J Plant Growth Regul (2010) 29:164–170
DOI 10.1007/s00344-009-9130-7

Nitrogen-Use Efficiency in Relation to Different Forms and Application Rates of Se in Lettuce Plants

Juan J. Ríos · Begoña Blasco · Luis M. Cervilla · Maria M. Rubio-Wilhelmi · Miguel A. Rosales · Eva Sanchez-Rodriguez · Luis Romero · Juan M. Ruiz

Received: 11 June 2009 / Accepted: 26 October 2009 / Published online: 20 December 2009

Abstract The main objective of this work was to determine whether nitrogen-use efficiency (NUE) was affected by the application of different forms and dosages of selenium (Se), to ascertain the influence of this trace element in a biofortification program in lettuce plants. The parameters analyzed were biomass, NO_3^- concentration, and total reduced N as well as those defining NUE in plants: total nitrogen accumulation (TNA), nitrogen efficient ratio (NER), nitrogen-utilization efficiency (NUtE), and nitrogen-uptake efficiency (NUpE). According to our results, application of Se affected NUE in lettuce plants. With the application of selenite as well as selenate NO_3^- uptake was reduced, thus diminishing the NUpE and the foliar concentration of this anion. In addition, selenate application at a rate of 20 μM and selenite at 5 μM induced N utilization, reflected by an increase in NER and NUtE; this result coincides with augmented biomass production. Notably, our results indicate that when Se is applied at high rates, selenite is far more phytotoxic, this being associated with a higher reduction of NUE in these plants.

Keywords Biofortification · Lettuce · Selenate · Selenite · NUE

Introduction

Selenium (Se) has been deemed essential to animal nutrition since 1957, and humans have a daily requirement of 50–70 μg day^{-1} (U.S. Department of Agriculture 2003). It is a nutritional component of the enzymes glutathione peroxidase, selenoprotein P, and tetraiodothyronine 5′-deiodinase. In addition, studies have shown that a dietary Se supplement of 100-200 μg day^{-1} results in a decreased incidence of lung and prostate cancers (Ip and others 1991; Ip and Ganther 1992; Läuchi 1993; Clark and others 1996).

Dietary Se deficiency in humans is caused by the ingestion of plant foods with an imperceptible concentration of this element as a result of its low bioavailability in many crop soils (Smorklji and others 2005; Pedrero and others 2006), especially in parts of China and Australia, UK, East Europe, and Africa (Chen and others 2002; Lyons and others 2005). Therefore, given this low bioavailability and the fact that plants are the main dietary source of this element, studies have investigated ways to increase the Se content in plants used for human consumption.

Biofortification has been defined as the process of increasing the bioavailable concentrations of essential elements in edible portions of plants through agricultural intervention or genetic selection (White and Broadley 2005). There are currently works such as those of Chen and others (2002) on wheat varieties and more recently Pedrero and others (2006) on radish that show that fertilization with Se raises the content of this trace element in plants; all these works focused on potential increases in daily Se ingestion in humans.

However, none of these studies has thoroughly analyzed the possible effect of the application of Se on the essential nutrients for plant growth and development such as nitrogen (N), which is the determining factor in crop yield. Crop yield depends heavily on the quantity of N available in the medium (Lea and Azevedo 2006) as approximately 30-40% of the N applied can be converted by plants into crops for human consumption. Moll and others (1982) have defined

J. J. Ríos (✉) · B. Blasco · L. M. Cervilla · M. M. Rubio-Wilhelmi · M. A. Rosales · E. Sanchez-Rodriguez · L. Romero · J. M. Ruiz
Department of Plant Physiology, Faculty of Science, University of Granada, 18071 Granada, Spain
e-mail: jjrios@ugr.es

NUE as biomass production with available unit N in the medium. The NUE concept can be split into two aspects: uptake efficiency, the ability of the plant to remove N from the soil normally present as a nitrate or ammonium ions, and utilization efficiency, the ability of the plants to transfer N to the shoot (Lea and Azevedo 2006). At present, there is great interest in identifying the processes involved in the regulation of N uptake and metabolism within plants (Andrews and others 2004), and one of the main aims of using NUE in plants for human consumption is to reduce foliar levels of NO_3^-.

