia del fotosistema II y tasa de transporte electrónico) ni los que se asocian a su bioquímica (tasa de carboxilación y regeneración de la Rubisco) se encuentran significativamente afectados. En cambio, las conductancias estomática y del mesófilo disminuyen en un 70% y en un 44%, respectivamente. Esto lleva a que las concentraciones de CO_2 en la cámara subestomática y en el cloroplasto desciendan más de un 30% y 35%, respectivamente (Sagardoy *et al*., 2010). Es decir, en remolacha crecida en exceso de Zn se reduce la fijación de C en las hojas principalmente porque no llega el CO_2 en cantidad suficiente al cloroplasto. La razón que impide el paso de CO_2 a través de los estomas parece residir en los cambios fisiológicos que sufren los mismos con un exceso de Zn. Por otro lado, comprobamos que la apertura estomática no respondía a estímulos ambientales ni químicos, y las imágenes de microscopía electrónica de la superficie foliar mostraban estomas más pequeños, de forma más redondeada y menos numerosos que los observados en hojas controles. Además de estas alteraciones, en muchos casos los estomas se encontraban recubiertos por

una sustancia desconocida, que pareció ser de naturaleza grasa, y que sellaba la abertura estomática. Las imágenes de microscopía de cortes transversales de hoja mostraban un mesófilo más compacto que en las hojas controles, con menos espacios intercelulares, que podría obstaculizar la difusión de CO_2 hasta el interior de las células y, por tanto, ser causa de la disminución en la conductancia del mesófilo (Sagardoy *et al*., 2010). Las remolachas crecidas con exceso de Zn deben enfrentarse a una menor disponibilidad de CO_2 en las hojas, y el metabolismo de los carboxilatos se ve alterado en hojas y raíces.

El estudio de las actividades enzimáticas en extractos de raíces y hojas y el análisis de ácidos orgánicos en raíces, xilema y hojas nos dan una visión general de lo que puede ocurrir. En plantas crecidas con 50 y 100 µM de Zn se observa un comportamiento similar para hacer frente al estrés. En las hojas no hay cambios en la actividad de la anhidrasa carbónica (CA), la fosfo*enol*piruvato carboxilasa (PEPC) muestra una ligera tendencia a la baja, mientras que la malato deshidrogenasa (MDH) reduce su actividad entre un 38% (50 µM) y un 79% (100 µM). Sin embargo, aumentan las actividades de las enzimas citrato sintasa (CS), isocitrato deshidrogenasa (ICDH) y fumarasa, involucradas en el ciclo de Krebs (Sagardoy *et al*., 2011). Sabemos que la fijación de C se ha visto reducida, pero el ciclo de Krebs aumenta su actividad al igual que el proceso de respiración, que es el doble de lo normal. La energía que obtiene de estos procesos podría usarse en el transporte y compartimentación del Zn para minimizar el impacto que tiene el exceso del metal. Las concentraciónes de ácido cítrico y málico se multiplicaron por dos en las hojas, probablemente actuando como esqueletos carbonados necesarios para la obtención de energía. Se comprobó que existe un flujo de los carboxilatos que se sintetizan en las raíces mediante una ruta anaplerótica, transportándose *via* xilema a las hojas. En raíces, tanto PEPC como CS aumentan su actividad, hay una tendencia al descenso de ICDH y fumarasa y no se observan cambios significativos en MDH. Esto indica que se está aumentando la fijación de C en las raíces y que el ciclo de Krebs se encuentra alterado: se sintetizan ácidos que no se utilizan allí sino que se envían a las hojas. Cuando se compara la concentración total de carboxilatos en raíces con la del control, no es diferente en las plantas con 50 µM Zn, mientras que se encuentra algo aumentada en las de 100

μM Zn debido al aumento de los ácidos cítrico y málico. En cambio, hay un fuerte incremento en las concentraciones de ácidos cítrico y málico en el xilema en ambos tratamientos de Zn, y también aumentan el oxálico y succínico en el de 50 μM Zn. Este proceso de fijación de C en raíces y transporte a hojas para obtención de poder reductor podría ser una estrategia común en las plantas que se enfrentan a muchos estreses que provoquen daños en el proceso fotosintético (Andaluz *et al*.,2002).

