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Research Report

Concentrations of Minerals and Phenolic Compounds in Three Edible Sprout Species Treated with Iron-chelates during Imbibition

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Abstract. Iron (Fe) is an essential micronutrient involved in fundamental biological processes in both humans and plants. Iron deficiency is common in humans, making iron supplementation of foods an important area of research. Edible sprouts are a rich source of minerals and phenolic compounds beneficial to human health; our objective was therefore to investigate the effects of iron supplementation in sprouts. We supplemented iron concentrations in three species of edible sprouts (alfalfa, broccoli, and radish) by soaking the seeds in a high-iron solution, and subsequently measured the concentration of minerals and of phenolic compounds. Seeds were soaked in either Fe(III)-EDTA or Fe(III)-citrate at concentrations of 2.5, 5.0, or 10 mM for 5-8 h, and then were maintained with distilled water in a commercial sprouter for 5 days. The soaking treatment significantly increased the iron concentration in 5-day-old alfalfa sprouts by up to 1.8 times the concentration observed in the controls. For broccoli and radish sprouts, an insignificant trend toward higher Fe concentrations was observed. The accumulated iron in treated alfalfa sprouts was negatively associated with concentrations of other minerals such as Ca, Mg, Mn, and Na. Treated alfalfa sprouts showed a significant increase of 8.0-36.4% in total phenolic concentrations compared to the controls, whereas broccoli and radish sprouts showed no significant change in phenolic concentrations. In summary, soaking seeds with iron chelates enhanced the iron concentration of sprouts, especially alfalfa sprouts, and had a positive or neutral impact on the concentration of phenolic compounds, suggesting that this treatment could be used to improve the nutritional quality of some types of edible sprouts.

***Additional key words*:** alfalfa, broccoli, Fe(III)-citrate, Fe(III)-EDTA, radish, total phenolic concentration

Introduction

Iron is an essential micro-nutrient that is involved in fundamental biological processes in plants, such as respiration, photosynthesis, and chlorophyll formation (Marschner, 1995; Pushnik and Miller, 1989). Healthy plants thus typically maintain iron homeostasis through the controlled uptake and transport of iron (Jeong and Guerinot, 2009). Iron deficiency induces chlorosis as a result of the inactivation of the enzyme Mg-protoporphyrin IX monomethyl ester cyclase, which prevents the biosynthesis of protochlorophyllide, a chlorophyll precursor (Bollivar and Beale, 1996; Spiller et al., 1982). Excess iron in plants, on the other hand, results in cellular damage due to oxidative stress as a result of the Fenton reaction (Kampfenkel et al., 1995).

In human nutrition, iron is similarly indispensable in maintaining and promoting health, but iron deficiency is nevertheless common due to both insufficient uptake and low bioavailability (Cakmak, 2002). Iron deficiency can cause various health problems, such as anemia, lack of energy, immunodeficiency, problems during pregnancy, stunted growth, impaired motor neural development, and long-term impairment of mental function (Welch, 2002). Anemia due to iron deficiency is the most common health disorder in humans, affecting 1.6 billion people-24.7% of the world's population (WHO, 2008).

Edible sprouts from cruciferous plants such as radish and broccoli, and from legumes such as alfalfa, are a common and beneficial component of a healthy diet because they are

rich in health-promoting nutrients such as vitamins, proteins, minerals, and phytochemicals, including antioxidants (Hesterman et al., 1981; Plum et al., 1997; Takaya et al., 2003; Zieliński et al., 2007). In cruciferous plants, for example, sprouts exhibit concentrations of glucosinolates more than 10 times higher than those found in mature plants, and the anticancer effects of glucosinolates have been demonstrated by both in vitro and human-intervention studies (Fahey et al., 1997; Jeffery and Araya, 2009; Vasanthi et al., 2009). Many phytochemicals, including glucosinolates, appear to play a crucial role in human health; an overwhelming body of epidemiological evidence shows that a diet of fruits and vegetables high in phytochemicals maintains health and reduces the risk of many chronic and degenerative diseases (Birt et al., 2001; Formica and Regelson, 1995; Hu, 2003). In recent years, several studies have explored the possibility of enhancing phytochemical concentrations in several common types of edible sprouts using abiotic stress treatments (Lee et al., 2012; Oh and Rajashekar, 2009; Pérez-Balibrea et al., 2008). Compared to this body of research on phytochemical supplementation in sprouts, however, supplementation of sprouts with essential minerals like iron has received little attention.

