

Isotopic fractionation of zinc in field grown tomato

T.R. Cavagnaro and L.E. Jackson

Abstract: Many of the world's soils are deficient in zinc (Zn), and this has implications for plant and human nutrition. Consequently, there is a need to better understand plant uptake and allocation of Zn. Natural abundances of stable isotopes have been used to gain insight into uptake, assimilation, and allocation of various elements by plants. Inductively coupled plasma mass spectrometry was used to study the fractionation of Zn isotopes in the shoots and fruits of mature tomato plants (*Solanum lycopersicum* L.) grown on an organic farm. Effects of mycorrhizal colonization of roots on Zn fractionation were studied by growing a tomato mutant with reduced mycorrhizal colonization, and its mycorrhizal wild-type progenitor. Fruits of both genotypes were enriched in ^{64}Zn and ^{66}Zn and depleted in ^{67}Zn and ^{68}Zn isotopes, based on calculations that expressed the concentration of each isotope as a percentage of total Zn. The reverse was true of the shoots. Furthermore, shoots of the mycorrhizal genotype were very slightly enriched in ^{64}Zn and ^{66}Zn isotopes relative to those of the reduced mycorrhizal colonization genotype. Possible explanations for fractionation of Zn between shoots and fruits, including differential bonding of Zn to cellular components, processes affecting Zn–phytate–protein complexes, and Zn transport and translocation processes are discussed.

Key words: ICP-MS, isotopic fractionation, *Solanum lycopersicum* L. (tomato), mycorrhiza mutant, stable isotopes, zinc isotopes.

Résumé : Plusieurs sols du monde sont déficients en zinc (Zn), ce qui a des implications pour la nutrition des plantes et des humains. Conséquemment, on doit mieux comprendre l'absorption et l'allocation du Zn chez les plantes. Les auteurs ont utilisé l'abondance des isotopes stables, afin d'examiner l'absorption, assimilation et l'allocation de divers éléments chez les plantes. Ainsi, on a utilisé la spectroscopie de masse inductivement couplée à celle des plasmas, pour étudier le fractionnement des isotopes stables du Zn, dans les tiges et les fruits de plants de tomates (*Solanum lycopersicum* L.) cultivés sur une ferme organique. On a également étudié les effets de la colonisation mycorrhizienne des racines sur le fractionnement de Zn, en cultivant un mutant de la tomate ayant une colonisation mycorrhizienne réduite ainsi que son progéniteur mycorrhizien sauvage. Les fruits des deux génotypes sont enrichis en isotopes ^{64}Zn et ^{66}Zn et dépourvus de ^{67}Zn et ^{68}Zn , sur la base de calculs qui expriment la teneur de chaque isotope en pourcentage du Zn total. Cette situation est inversée dans les tiges. De plus, les tiges du génotype mycorrhizien sont faiblement enrichies en ^{64}Zn et ^{66}Zn , comparativement à celles du génotype arborant une faible mycorrhization. On discute comment on pourrait expliquer le fractionnement du Zn entre les tiges et les fruits, incluant l'attachement différentiel du Zn aux composantes cellulaires, des processus qui affectent les complexes Zn–phytates–protéines ainsi que les processus de transport et de l'allocation du Zn.

Mots-clés : fractionnement isotopique, *Solanum lycopersicum* L. (tomato), mutant mycorrhizien, isotopes stables, isotopes du zinc.

[Traduit par la Rédaction]

Introduction

Globally, 30% of cultivated soils are deficient in zinc (Zn) (Kochian 2000), which has implications for plant growth and nutrition and the nutritive value of crops. With

almost half of the world's population at risk of inadequate Zn intake (Brown and Wuehler 2000), there is a need to increase dietary intake in many regions. To this end, crop-breeding programs to improve the density of micronutrients, including Zn, have been initiated. The success of these programs will depend in part upon the development of efficient screening methods that can detect genotypic variation in Zn acquisition and metabolism. To facilitate this, a thorough understanding of plant Zn uptake, allocation, and metabolism is required.