NO_3^- is taken up by plant roots, translocated to the shoot by transpiration, stored in vacuoles, and/or transformed into assimilation products such as amino acids and proteins required for biomass synthesis. However, foliar accumulation of this nutrient can pose a problem for crops when both the application and the NO_3^--uptake capacity exceed the demands of plant growth (Ruiz and Romero 1999; Prasad and Chetty 2008). However, NO_3^- also represents a risk to human health because on ingestion it is rapidly transformed into nitrite and *N*-nitrose compounds. These forms are toxic and can provoke serious pathologies in humans, such as methemoglobinemia and blue-baby syndrome, or magnify the risk of cancer as the nitrites are transformed into nitrosamines (Mensinga and others 2003; Santamaria 2006).

Due to the increase in the use of nitrogenous fertilizers, cultivated vegetables normally contain high concentrations of NO_3^- (Santamaria 2006), and given the high concentrations of this anion in vegetables cultivated for human consumption, they can be harmful to health and are closely regulated by law. The Joint Expert Committee of Food and Agriculture (JECFA) and the European Commission's Scientific Committee on Food (SCF) have determined an acceptable daily intake (ADI) of less than 3.7 mg kg^{-1} body weight (bw) for NO_3^- (Speijers and van den Brandt 2003). On the other hand, the U.S. Environmental Protection Agency (EPA) has estimated Reference Doses (RfD) for NO_3^- of 7.0 mg NO_3^- kg^{-1} bw per day (Mensinga and others 2003). Many plants tend to accumulate large amounts of NO_3^-, for example, lettuce, spinach, beets, radishes, and celery (MAFF 1998). European Union legislation allows lettuce to contain no more than 4,500 mg NO_3^- kg^{-1} fresh weight.

As mentioned above, many studies are focused on biofortification programs using trace elements, including Se, with the aim of boosting the intake of Se in humans through plant consumption (Cartes and others 2005; Pedrero and others 2006; Rios and others 2008). However, very few of these studies analyzed the impact of this element on plant physiology. Therefore, the aim of the present work was to determine whether NUE and foliar NO_3^- concentration are affected by the application rates and forms of Se.

Materials and Methods

Plant Material and Growing Conditions

Seeds of *Lactuca sativa* L. cv Philipus were germinated and grown for 35 days in cell flats (cell size = 3 cm × 3 cm × 10 cm) filled with perlite mixture, and the flats were placed on benches in an experimental greenhouse in southern Spain (Saliplant S.L., Motril, Granada). The 35-day-old seedlings were transferred to a cultivation chamber under controlled environmental conditions with relative humidity of 60-80%, temperature of 25/15°C (day/night), and a 12-h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). The plants were grown in individual 8-l pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height) filled with vermiculite. Until the end of the experiment the plants received a growth solution composed of 4 mM $Ca(NO_3)_2$, 6 mM KNO_3, 2 mM $MgSO_4{\cdot}7H_2O$, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 50 μM H_3BO_3, 2 μM $MnCl_2{\cdot}4H_2O$, 1 μM $ZnSO_4{\cdot}7H_2O$, 0.1 μM $Na_2MoO_4{\cdot}2H_2O$, 0.25 μM $CuSO_4{\cdot}5H_2O$, and 10 μM Fe-EDDHA. The nutrient solution (pH 5.5-6.0) was renewed every 3 days and the vermiculite was partly rinsed with millipore-filtered water to avoid nutrient accumulation.

At 45 days after germination, the different treatments were applied together with the nutrient solution described above and maintained for 21 days. The treatments consisted of applying SeO_4 at different rates (5, 10, 20, 40, 60, 80, 120 μmol l^{-1} as Na_2SeO_4) and SeO_3 at different rates (5, 10, 20, 40, 60, 80, 120 μmol l^{-1} as Na_2SeO_3). The selected Se application rates ranged from the recommended rate for biofortification programs to rates causing phytotoxicity (Ríos and others 2008). In addition to these treatments, a control treatment was used consisting of the application of the complete growth solution without a Se supplement. The experimental design was a randomized complete block with 15 treatments, arranged in individual pots with six plants per treatment, and three replications. The experiment was repeated three times under the same conditions ($n = 9$).