En las plantas crecidas con 300 μM de Zn no encontramos este mecanismo, debido probablemente a que los daños causados por el exceso de Zn son demasiado grandes. No hay fijación anaplerótica de C en las raíces vía PEPC y, aunque aumenta la concentración de cítrico y málico en xilema respecto a las controles, la tasa de transpiración en estas plantas es muy baja y el total de carboxilatos en hojas disminuye (Sagardoy *et al*., 2011). Sólo la CS tiene una actividad mayor en raíces y hojas que en las controles. En hojas, la fumarasa tiene una actividad casi 6 veces mayor que en los controles, y tasa de respiración es también muy alta. Estos hechos pueden contribuir a la obtención de poder reductor con el que hacer frente a la toxicidad por Zn, utilizando esta energía para compartimentar el metal y reparar el daño oxidativo celular.

El tomate y la toxicidad por Cd

El tomate, *Lycopersicon esculentum*, es un cultivo de gran importancia cuyo destino es el consumo humano. Esta especie es capaz de absorber y movilizar metales pesados aunque la acumulación final no sea alta en el fruto (Introducción, Tabla 2). Hemos comprobado que el tomate puede ser un buen modelo para investigar ligandos de unión a Cd en xilema, ya que transporta altas concentraciones de este metal y además la obtención de xilema es relativamente sencilla. Los datos obtenidos también han mostrado que el tomate se enfrenta de diferente manera a la toxicidad por Cd que la remolacha (Larbi *et al*., 2002), siendo mucho más sensible a este estrés.

En presencia de Cd, el crecimiento de las plantas de tomate se ve reducido y se observa un oscurecimiento de las raíces. Las hojas tienen síntomas de clorosis cuando crecen con 10 μM Cd y zonas necróticas cuando lo hacen con 100 μM Cd.

La entrada de Cd en las raíces se realiza a través de transportadores de otros metales y esto afecta a la absorción de micronutrientes esenciales. En raíces encontramos menores concentraciones de K y Mn y una mayor concentración de Mg. En plantas cultivas con 100 µM de Cd también aumentan las concentraciones de Fe, Zn y Cu, quizá por un efecto sinérgico. La distribución del Cd en el tomate es diferente según a qué concentración se haya cultivado la planta, siendo el Cd en raíces el 66% del total a 10 µM de Cd y el 39% a 100 µM (López-Millán *et al*., 2009). Esto indica que a bajas concentraciones de Cd el tomate utiliza la estrategia de acumular el metal en la raíz y disminuir su transporte a la hoja, algo que no se veía en la remolacha crecida con el mismo tratamiento. Sin embargo, a altas concentraciones la raíz no parece ser capaz de mantener esa estrategia de almacenamiento y no puede evitar una alta movilización de Cd hacia la hoja. Según los análisis de xilema, la concentración de Cd a los 4 días de tratamiento es el doble que la concentración en solución nutritiva, pero a los 8 días disminuye hasta valores similares a los de la solucion nutritiva, lo que sugiere que en ningún momento los mecanismos de exclusión son efectivos en esta especie. Además, la presencia de Cd afecta al transporte de Fe y Zn en tomate, ya que cuando aumenta la concentración de Cd en xilema aumenta también la de Zn y disminuye la de Fe, y cuando disminuye el Cd disminuye el Zn y aumenta el Fe. La interacción del Cd con estos dos metales es diferente, ya que la absorción de Fe en raíz está dificultada, y que la concentración de Fe en hoja es sólo la mitad de la de una planta control. Por otro lado, el efecto sinérgico del Cd en el transporte de Zn en xilema no lleva a cambios en la concentración final de Zn en hoja, lo que nos indica una regulación fina de la descarga de Zn de xilema a hoja y/o en la toma de Zn por la célula del mesófilo.