Zn is an essential component of over 300 enzymes (Hacisalihoglu and Kochian 2003). It plays catalytic, co-catalytic, or structural roles in many plant enzymes (Marschner 1995). Zinc-efficient genotypes are those that are able to grow and yield well under Zn-deficient soil con-

Received 8 January 2007. Published on the NRC Research Press Web site at canjbot.nrc.ca on 3 May 2007.

T. Cavagnaro.¹ School of Biological Sciences, Monash University, Clayton, Victoria, 3800, Australia.

L. Jackson. Department of Land, Air and Water Resources, University of California Davis, One Shields Avenue, Davis, CA 95616-8627, USA.

¹Corresponding author (e-mail: tim.cavagnaro@sci.monash.edu.au).

ditions (Graham and Rengel 1993). While the mechanisms underlying Zn-uptake efficiency are not yet clear, it is likely a function of shoot remobilization, compartmentalization, biochemical utilization and (or) root uptake, and remobilization processes (Hacisalihoglu and Kochian 2003). Responses of genotypes to edaphic conditions and their interactions with other organisms are also important. We have demonstrated that the Zn contents in the shoots and fruits of tomatoes can be up to 50% higher when colonized by arbuscular mycorrhizal (AM) fungi than when they are not colonized (Cavagnaro et al. 2006). The majority of terrestrial plant species, including many crop plants, form associations with AM fungi (Marschner and Dell 1994; Smith and Read 1997), but their effect on Zn-uptake efficiency is not consistent across genotypes (Hacisalihoglu and Kochian 2003).

Much insight has been gained into the uptake, assimilation, and allocation of elements by plants through studies of the natural abundances of stable isotopes (Farquhar et al. 1982; Marschner 1995; Dijkstra et al. 2003; Weiss et al. 2004). Inductively coupled plasma mass spectrometry (ICP-MS) has been used to study the natural abundance of Zn isotopes. Weiss et al. (2004) observed fractionation of $^{66/64}\text{Zn}$ between the shoots and roots of solution culture grown tomato, lettuce, and rice. Such isotopic fractionation may occur during active and (or) passive plant processes (Weiss et al. 2004).

The aim of the work presented here was to study the isotopic composition of Zn (four stable isotopes: ^{64}Zn , ^{66}Zn , ^{67}Zn , ^{68}Zn) in the shoots and fruits of mature, field-grown, mycorrhizal and non-mycorrhizal tomato plants. Mycorrhizal effects were studied using a mycorrhiza-defective tomato mutant (named *rmc*) and its mycorrhizal wild-type progenitor (named 76R MYC+) (Barker et al. 1998). The two genotypes grow well under field conditions (Cavagnaro et al. 2006), and their growth is matched in the absence of AM fungi (Cavagnaro et al. 2004; Poulsen et al. 2005). This experimental system, therefore, allows for detailed study of AM functioning under field conditions.

Materials and methods

Field-experiment design

Samples analyzed in the present study were collected as part of a larger study of the role of AM fungi on plant growth and nutrition and the wider soil biota (Cavagnaro et al. 2006). Briefly, an experiment was established in a tomato (*Solanum lycopersicum* L.) field on an organically managed farm, Jim & Deborah Durst Farming, in Esparto, Yolo County, California. A detailed description of the field site and results of the larger study are reported in Cavagnaro et al. (2006). The mycorrhiza-defective tomato mutant, *rmc*, and its mycorrhizal wild-type progenitor, 76R MYC+ (Barker et al. 1998), were planted in the field on 20 May 2003. The experiment was a randomized complete block design with four blocks containing eight plots, with five experimental plants per plot (Cavagnaro et al. 2006). The experiment included N- and P-addition treatments; however, the results from samples receiving no added nutrients are presented here. One week following planting, in-growth cores containing 505 g of field soil were installed between the experimental plants at a depth of 20–22.5 cm. The ends of the in-growth cores were covered by

mesh with 1 mm × 2 mm openings, which allowed growth of roots into the in-growth cores.