Plant Sampling

Lettuce leaves were sampled on day 66 after germination. Leaf samples were standardized by using only fully expanded leaves from the middle part of plants in each replicate because they reflect most clearly, from the nutritional and metabolic standpoints, the treatment effects. The material was rinsed three times in distilled water after disinfection with nonionic 1% detergent and then blotted on filter paper. The plant material (edible leaves) was lyophilized and used to determine the biomass (dry weight,

DW), NO_3^- concentration, total reduced N concentration, and the different parameters that define the NUE (as described below).

Plant Analysis

NO_3^- was analyzed from an aqueous extraction of 0.2 g of dried and ground leaf material in 10 ml of millipore-filtered water. A 100-µl aliquot was taken for determination of NO_3^- and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, and the NO_3^- concentration was measured by spectrophotometry following the method of Cataldo and others (1975). The results were expressed as mg kg^{-1} fresh weight (FW).

For the total reduced-N determination, a sample of 0.1 g dry weight (DW) was digested with sulfuric acid and H_2O_2. After dilution with deionized water, a 1-ml aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 µM sodium phosphate, and 5.4% [w/v] sodium hydroxide), 15% (w/v) sodium silicate/0.03% (w/v) sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37°C for 15 min, and total reduced N was measured by spectrophotometry according to the method of Baethgen and Alley (1989). Total nitrogen content (TNC) was assumed to represent the sum of organic N and NO_3^-. The total reduced and total N were expressed as mg g^{-1} DW.

For measuring the total Se content, the dried plant matter and 25 mg of sample were digested with 2.5 ml of concentrated HNO_3 and 1 ml of H_2O_2 in an analytical microwave oven. The resulting solution was diluted to 25 ml with deionized water and the metal concentration determined by ICP-MS according to the method of Pedrero and others (2006).

Total nitrogen accumulation (TNA) was calculated using both the TNC and DW of the total leaves (Sorgona and others 2006), with the result expressed as mg N:

$$\text{TNA} = \text{TNC} \times \text{total leaves DW}.$$

Nitrogen-utilization efficiency (NUtE) was calculated as follows (Siddiqi and Glass 1981) with the results expressed as g^2 DW mg^{-1} N:

$$\text{NUtE} = \text{Total leaves DW}/\text{TNC}.$$

Nitrogen-uptake efficiency (NUpE) was calculated as follows (Elliot and Läuchli 1985):

$$\text{NUpE} = \text{TNA}/\text{RDW}$$

where RDW is the root dry weight. The results are expressed as mg N g^{-1} DW. Nitrogen efficient ratio (NER) was calculated as follows (Elliot and Läuchli 1985):

$$\text{NER} = \text{DW}/\text{TNA}$$

Statistical Analysis

The data were subjected to a simple analysis of variance (ANOVA) at 95% confidence using the program Statgraphics 6.1. A two-way ANOVA was applied to ascertain whether the Se application rate and the forms of Se used significantly affected the results, and the means were compared by Fisher's least-significant differences (LSD). The significance levels for both analyses were expressed as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ and not significant (ns). Data are given as mean ± standard error ($n = 9$).

Results and Discussion

In plants, stress negatively affects growth and development and also generates reactive oxygen species (ROS), which damage numerous macromolecules and cell structures. Consequently, under adverse conditions, one of the most reliable and widely used indicators of stress is plant biomass (Moran and others 2002; Blasco and others 2008). Our results with respect to shoot biomass reveal significant differences depending on the form and rate of Se application (Fig. 1), with, in general, greater growth when selenate was applied instead of selenite ($p < 0.001$, Fig. 1). In general, the results indicate that selenate is less toxic than selenite, given that the plant tolerated up to 80 µM selenate with no growth reduction, whereas for selenite all doses above 5 µM reduced growth progressively. The highest values for shoot biomass were registered at the rates of 20 µM for selenate and 5 µM for selenite. These values resulted in marginally greater plant growth than in the control (Fig. 1).