Este comportamiento también es diferente del observado en remolacha con los mismos tratamientos de Cd, ya que la remolacha puede acumular en hoja entre el 50 y el 74% del Cd cuando crece en concentraciones de 10 y 50 µM Cd, respectivamente. El aumento de la concentración del metal en xilema en remolacha es proporcional a la concentración de Cd en solución nutritiva y al tiempo de exposición, y además no afecta a la concentración de Zn en el xilema aunque tiene un efecto sinérgico con el Fe. En remolacha, el Cd sí que disminuye la concentración de Zn en hoja, pero esto

no ocurre ni en xilema ni en raíz, por lo que podemos especular que en esta especie existe una competición entre Cd y Zn (no entre Cd y Fe como en tomate), y que esta competición podría ocurrir en el paso de descarga de xilema a hoja.

La toxicidad por Cd afecta a la fotosíntesis en hoja de tomate reduciendo progresivamente la fijación de C y la conductancia estomática. La concentración de pigmentos fotosintéticos también disminuye, aunque de manera más marcada en los tratamientos más bajos de Cd, como también ocurre en remolacha tratada con Cd (Larbi *et al*., 2002). Al igual que en remolacha con exceso de Zn, se midieron las actividades de PEPC, MDH, CS, ICDH y fumarasa en raíces y hojas de tomate con toxidad de Cd. En las raíces de ambos tratamientos de Cd encontramos que la actividad de todas estas enzimas se incrementaba respecto de la situación control, lo que nos indica que se está produciendo una fijación de C por esta vía anaplerótica. De hecho, la concentración de ácido málico en xilema aumenta con el tiempo de exposición a Cd, la actividad de PEPC en hoja no cambia respecto de las controles, mientras que las actividades de CS y MDH en hoja aumentan progresivamente (López-Millán *et al*., 2009). Nos encontraríamos de nuevo con una fijación de C en raíces y un flujo de carboxilatos hacia las hojas para la obtención de energía, al estar la fotosíntesis gravemente afectada por la toxicidad de Cd. El poder reductor obtenido podría emplearse en detoxificación del metal, sintetizando ligandos de unión y transportándolo a vacuolas, así como en la síntesis de moléculas antioxidantes para proteger a las células del daño oxidativo causado por el Cd.

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Capítulo 9

Conclusiones

1. Concentraciones elevadas de Zn añadido en forma de sal en solución nutritiva reducen el crecimiento, la tasa fotosintética y la transpiración de plantas de remolacha.

2. La remolacha es una especie tolerante al exceso de Zn. Es capaz de transportar y almacenar el metal en la parte aérea pero mantiene una concentración de Zn en hoja constante frente al aumento de metal en solución, posiblemente gracias a mecanismos de exclusión en la raíz.

3. Las plantas de remolacha cultivadas con un exceso moderado de Zn, exhiben síntomas de una deficiencia moderada de Fe. Si bien la toma y transporte *via* xilema del Fe no se ven afectados, su concentración en hoja se reduce a la mitad, indicando un posible mecanismo de competición en la descarga a hoja entre ambos metales.

4. A concentraciones elevadas de Zn, las plantas de remolacha no presentan clorosis, sino que muestran una fuerte reducción del crecimiento y de la tasa fotosintética. La tasa de respiración se dobla, probablemente para aumentar la capacidad de la compartimentación/exclusión del metal.

5. En remolacha, la principal limitación de la fotosíntesis en condiciones de elevado exceso de Zn es la reducción de las conductancias estomática y del mesófilo. En estas condiciones los cambios fisiológicos en los estomas y la estructura interna del mesófilo dificultan la difusión del CO_2.

6. El tomate es una especie sensible a la toxicidad de Cd. En plantas cultivadas con bajas concentraciones de Cd la estrategia de detoxificación consiste en la acumulación de Cd en las raíces, mientras que a concentraciones altas se produce una movilización masiva de Cd a las hojas. El exceso de Cd disminuye el crecimiento y la tasa fotosintética.

7. El exceso de Cd en tomate altera la homeostasis de otros nutrientes, interfiriendo el paso de Fe al xilema y facilitando el de Zn.

8. Tanto la toxicidad de Zn en remolacha como la de Cd en tomate provocan un aumento de la fijación anaplerótica de C en la raíz en forma de ácidos orgánicos, que tiende a compensar la carencia de esqueletos carbonados asociada con la disminución de la fotosíntesis.

9. En ambas especies, la toxicidad de Zn y la de Cd aumentan la actividad del ciclo de Krebs y la respiración, aumentando la disponibilidad de poder reductor para hacer frente al exceso de metal y los daños oxidativos producidos en la célula.