The plants used for the experiment were destructively harvested on the 30 July 2003. Soil was removed from the in-growth cores, and roots were extracted by wet sieving. These roots were cleared with 10% KOH (*m/v*) and stained with trypan blue using a modification of the method of Phillips and Hayman (1970), omitting phenol from all reagents. AM colonization of roots was then determined using the gridline-intersect method (Giovannetti and Mosse 1980). Aboveground plant biomass (five plants per plot) was harvested, and fruit dry-mass and shoot dry-mass were determined after oven-drying at 60 °C. Following dry-mass determination, plant material was ground, and a composite sample of shoot material and a second composite sample of fruit material were prepared for each plot and used in nutrient analyses. Plant material was digested with nitric acid in a microwave (Sah and Miller 1992) for analysis by ICP-MS.

Analytical methods

Dissolved tomato fruit and shoots were analyzed by the Interdisciplinary Center of Plasma Mass Spectrometry at the University of California at Davis (icpms.ucdavis.edu/) using an Agilent Technologies (USA) quadrupole 7500ce inductively coupled plasma mass spectrometer. Instrumentation and method parameters are summarized in Table 1. External calibration was done using a serial dilution of a Zn SPEX[®] standard (SPEX Certiprep Group, Metuchen, N.J.) that bracketed the concentrations in the samples. Standards, blanks, and samples were spiked with an internal standard solution of 100 ppb germanium (Ge). Isobaric interferences of ^{70}Ge with ^{70}Zn prevented the use of this particular Zn isotope for sample concentrations or isotopic ratios. In addition, the naturally low abundance of ^{70}Zn (0.62 atom.%) would likely have produced highly variable results, and indicated inaccurate enrichments or depletion when used in isotopic ratios.

Standards, blanks, and samples were first corrected for instrumental drift by normalizing isotope intensities (counts per second) to their respective internal standard (^{72}Ge) intensity. Blank sensitivities were then subtracted from the samples and standards. Sample concentrations were then determined by normalizing to the sensitivity calibrations established using the Zn external standards. R^2 values were 0.9991 or higher.

Calculations and statistical analysis

All isotopic analyses were performed twice on separate new digests of plant material. Calculations were performed using the mean sample values of the two analytical runs of each sample. All isotopic data are presented as the amount of Zn (μmol) that was in the form of a given isotope (^{64}Zn , ^{66}Zn , ^{67}Zn , or ^{68}Zn) as a percentage of the total amount of Zn (μmol) in the shoots or fruits (eq. 1).

$$[1] \quad \frac{\frac{a}{b}}{\frac{^{64}\text{Zn}}{64} + \frac{^{66}\text{Zn}}{66} + \frac{^{67}\text{Zn}}{67} + \frac{^{68}\text{Zn}}{68}} \times 100$$

Where: *a* is the amount ($\mu\text{g/g}$) of Zn isotope ^{64}Zn , ^{66}Zn ,

Table 1. Agilent Technologies quadrupole 7500ce inductively coupled plasma mass spectrometer (ICP-MS) and analytical parameters.

	Value
RF power (Babington nebulizer)	1500 W
Ar plasma gas flow rate	15 L/min
Ar carrier gas flow rate	1.05 L/min
Oxide production (m/z of 156/140)	~ 1.5%
Doubly charged production (m/z of 70/140)	<3%
Measured isotopes	⁶⁴ Zn, ⁶⁶ Zn, ⁶⁷ Zn, ⁶⁸ Zn, ⁷² Ge
Dwell time for each isotope	0.3 s
No. replicates	10
Total integration for each isotope	6.66 s
Total acquisition time	66.6 s

⁶⁷Zn, or ⁶⁸Zn, in a given plant sample; *b* is the molecular weight of Zn isotope *a* (⁶⁴Zn, ⁶⁶Zn, ⁶⁷Zn, or ⁶⁸Zn).