In this study, we, therefore, investigated the effects of a possible iron supplementation treatment on alfalfa, broccoli, and radish sprouts. After treating seeds by soaking them in various iron-chelate solutions, we measured iron concentrations in the resulting sprouts to determine the efficacy of the treatments relative to untreated control plants. We also examined changes in the concentrations of other minerals and of phenolic compounds as a result of these iron treatments, since little is known about the effects of iron supplementation on these important nutrients.

Materials and Methods

Alfalfa (*Medicago sativa* L.), broccoli (*Brassica oleracea* L.), and radish (*Raphanus sativus* L.) (Johnny's Selected Seeds, Winslow, ME, USA) seeds were used in this study. After determination of the ideal soaking duration, seeds were soaked in one of six iron-chelate solutions (or received a control treatment), with approximately 2000 (alfalfa), 1000 (broccoli), or 500 (radish) seeds used per treatment. The seeds were then sown, germinated, and grown for 5 days. At the end of this period, their growth and nutrient concentrations were measured and analyzed. These methods are detailed in the following sections.

Determination of Soaking Duration

Prior to the treatment of seeds with iron solutions, the imbibition characteristics of the three species were tested to determine the optimal soaking duration. First, small seeds were removed by sifting to reduce variation. Since the seeds of cruciferous species are relatively impermeable to water due to their thick seed coats, the seeds were scarified with very fine sandpaper attached to the bottom of a petri dish. To accomplish this, petri dishes containing the seeds were shaken in a shaking incubator (Innova 4000; New Brunswick Scientific, Edison, NJ, USA) set at 250 rpm and 20°C for 5 h (radish) or 8 h (broccoli). After washing the scarified seeds with distilled water, seeds of each species (including alfalfa) were placed in petri dishes containing distilled water. Every 30 min for the first 7 h, and once at 24 h, excess water was removed from the seeds with a paper towel and then each seed (5 seeds/species) was weighed (Baskin and Baskin, 1998). At the end of this procedure, the seeds used were discarded.

Iron Treatment

Stock solutions of Fe(III)-EDTA and Fe(III)-citrate (10 mM) were diluted under dark conditions with distilled water to 10, 5.0, and 2.5 mM, producing six different treatment solutions (three concentrations of two iron chelates). These solutions were then adjusted to a pH of 6.0 with 0.5 mM KOH. Seeds were soaked in distilled water for 30 min. Seeds other than those in the control, where seeds were just soaked in double distilled water, were then soaked, under dark conditions, in one of the six treatment solutions for a duration determined by the imbibition tests (7 h for alfalfa sprouts, 8 h for broccoli sprouts, and 5 h for radish sprouts). Seeds were then immediately rinsed with distilled water twice and sown.

Growing Conditions

Treated seeds (and the controls) were sown onto 4 plastic trays (18 cm × 9 cm) and germinated in an automated sprout-growing system (EasyGreen MikroFarm, Seed and Grain Technologies, Albuquerque, NM, USA) for 5 days. A mist generator within the system supplied sprouts with distilled water every 15 min, 4 times per day. Trays placed on the system were rotated once per day to ensure even distribution of water. To control the growth environment, the system was placed inside a growth chamber (Controlled Environments Inc., Pembina, ND, USA) set to 20°/15°C (day/night) under a 15-h photoperiod, with a photosynthetic photon flux (PPF) of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ using fluorescent lamps.

Mineral Concentration

After the 5-day growth period, digestion and mineral analysis was carried out as described by Vasconcelos et al. (2006) with a minor modification. Sprout samples (0.5 g each), omitting seed coats, were dried at 70°C in an oven for 3 days, after which dry weights of the samples were recorded.