10. Se han definido las condiciones óptimas de toxicidad de Cd y Zn en tomate y remolacha que permiten obtener xilema en concentraciones adecuadas para abordar la especiación de estos elementos en dicho compartimento.

Anexo

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Nutrición vegetal, metales pesados en plantas, cromatografía líquida de alta resolución, fotosíntesis, remolacha, tomate, cinc, cadmio, hierro.

FORMACIÓN ACADÉMICA

Máster	**Centro**	**Fecha**
Biología Molecular y Celular	Universidad de Zaragoza	2007
Postgrado	**Centro**	**Fecha**
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ACTIVIDADES ANTERIORES DE CARÁCTER CIENTÍFICO O PROFESIONAL

Fechas	**Puesto**	**Institución**
Agosto 2004	Prácticas	Hospital Clínico Universitario Zaragoza
Sept-Oct 2004	Prácticas	EEAD-CSIC
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Enero 05 – Dic 09	Doctorando	EEAD-CSIC

IDIOMAS DE INTERÉS CIENTÍFICO (R = regular, B = bien, C= correctamente)

Idioma	**Habla**	**Lee**	**Escribe**
Inglés	C	C	C

PARTICIPACIÓN EN PROYECTOS DE I+D FINANCIADOS EN CONVOCATORIASPÚBLICAS

Título del proyecto: *Estudios sobre la homeostasis de metales en plantas*
Entidad financiadora: MEC (Plan Nacional de Investigación)
Duración desde: Oct 2007 Hasta: Oct 2010
Investigador principal: Javier Abadía Bayona

Título del proyecto: *Adquisición y transporte de metales en plantas*
Entidad financiadora: CICYT (Plan Nacional de I+D)
Duración desde: Dic 2004 Hasta: Dic 2007
Investigador principal: Javier Abadía Bayona

Título del proyecto: *Modeling biochemical proceses in orchards at leaf and canopy levels using hyperspectral data (HyperPeach)*
Entidad financiadora: Belgium Science Policy Office (BELSPO), Belgium
Duración desde: 2005 Hasta: 2006
Investigador principal: Basjan Van Aardt

Título del proyecto: *Nuevos métodos de detección de estrés en vegetación mediante sensores remotos hiperespectrales*
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Duración desde: 2004 Hasta: 2005
Investigador principal: Pablo J. Zarco-Tejada

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ESTANCIAS EN CENTROS EXTRANJEROS

CLAVE: D = doctorado, P = postdoctoral, I = invitado, C = contratado, O = otras (especificar).

CENTRO:	**Okayama University** (Research Institute for Bioresources)		
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CLAVE: D = doctorado, P = postdoctoral, I = invitado, C = contratado, O = otras (especificar)

.

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AÑO:	2007	DURACION:	1 mes
TEMA:	Estudio del aparato fotosintético.	CLAVE:	D

CONGRESOS

Organización de congresos

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OTROS MÉRITOS O ACLARACIONES QUE SE DESEE HACER CONSTAR

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- 2003-2004. Miembro de la asociación: "Ciencias en Acción", organizadora del 2º Foro para Científicos "Empleo-Facultad 2004" en la Universidad de Zaragoza.
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• 2005 Curso de Especialización Postuniversitaria del Programa Master en Mejora Genética Vegetal. Instituto Agronómico Mediterráneo de Zaragoza, CHIEAM.

• 2007 Curso de Estrés Abiótico del Master Internacional de Mejora Genética Vegetal. Instituto Agronómico Mediterráneo de Zaragoza, CHIEAM.

Cursos y Seminarios Recibidos

- 2003 I Seminario de Formación en Genética y Biología Molecular, por Colegio Mayor Universitario Santa Isabel, Zaragoza.
- 2004 VIII Curso nacional de Transplante de Órganos, Células Madre y Terapia Celular. Hospital Clínico Universitario, Facultad de Medicina de la Universidad de Zaragoza.
- 2006 Curso "Fotosíntesis y teledetección de la vegetación". Cátedra UNESCO2006 de la Universidad de Valencia.

Plant Stress Physiology

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