As an example of delta values showing discrimination between light and heavy isotopes, ⁶⁴Zn and ⁶⁸Zn, were chosen. Calculations were as follows (eq. 2). The standard value was 48.98% for ⁶⁴Zn and 18.57% for ⁶⁸Zn, as used by Weiss et al. (2004)

$$[2] \quad \left[\left(\frac{(^{68}\text{Zn}/^{64}\text{Zn})_{\text{sample}}}{(^{68}\text{Zn}/^{64}\text{Zn})_{\text{standard}}} \right) - 1 \right] \times 1000$$

All data were analyzed using SAS[®] version 8.02 (SAS Institute Inc. Cary, N.C.). Comparisons between shoots and fruits of the same genotype (i.e., *rmc* and 76R MYC+) were made using repeated-measures analysis. Comparisons between genotypes of the same plant part (i.e., shoots and fruits) were made using the GLM procedure. Means and standard errors of isotopic abundances are reported. The percentage of the root colonized by mycorrhizal fungi was arcsine-transformed prior to analysis.

Results

The isotopic composition of the Zn in shoots was significantly different from that in the fruit (Figs. 1a–1d). For both genotypes, the micromole amount of Zn in the form of either ⁶⁴Zn or ⁶⁶Zn as a percentage of the total micromole amount of Zn in the shoots was significantly less than in the fruit; the reverse was true of the heavier isotopes, ⁶⁷Zn and ⁶⁸Zn. Relative to the *rmc* plants, shoots of the 76R MYC+ plants were enriched in ⁶⁴Zn and ⁶⁶Zn and depleted in ⁶⁷Zn and ⁶⁸Zn. The fruits of the 76R MYC+ plants were enriched in ⁶⁶Zn and depleted in ⁶⁸Zn relative to the *rmc* plants; however, the differences were very small (<0.1%). Total Zn content was significantly higher in the shoots and fruits of the 76R MYC+ plants than the *rmc* plants (data not shown; see Cavagnaro et al. 2006).

The delta value of the most extreme isotopic ratio (^{68/64}Zn) shows marked discrimination against ⁶⁴Zn among shoots of 76R MYC+, compared with plants with reduced mycorrhizal colonization (198 atom.% for *rmc* vs. 126 atom.% for 76R MYC+). Fruit had similar delta values for the two genotypes (26 and 24, respectively), but the much lower values indicate that there is preferential accumulation of ⁶⁴Zn in the fruit. Quite similar delta values occurred for ^{68/66}Zn; values were

95%–100% of the ^{68/64}Zn values for the shoots, and 63%–70% of the ^{68/64}Zn values for the fruit (data not shown). Less-pronounced differences were found for the other ratios, especially ^{66/64}Zn and ^{68/67}Zn. It should be noted that these delta values are not absolute isotope abundances, but differences between sample readings using reported standard values of isotope abundance (Weiss et al. 2004).