Samples were then pre-digested in borosilicate glass tubes with 3 mL of 70% HNO_3 overnight. Samples were further digested using a heating block (Martin Machine; Ivesdale, IL, USA) at 125°C for 1.5 h. Subsequently, 1.5 mL of H_2O_2 was added to each sample and a temperature of 125°C was maintained for 1 h (repeated twice). The temperature was then raised to 200°C to dry out the samples. Samples were then re-suspended in 5 mL of 2% HNO_3 for analysis. Elemental analysis was performed using an inductively coupled plasma-optical emission spectroscopy (CIROS ICP Model FCE12, Spectro, Kleve, Germany). Tomato leaf standard (0.2 g) (SRM 1573A, National Institute of Standards and Technology, Gaithersburg, MD, USA) and two blanks of HNO_3 were digested and analyzed to verify the reliability of analytical procedures in parallel with the samples.

Total Phenolic Concentration

After the 5-day growth period, fresh samples (0.2 g each) were harvested, omitting seed coats, and were stored at -20°C until use. Samples from five sprouts were combined and analyzed jointly to determine the total phenolic concentration for each treatment, with three replications. The total phenolic concentration of each combined sample was measured using the modified Folin-Ciocalteu method (Ainsworth and Gillespie, 2007) as described by Oh and Rajashekar (2009). The optical density of samples was assessed as their absorbance at 765 nm using a UV-Vis spectrophotometer (Cary50, Varian, Palo Alto, CA, USA). A freshly prepared 1 $mg \cdot mL^{-1}$ solution of gallic acid (Acros Organics, Geel, Belgium) was used as a standard.

Growth Characteristics

After the 5-day growth period, sprout growth was measured by combining and weighing 10 sprouts per treatment, replicated 10 times (using different sprouts). Each set of 10 sprouts was blotted with paper towels prior to the measurement of its fresh weight. Samples were then dried at 70°C in an oven (FS-420, Advantec, Kashiwa, Japan) for 3 days, after which the dry weight of each set of 10 sprouts was measured.

Statistical Analysis

Two-way analysis of variance (ANOVA) was performed using the Statistical Analysis System (SAS) program (SAS Institute, Cary, NC, USA). The significance of differences in means was assessed using the Duncan's multiple range test.

Results

Imbibition of Water

Typical imbibition curves of seeds were obtained for all three sprout species (Fig. 1). Seed weights increased for the first several hours and then plateaued; the time to this plateau depended on the permeability of the seeds of each species. On the basis of these imbibition curves, treatment-solution soaking times for alfalfa, broccoli, and radish seeds were determined to be 7 h, 8 h, and 5 h, respectively. The goodness-of-fit (R^2) of the imbibition curves for broccoli and radish seeds was lower than that for alfalfa seeds, despite the scarification of the broccoli and radish seeds with sandpaper.

Iron Concentration

Iron concentrations in the alfalfa sprouts were positively correlated with the iron concentrations of their treatment