In plants, Se uptake and accumulation are important for the effectiveness of a biofortification program. Furthermore, the possible involvement of this element in NUE, and consequently the concentration of total reduced N,

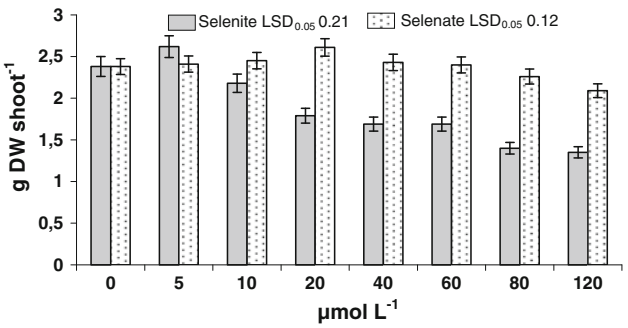

Fig. 1 Shoot biomass in lettuce *Lactuca sativa* L. treated with different forms and application rates of Se. Data are mean ± standard error ($n = 9$)

offers some idea of the interaction between Se and N and therefore of the potential repercussions for plant growth and development. Thus, Fig. 2 shows that the foliar Se concentration increased with the application rate ($p < 0.001$), peaking in the 120-μM treatment for both Se forms applied (Fig. 2). As reflected in Fig. 2, it is striking that for most of the application rates, the foliar concentration of Se was higher with SeO_4 than with SeO_3.

The NO_3^- concentration is important in plants grown for human consumption because a high intake of this nutrient can be harmful to human health (Santamaria 2006) and therefore diminishes the quality of the products obtained. Figure 3 shows the results for the foliar concentration of NO_3^-, with a significant decline ($p < 0.001$) as the application rate of Se increased, for both selenite and selenate (Fig. 3). In general, it was also noteworthy that NO_3^- values were lower when selenite rather than selenate was applied (Fig. 3). It should be mentioned that NO_3^- concentrations in our work that were below the limit imposed by the SCF, regardless of the rate and form of Se applied (Fig. 3), suggest that application of this element increases the quality of the lettuce obtained. In the case of selenate, this decline in NO_3^- concentration may be due to the antagonistic effect of NO_3^- at the level of root uptake of the selenate, whereas in the case of selenite, the decrease in the foliar concentration of NO_3^- could be due to a toxic effect of selenite inhibiting or affecting the root transporters of NO_3^-. Another determining factor in the decrease in foliar NO_3^- is reduction through the enzyme nitrite reductase (Aslam and others 1990). In our work, only selenite induced this enzymatic activity (data not shown), a situation that could explain why selenite caused a greater reduction of the foliar concentration of NO_3^- than did selenate (Fig. 3).

The result of NO_3^- assimilation is reflected in the foliar concentration of total reduced N, which can be seen in Fig. 4. The data indicate significant differences between the various rates of Se added ($p < 0.01$), although not between the different forms ($p > 0.05$; Fig. 4). The highest total value for reduced N was found for the rate of 60 μM of selenite and 80 μM of selenate (Fig. 4), with both treatments registering values higher than those of control (Fig. 4). These data suggest that the application of both forms of Se increased N assimilation. In general, considering the data described to date, the application of selenate up to a rate of 20 and 5 μM of selenite proved beneficial for the cultivation of lettuce because (1) it did not affect biomass production, (2) it diminished the foliar concentration of NO_3^-, and (3) it increased the foliar concentration of total reduced N.