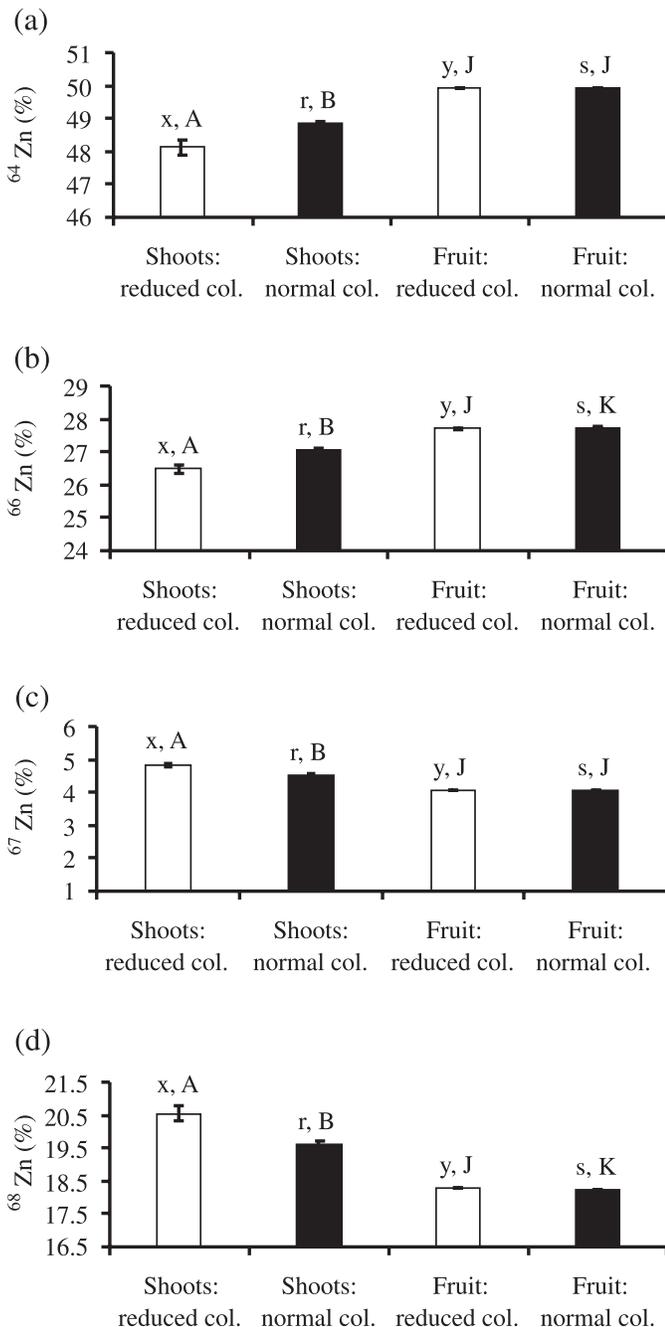
The arcsine transformed percentage of root length (mean ± SE) colonized by AM fungi was 0.22 ± 0.04 and 0.07 ± 0.02 for the 76R MYC+ and *rmc* plants, respectively. Colonization of *rmc* plants was restricted to the root surface and epidermis, whereas colonization of 76R MYC+ plants was complete, including formation of arbuscules, hyphal coils, vesicles, and intercellular hyphae in the cortical cell layers.

Discussion

The fruits and shoots of field-grown tomatoes were enriched in light (⁶⁴Zn, ⁶⁶Zn) and heavy (⁶⁷Zn, ⁶⁸Zn) isotopes, respectively. These data support earlier observations of Zn-isotope fractionation (in that case ^{66/64}Zn) in the shoots and roots of solution culture grown tomato, rice, and lettuce (Weiss et al. 2004). Differences in the diffusion of isotopes can lead to fractionation; however, given the magnitude of the differences between shoots and fruits seen here, we consider it unlikely that this is the sole causal mechanism. Differential bonding of Zn isotopes to cellular components is one explanation for the fractionation. Zn bonds covalently to carbonyl and hydroxyl groups in cell walls (Weiss et al. 2004), as well as to peptides, phospholipids, and phytic acid (Marschner 1995). Such differential bonding of isotopes to other compounds is used in isotope separation for analytical purposes; for example, heavy magnesium (Mg) isotopes preferentially bind to synthetic peptide resins, while lighter isotopes remain in solution (Kim et al. 2003).

Zn plays catalytic, co-catalytic, or structural roles in many plant enzymes (Marschner 1995; Hacisalihoglu and Kochian 2003), all of which could be involved in fractionation. Much of the Zn in a plant is stored as Zn–protein–phytate complexes, which can be remobilized later during plant development (Marschner 1995) and potentially play an important role in determining plant Zn-assimilation efficiency. Zn isotopes may be fractionated either during the formation of these complexes or during Zn remobilization. The ZAT superfamily of cation-diffusion facilitators are likely to be

Fig. 1. Isotopic fractionation in shoots and fruit of 76R MYC+ (normal colonization; solid bars) and *rnc* (reduced colonization; open bars) genotypes of tomato of ^{64}Zn (a), ^{66}Zn (b), ^{67}Zn (c), and ^{68}Zn (d). Values are the mean (\pm SE) amount (μmol) of Zn that was in the form of a given isotope (^{64}Zn , ^{66}Zn , ^{67}Zn or ^{68}Zn) as a percentage of the total amount (μmol) of Zn in the shoots or fruit ($n = 4$). Means followed by the same letter are not significantly different; valid statistical comparisons can only be made between the shoots and fruits of *rnc* (denoted *x* and *y*), the shoots and fruits of 76R MYC+ (denoted *r* and *s*), between the shoots of the two genotypes (denoted A and B), and the fruits of the two genotypes (denoted J and K). The coefficient of variation across all samples was $<0.4\%$. Each graph shows a range of 5% of the total Zn content on the y axis.