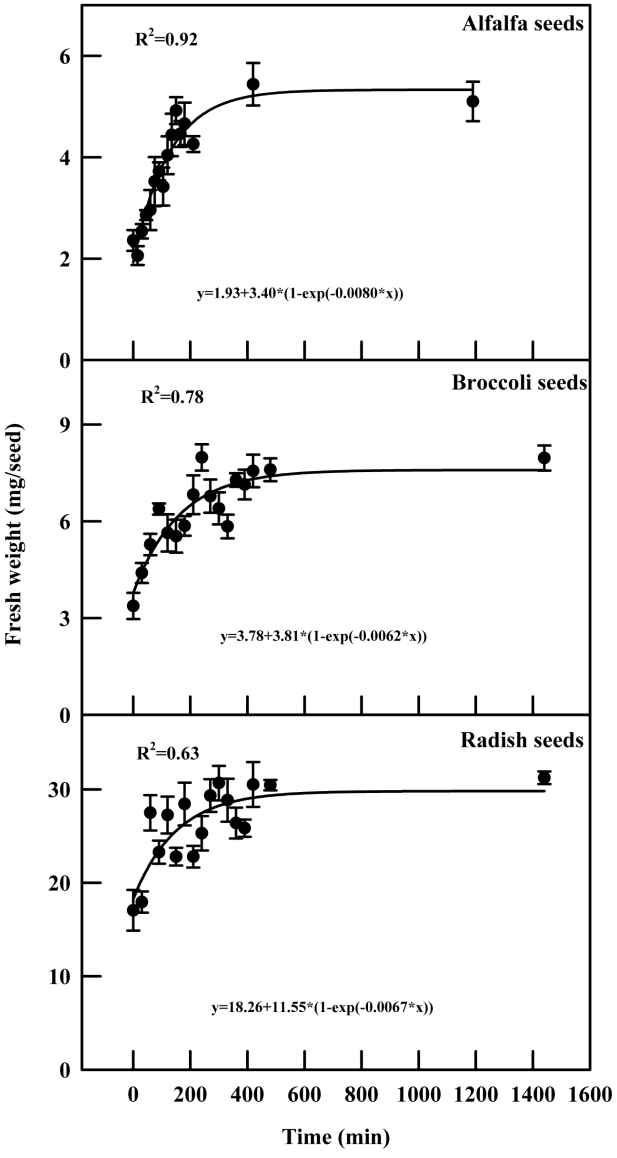

Fig. 1. Imbibition curves for seeds of alfalfa, broccoli, and radish. The vertical bars indicated standard errors.

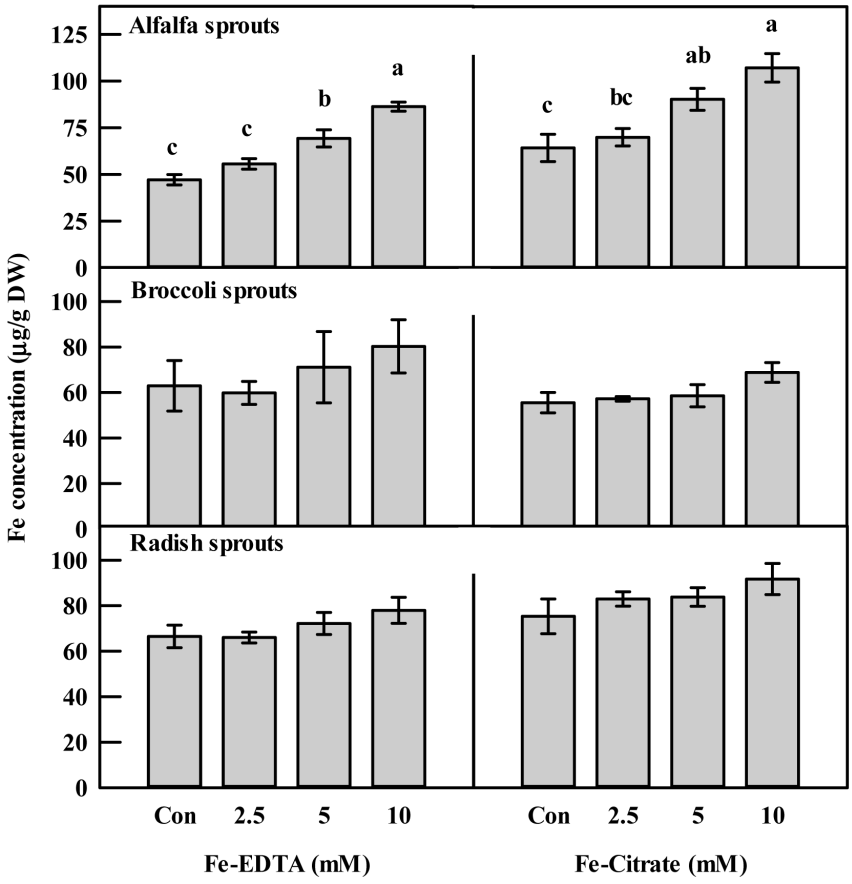

Fig. 2. Iron concentrations in the sprouts of alfalfa, broccoli, and radish, measured 5 days after sprouting. The vertical bars indicated standard errors. Different letters in bars indicate significant difference at $p < 0.05$.

