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Short communication

# Carboxylate metabolism in sugar beet plants grown with excess Zn

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ABSTRACT

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



## Introduction

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

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

## Materials and methods

### Plant material

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

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trol, 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 °C.

*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 °C, 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 °C. All samples were filtered (0.22 μm, 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).

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).

**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 ± S.E. 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.

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**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 ± S.E. of four plants per treatment (from one batch). For xylem sap, data are mean ± S.E. 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 ± 3 a |
| 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 |

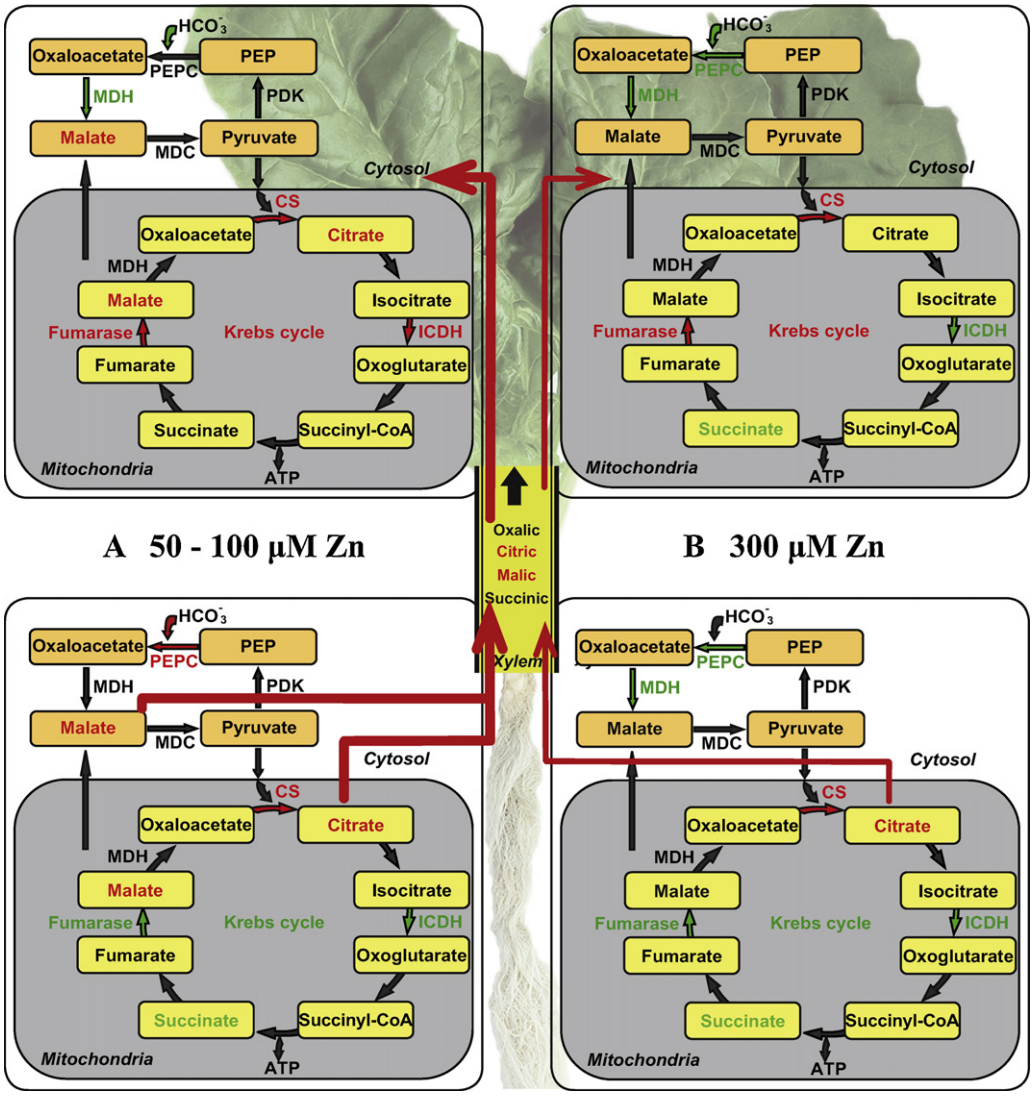


**Fig. 1.** Metabolic model for carboxylate metabolism in sugar beet plants grown with Zn excess. Colors indicate either increases (red) or decreases (green). The treatments of 50 and 100 μM Zn cause an enhancement in C assimilation in the root cytosol *via* increases in PEPC activity. The carboxylates 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. Phospho*enol*pyruvate, PEP; phospho*enol*pyruvate carboxylase, PEPC; malate dehydrogenase, MDH; malate decarboxylase, MDC; citrate synthase, CS; isocitrate dehydrogenase, ICDH; pyruvate dehydrogenase kinase, PDK.

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

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

*Effects of 300 μM Zn*

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

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

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

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

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

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## References

Benton-Jones Jr J, Wolf B, Mills HA. Plant analysis handbook. Athens: Micro-Macro Publishing Inc; 1991.
Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. New Phytol 2007;173:677–702.
Callahan DL, Baker AJM, Kolev SD, Wedd AG. J Biol Inorg Chem 2006;11:2–12.
Jiménez S, Ollat N, Deborde C, Maucourt M, Rellán-Álvarez R, Moreno MA, et al. J Plant Physiol 2010., doi:10.1016/j.jplph.2010.08.010.
Johnson JF, Allan DL, Vance CP. Plant Physiol 1994;104:657–65.
Krämer U, Talke IN, Hanikenne M. FEBS Lett 2007;581:2263–72.
Larbi A, Morales F, Abadía A, Gogorcena Y, Lucena JJ, Abadía J. Funct Plant Biol 2002;29:1453–64.
López-Millán AF, Morales F, Abadía A, Abadía J. Plant Physiol 2000a;124:873–84.
López-Millán AF, Morales F, Andaluz S, Gogorcena Y, Abadía A, De Las Rivas J, et al. Plant Physiol 2000b;124:885–97.
López-Millán AF, Morales F, Abadía A, Abadía J. Physiol Plant 2001;112:31–8.
Pilon M, Cohu CM, Ravet K, Abdel-Ghany SE, Gaymard F. Curr Opin Plant Biol 2009;12:347–57.
Puig S, Peñarrubia L. Curr Opin Plant Biol 2009;12:299–306.
Sagardoy R, Morales F, López-Millán AF, Abadía A, Abadía J. Plant Biol 2009;11:339–50.
Sagardoy R, Vázquez S, Flórez-Sarasa I, Albacete A, Ribas-Carbó M, Flexas J, et al. New Phytol 2010;187:145–58.
Trampczynska A, Küpper H, Meyer-Klaucke W, Schmidt H, Clemens S. Metallomics 2010;2:57–66.
Wei Z, Wong JW, Hong F, Zhao H, Li H, Hu F. Microchem J 2007;86:53–9.
White PJ, Broadley MR. Trends Plant Sci 2005;10:586–93.
White PJ, Broadley MR. New Phytol 2009;182:49–84.