involved in storing Zn in the vacuole (Otegui et al. 2002; Ghandilyan et al. 2006), and may also play an important role. Fractionation of Zn isotopes may also occur during transport and (or) translocation. Zn appears to be taken up by divalent cation transporters from the plasma membrane belonging to the ZIP family in roots (Epstein and Bloom 2005; Ghandilyan et al. 2006), after which it is transferred to the xylem, most likely by heavy-metal ATPases (Eren and Arguello 2004; Ghandilyan et al. 2006) for translocation. Transporters located in the vacuole, including ZAT and MHX, also play an important role in Zn transport (Ramesh et al. 2004). Although the role of specific enzymes in Zn fractionation is unknown, the potential for such fractionation is supported by the role of enzymes in the fractionation of other isotopes. For example, the heavier N isotope (^{15}N) is discriminated against by plant enzymes including nitrate reductase and glutamine synthase (Dijkstra et al. 2003), and carbon (C) isotopes are fractionated by plants during photosynthesis and periods of intrinsic water stress (Farquhar et al. 1982). Weiss et al. (2004) suggested that Zn could be fractionated via preferential binding of heavy Zn to plant cell walls in nonexchangeable forms, owing to covalent bonding to carbonyl and hydroxyl groups in the cell walls. Such fractionation processes could be especially important in the long-distance transport of Zn in the xylem or phloem (e.g., shoots to roots), since over 75% of Zn is typically transferred to shoots (Weiss et al. 2004); such processes of fractionation will be diffusion-limited.

Our results show different Zn signatures in the shoots, and to a lesser extent fruits, of mycorrhizal and nonmycorrhizal plants. The fractionation observed here may be due to discrimination against heavier isotopes during supply of Zn to plants via the mycorrhizal pathway. This could occur during hyphal uptake, translocation within hyphae, transfer into the fungal/plant apoplastic space, and (or) plant uptake of Zn from the plant/fungal apoplastic space. A more complete understanding of Zn metabolism in AM is needed before the biological significance of the differences seen here can be fully appreciated, for example, identification of specific Zn transporter genes, mechanisms of Zn translocation, the nature of the active versus passive transport processes, and additional information on the regulation of Zn transporters by AM fungi (e.g., Burleigh et al. 2003). The higher phosphorus (P) concentration of mycorrhizal plants (see Cavagnaro et al. 2006) may lead to a higher phytate content (Lu and Koide 1991), which may influence Zn fractionation (see above). The higher Zn concentration of the mycorrhizal plants (Cavagnaro et al. 2006) may also be important. To fully explore the mycorrhizal contribution to Zn fractionation it will be necessary to construct a complete plant Zn-isotope budget, including roots and arbuscular mycorrhizal fungi (AMF). This will be challenging for the fungi, especially in field settings.

The delta values observed here between $^{68}/^{64}\text{Zn}$ and $^{68}/^{66}\text{Zn}$ showed much greater discrimination against the heavier isotope compared with the differences found by Weiss et al. (2004) for $^{66}/^{64}\text{Zn}$ ranged from -0.5 to 0.2 atom.% for the shoots and roots of solution culture grown nonmycorrhizal tomato plants, and which was similar to our value for the shoots with reduced mycorrhizal colonization (0.7 atom.%). The difference between these sets of delta values suggest

that mass differences are less important than the behavior of a given isotope; enzymatic discrimination and differences in kinetic characteristics and equilibria may favor specific isotopes of Zn.

Fractionation of Zn isotopes between plant parts has the potential to be a useful tool, as with other stable isotopes, with potential use as simple screening methods, for example, water use efficiency as indicated by $^{13}\text{C}/^{12}\text{C}$ ratios (Araus et al. 2002). Zn metabolism and its biogeochemical cycling by plants are generally not as well understood as for many other nutrients, so that more detailed mechanistic studies are necessary. However, given the importance of Zn in human nutrition, and its widespread deficiency in the diet of much of the world's population, such activities should be a high priority.