Although there are several factors that affect growth of lettuce, N is a determining element for yield of this crop. For lettuce cultivation, which is highly dependent on N availability, apart from knowing the concentration of NO_3^- and of total reduced N, it is important to determine the parameters of NUE to assess the need for nitrogenous fertilizers (Lawlor 2002). The data compiled show an increase in TNA when Se was applied in the form of selenate as the concentration is increased (Table 1). However, when the applied form was selenite, the values for NUE fell with increasing application rates, with the lowest value

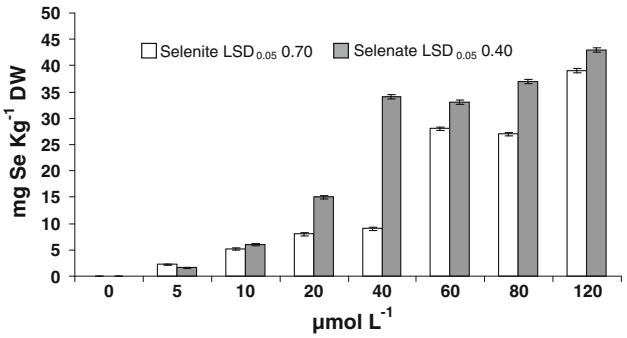

Fig. 2 Foliar concentration of Se found in lettuce plants treated with different concentrations and forms of Se. Data are mean ± standard error ($n = 9$)

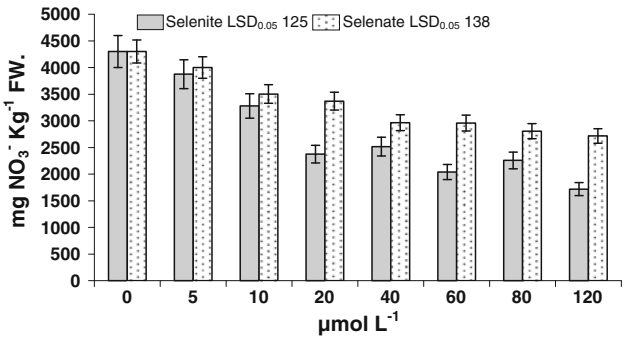

Fig. 3 Concentration of NO_3^- in lettuce plants treated with different forms and application rates of Se. Data are mean ± standard error ($n = 9$)

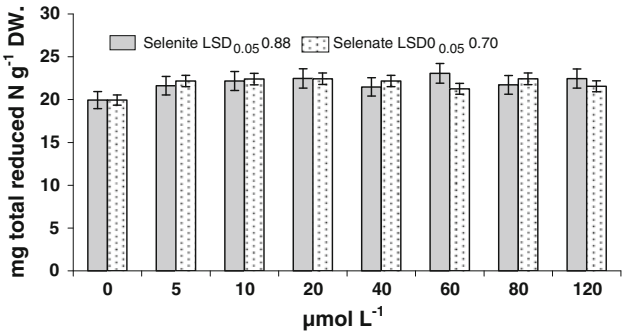

Fig. 4 Concentration of total reduced N in the shoots of lettuce plants treated with different forms and application rates of Se. Data are mean ± standard error ($n = 9$)

Table 1 Specific parameters of NUE in lettuce plants treated with different forms and application rates of Se

Treatment	TNA		NER		NUtE		NUpE	
	SeO_3	SeO_4	SeO_3	SeO_4	SeO_3	SeO_4	SeO_3	SeO_4
Application rate (μM)								
0	53.29 ± 3.66	53.29 ± 3.66	0.044 ± 0.007	0.044 ± 0.007	0.018 ± 0.002	0.018 ± 0.002	292 ± 9.02	292 ± 9.02
5	52.90 ± 3.66	47.09 ± 3.62	0.055 ± 0.008	0.045 ± 0.007	0.021 ± 0.002	0.019 ± 0.002	182 ± 7.94	211 ± 8.21
10	50.64 ± 3.65	52.46 ± 3.66	0.041 ± 0.007	0.048 ± 0.007	0.019 ± 0.002	0.019 ± 0.002	198 ± 8.12	186 ± 7.96
20	43.48 ± 3.47	58 ± 3.91	0.030 ± 0.006	0.060 ± 0.009	0.017 ± 0.002	0.023 ± 0.002	235 ± 8.62	149 ± 7.62
40	44.13 ± 3.49	60 ± 4.02	0.027 ± 0.006	0.055 ± 0.008	0.016 ± 0.002	0.022 ± 0.002	218 ± 8.31	179 ± 7.94
60	44.93 ± 3.49	52.88 ± 3.66	0.031 ± 0.006	0.053 ± 0.008	0.019 ± 0.002	0.022 ± 0.002	198 ± 8.12	161 ± 7.74
80	45.47 ± 3.51	65.90 ± 4.23	0.021 ± 0.002	0.049 ± 0.007	0.015 ± 0.001	0.021 ± 0.002	270 ± 8.79	162 ± 7.74
120	48.16 ± 3.62	60.77 ± 4.02	0.022 ± 0.002	0.043 ± 0.007	0.016 ± 0.002	0.020 ± 0.002	202 ± 8.14	177 ± 7.89
p value	**	**	**	**	**	**	***	***
LSD	1.91	3.01	0.006	0.009	0.004	0.005	17.21	16.44
Statistical analysis								
Form	***		**	ns		***		
Application rate	***		ns	ns		***		
FxD	***		ns	ns		***		
LSD	1.71		0.014	0.005		4.64		