Acknowledgements

This work would not have been possible without the ongoing support of our farmer cooperators, Jim and Deborah Durst. We thank Prof. Sally E. Smith (University of Adelaide) and Dr. Susan J. Barker (University of Western Australia) for allowing us to use the *rmc* tomato mutant/wild-type system. Thanks also to Ms. Michelle Gras of the Interdisciplinary Center of Plasma Mass Spectrometry at University of California, Davis, for performing isotopic analyses of plant samples and for advice on data presentation. We also thank Dr. Jay Black, Prof. Jim Richards, and Mr Felipe Barrios Masias for valuable discussions and Prof. Arnold Bloom for reading an earlier version of the manuscript. The comments from two anonymous reviewers are appreciated and strengthened the manuscript. Our research is funded by the California Department of Food and Agriculture Specialty Crops Program (SA6674) and the United States Department of Agriculture National Research Initiative Soils and Soil Biology Program (2004-03329).

References

- Araus, J.L., Slafer, G.A., Reynolds, M.P., and Royo, C. 2002. Plant breeding and drought in C3 cereals: what should we breed for? *Ann. Bot. (Lond.)*, **89**: 925–940. doi:10.1093/aob/mcf049. PMID:12102518.
- Barker, S.J., Stummer, B., Gao, L., Dispain, I., O'Connor, P.J., and Smith, S.E. 1998. A mutant in *Lycopersicon esculentum* Mill. with highly reduced VA mycorrhizal colonization, isolation and preliminary characterization. *Plant J.* **15**: 791–797. doi:10.1046/j.1365-313X.1998.00252.x.
- Brown, K.H., and Wuehler, S.E. 2000. Zinc and human health: results of recent trials and implications for program interventions and research. International Development Research Centre, Ottawa, Ont.
- Burleigh, S.H., Kristensen, B., and Bechmann, I.E. 2003. A plasma membrane zinc transporter from *Medicago truncatula* is up-regulated in roots by Zn fertilization, yet down-regulated by arbuscular mycorrhizal colonization. *Plant Mol. Biol.* **52**: 1077–1088. doi:10.1023/A:1025479701246. PMID:14558666.
- Cavagnaro, T.R., Smith, F.A., Hay, G., Carne-Cavagnaro, V.L., and Smith, S.E. 2004. Inoculum type does not affect overall resistance of an arbuscular mycorrhiza-defective tomato mutant to colonisation but inoculation does change competitive interactions with wild-type tomato. *New Phytol.* **161**: 485–494. doi:10.1111/j.1469-8137.2004.00967.x.
- Cavagnaro, T.R., Jackson, L.E., Six, J., Ferris, H., Goyal, S., Asami, D., and Scow, K.M. 2006. Arbuscular mycorrhizas, microbial communities, nutrient availability, and soil aggregates in organic tomato production. *Plant Soil*, **282**: 209–225. doi:10.1007/s11104-005-5847-7.
- Dijkstra, P., Williamson, C., Menyailo, O., Douchett, R., Koch, G., and Hungate, B.A. 2003. Nitrogen stable isotope composition of leaves and roots of plants growing in a forest and meadow. *Isotopes Environ. Health Stud.* **39**: 29–39.
- Epstein, E., and Bloom, A.J. 2005. Mineral nutrition of plants: principles and perspectives. Sinauer Associates, Sunderland, Mass.
- Eren, E., and Arguello, J.M. 2004. Arabidopsis HMA2, a divalent heavy metaltransporting PIB-type ATPase, is involved in cytoplasmic Zn^{2+} homeostasis. *Plant Physiol.* **136**: 3712–3723. doi:10.1104/pp.104.046292. PMID:15475410.
- Farquhar, G.D., O'Leary, M.H., and Berry, J.A. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust. J. Plant Physiol.* **9**: 121–137.
- Ghandilyan, A., Vreugdenhil, D., and Aarts, M.G.M. 2006. Progress in the genetic understanding of plant iron and zinc nutrition. *Physiol. Plant.* **126**: 407–417. doi:10.1111/j.1399-3054.2006.00646.x.
- Giovannetti, M., and Mosse, B. 1980. An evaluation of techniques for measuring vesicular–arbuscular mycorrhizal infection in roots. *New Phytol.* **84**: 489–500. doi:10.1111/j.1469-8137.1980.tb04556.x.
- Graham, R.D., and Rengel, Z. 1993. Genotypic variation in Zn uptake and utilization by plants. In *Zinc in soils and plants. Edited by D. Robson*. Kluwer Academic Publishers, Dordrecht, the Netherlands. pp. 107–114.
- Hacisalihoglu, G., and Kochian, L.V. 2003. How do some plants tolerate low levels of soil zinc? Mechanisms of zinc efficiency in crop plants. *New Phytol.* **159**: 341–350. doi:10.1046/j.1469-8137.2003.00826.x.
- Kim, D.W., Jeon, B.K., Kang, B.M., Lee, N.-S., and Ryu, H.-I. 2003. Adsorption and isotope effects by ion exchange with 1-aza-12-crown-4 bonded Merrifield peptide resin. *J. Colloid Interface Sci.* **263**: 528–532. doi:10.1016/S0021-9797(03)00287-X. PMID:12909044.
- Kochian, L.V. 2000. Molecular physiology of mineral nutrient acquisition, transport, and utilization. In *Biochemistry and molecular biology of plants. Edited by B.B. Buchanan, W. Gruissem, and R.L. Jones*. American Society of Plant Physiologists, Rockville, Md. pp. 1204–1249.
- Lu, X., and Koide, R.T. 1991. *Avena fatua* L. seed and seedling nutrient dynamics as influenced by mycorrhizal infection of the maternal generation. *Plant Cell Environ.* **14**: 931–939. doi:10.1111/j.1365-3040.1991.tb00962.x.
- Marschner, H. 1995. Mineral nutrition of higher plants. Academic Press Ltd., San Diego, Calif.
- Marschner, H., and Dell, B. 1994. Nutrient uptake in mycorrhizal symbiosis. *Plant Soil*, **159**: 89–102.
- Otegui, M.S., Capp, R., and Staehelin, L.A. 2002. Developing seeds of *Arabidopsis* store different minerals in two types of vacuoles and in the endoplasmic reticulum. *Plant Cell*, **14**: 1311–1327. doi:10.1105/tpc.010486. PMID:12084829.
- Phillips, J.M., and Hayman, D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **55**: 158–161.
- Poulsen, K.H., Nagy, R., Gao, L.-L., Smith, S.E., Bucher, M., Smith, F.A., and Jakobsen, I. 2005. Physiological and molecular

- evidence for Pi uptake via the symbiotic pathway in a reduced mycorrhizal colonization mutant in tomato associated with a compatible fungus. *New Phytol.* **168**: 445–454. doi:10.1111/j.1469-8137.2005.01523.x. PMID:16219083.
- Ramesh, S.A., Choimes, S., and Schachtman, D.P. 2004. Overexpression of an *Arabidopsis* zinc transporter in *Hordeum vulgare* increases short-term zinc uptake after zinc deprivation and seed zinc content. *Plant Mol. Biol.* **54**: 373–385. doi:10.1023/B:PLAN.0000036370.70912.34. PMID:15284493.
- Sah, R.N., and Miller, R.O. 1992. Spontaneous reaction for acid dissolution of biological tissues in closed vessels. *Anal. Chem.* **64**: 230–233. doi:10.1021/ac00026a026. PMID:1319690.
- Smith, S.E., and Read, D.J. 1997. *Mycorrhizal symbiosis*. Academic Press Ltd., Cambridge, UK.
- Weiss, D.J., Mason, T.F.D., Zhao, F.J., Kirk, G.J.D., Coles, B.J., and Horstwood, M.S.A. 2004. Isotopic discrimination of zinc in higher plants. *New Phytol.* **165**: 703–710.