TNA expressed as mg N. NER expressed as g DW mg^{-1} N (DW dry weight). NUtE expressed as mg N g^{-1} DW. NUpE expressed as g^2 DW mg^{-1} N. Levels of significance are represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *ns* not significant. Data are mean ± standard error ($n = 9$)

corresponding to 40 μM (Table 1). However, NER and NUtE behaved similar to TNA (Table 1). Selenite application lowered efficiency when the applied concentration was higher than 5 μM, reaching minimum values for NER and NUtE with an application rate of 120 μM (Table 1). However, when Se was applied in the form of selenate, we found an increase in NER and NUtE starting from the rate of 20 μM; this rate also registered the maximum value (Table 1). These parameters agree with the results found for biomass (Fig. 1), where selenate increased biomass with respect to selenite, indicating that this trace element, either as selenite at 5 μM or selenate at 20 μM, improved NUE and therefore biomass production in lettuce plants. Finally, the results for NUpE (Table 1) were similar to those found for the foliar concentration of NO_3^- given that both Se forms decreased NUpE (Table 1).

Conclusion

According to the different parameters analyzed in this work, Se application affected NUE in lettuce plants. Application of both selenite and selenate reduced NO_3^- uptake, thereby lowering the NUpE and the foliar concentration of this anion. In addition, the application of selenate at a rate of 20 μM and selenite at 5 μM induced N utilization, reflected by an increase in NER and NUtE, coinciding with a boost in biomass production. Notably, our results indicate that when selenite is applied at a high rate it is much more phytotoxic, which is associated with a greater reduction of NUE in these plants. In short, according to our results, the application of 20 μM of selenate would be highly beneficial for the cultivation of lettuce because apart from lowering the levels of NO_3^-, it would significantly improve NUE and therefore agricultural production of this crop.

Acknowledgments This work was supported by Plan Andaluz de Investigacion, research group AGR-161.

References

Andrews M, Lea PJ, Raven JA, Lindsey K (2004) Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. Ann Appl Biol 145(1):25–40

Aslam M, Harbit KB, Huffaker RC (1990) Comparative effects of selenite and selenate on nitrate assimilation in barley seeding. Plant Cell Environ 13:773–782

Baethgen WE, Alley MM (1989) A manual colorimetric procedure of measuring ammonium nitrogen in soil and plant. Commun Soil Sci Plant Anal 20:961–969

Blasco B, Rios JJ, Cervilla LM, Sanchez-Rodriguez E, Ruiz JM, Romero L (2008) Iodine biofortification and antioxidant capacity of lettuce patential benefits for cultivation and human health. Ann Appl Biol 152:289–299

Cartes P, Gianfreda L, Mora LM (2005) Uptake of selenium and its antioxidant activity in ryegrass when applied as selenate and selenite forms. Plant Soil 256:359–367

Cataldo DA, Haroon M, Schrader VL (1975) Young, rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal 6:71–80

Chen L, Yang F, Xu J, Yun H, Hu Q, Zhang Y, Pan G (2002) Determination of selenium concentration of rice in China and effect of fertilization of selenite and selenate on Se content of rice. J Agric Food Chem 50:5128–5130

Clark LC, Combs GF Jr, Trunbull BW, Slate EH, Slate DKC, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL Jr, Park HK, Sander BB Jr, Smith CL, Taylor JR (1996) Effect of Se supplementation for cancer prevention in patients with carcinoma of the skin. JAMA 276:1957–1963

Elliot GC, Läuchli A (1985) Phosphorous efficiency and phosphate-iron interaction in maize. Agron J 77:399–403

Ip C, Ganther HE (1992) Comparison of Se and S analogs in cancer prevention. Carcinogenesis 13:1167–1170

Ip C, Hayes C, Budnick RM, Ganther HE (1991) Chemical form of selenium, critical metabolites, and cancer prevention. Cancer Res 21:595–600

Läuchi A (1993) Selenium in plants: uptake, function, and environmental toxicity. Acta Bot 106:455–468

Lawlor DW (2002) Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production system. J Exp Bot 53:773–787

Lea PJ, Azevedo RA (2006) Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. Ann Appl Biol 149:243–247

Lyons G, Ortiz-Monasterio I, Stangoulis J, Graham R (2005) Selenium concentration in wheat grain. Is there sufficient genotypic variation to use in breeding? Plant Soil 269:369–380

MAFF: Ministry of Agriculture, Fisheries, Food (1998) 1997/8 UK monitoring programme of nitrate in lettuce and spinach, food surveillance information sheet No. 154, August 1998. MAFF, London

Mensinga TT, Speijers JGA, Meulenbelt J (2003) Health implications of exposure to environmental nitrogenous compounds. Toxicol Rev 14:576–584

Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron J 74:562–564

Moran JF, Becana M, Iturbe-Ormaetxe I, Frechillas S, Klucas RV, Aparicio-Tejo P (1994) Drought induces oxidative stress in pea plants. Planta 194:346–352

Pedrero Z, Madrid Y, Camara C (2006) Selenium species bioaccessibility in enriched radish (*Raphanus sativus*): a potential dietary source of selenium. J Agric Food Chem 54:2412–2417

Prasad S, Chetty AA (2008) Nitrate-N determination in leafy vegetables: study of the effects of cooking and freezing. Food Chem 106:772–780

Rios JJ, Rosales MA, Blasoc B, Cervilla LM, Romero L, Ruiz JM (2008) Biofortification of Se and induction of the antioxidant capacity in the lettuce plants. Sci Hortic 116:248–255

Ruiz JM, Romero L (1999) Cucumber yield and nitrogen metabolism in response to nitrogen supply. Sci Hort 82:309–316

Santamaria P (2006) Nitrate in vegetables: toxicity, content, intake and EC regulation. Sci Food Agric 86:10–17

Siddiqi MY, Glass AD (1981) Utilization index: a modified phosphorous nutrition of eight forms of two clover species: *Trifolium ambiguum* and *T. repens*. J Plant Nutr 4:289–302

Smorklji P, Pograje L, Hlaston-Ribic C, Stibilj V (2005) Selenium content in selected Slovenia foodstuffs and estimated daily intakes of selenium. Food Chem 90:691–697

Sorgona A, Abenavoli MA, Gringeri PG, Cacco G (2006) A comparison of nitrogen use efficiency definitions in citrus rootstocks. Sci Hortic 109(4):389–393

Speijers GJA, van den Brandt PA (2003) Nitrate and potential endogenous formation of N-nitroso compounds, WHO Food Additives Series 50. Available at http://www.inchem.org/documents/jecfa/jecmono/v50je06.htm

U.S. Department of Agriculture (2003) Dietary reference intakes: elements. http://warp.nal.usda.gov/fnic/etextA/000105.html. Accessed 5 March 2006

White PJ, Broadley MR (2005) Biofortifying crops with essential mineral elements. Trends Plant Sci 10:586–593