solutions (Fig. 2). Soaking seeds with Fe(III)-EDTA or Fe(III)-citrate supplemented the iron content of 5-day-old alfalfa sprouts, and this supplementation was significantly different from the controls for alfalfa sprouts treated with the 5 mM or 10 mM iron solutions. In particular, the iron concentrations in alfalfa treated with 10 mM of either Fe(III)-EDTA or Fe(III)-citrate was about twice as high as those of the controls. For broccoli and radish sprouts, the Fe(III)-EDTA and Fe(III)-citrate treatments both marginally increased iron concentrations compared to the controls, but these differences were not significant.

Other Mineral Concentrations

The iron-chelate soaking treatments affected the concentration of several other minerals in the alfalfa sprouts (Table 1). According to iron chelate, the concentration of several minerals such as K, Ca, Mg, S, Na, Cu, and Zn was changed. Alfalfa sprouts treated with Fe(III)-EDTA had higher concentration than those treated with Fe(III)-citrate. Regarding the concentration of iron, the concentrations of Ca, Mg, and Na were significantly lower than those of the controls for both the Fe(III)-EDTA and Fe(III)-citrate treatments. Most notably, 34% and 68% reductions in Ca concentration were observed for the 10 mM Fe(III)-EDTA and Fe(III)-citrate treatments, respectively. In addition, alfalfa sprouts receiving the both Fe(III)-EDTA and Fe(III)-citrate treatments showed a significantly higher K concentration as the iron concentration increased. For broccoli and radish sprouts a similar pattern of decreased mineral concentrations was also observed, with significantly lower concentrations of Ca, Mg, and/or Mn relative to the controls (data not shown).

Total Phenolic Concentration

Both iron-chelate treatments induced significantly higher total phenolic concentrations in alfalfa sprouts compared to the controls (Fig. 3). Alfalfa sprouts soaked with Fe(III)-EDTA showed an 8.0% to 13.5% increase in total phenolic concentration, whereas the Fe(III)-citrate treatment led to a 25.5% to 36.4% increase in total phenolic concentration. In broccoli and radish sprouts, an increase in total phenolic concentration relative to the controls was also observed for both treatments at high iron concentrations, but these differences were not significant.

Table 1. Concentrations of minerals other than Fe, measured 5 days after sprouting, in alfalfa sprouts soaked with Fe(III)-EDTA or Fe(III)-citrate.

Iron chelate (A)	Concentration (mM) (B)	Macronutrient					Micronutrient			
		P ($mg \cdot g^{-1}$ DW)	K ($mg \cdot g^{-1}$ DW)	Ca ($mg \cdot g^{-1}$ DW)	Mg ($mg \cdot g^{-1}$ DW)	S ($mg \cdot g^{-1}$ DW)	Na ($mg \cdot g^{-1}$ DW)	Cu ($\mu g \cdot g^{-1}$ DW)	Mn ($\mu g \cdot g^{-1}$ DW)	Zn ($\mu g \cdot g^{-1}$ DW)
Fe(III)-EDTA	2.5	7.17	12.19	0.83	2.07	3.22	0.83	14.41	15.93	89.53
	5	7.02	12.49	0.75	1.98	3.24	0.63	13.97	16.69	91.51
	10	7.17	13.00	0.64	1.79	3.23	0.53	14.76	16.69	91.74
Fe(III)-Citrate	2.5	6.99	10.52	0.54	1.60	2.45	0.43	9.92	16.17	74.98
	5	7.15	11.75	0.59	1.58	2.53	0.29	10.24	19.00	75.25
	10	7.34	12.76	0.31	1.40	2.50	0.17	10.03	17.24	70.05
Control		7.64	11.29	0.96	2.17	3.08	0.78	12.93	21.24	97.06
A		NS	*	***	***	***	***	***	NS	***
B		NS	*	**	*	NS	**	NS	NS	NS
A × B		NS	NS	NS	NS	NS	NS	NS	NS	NS

NS,*,**,***Nonsignificant and significant at p = 0.05, 0.01, and 0.001, respectively.

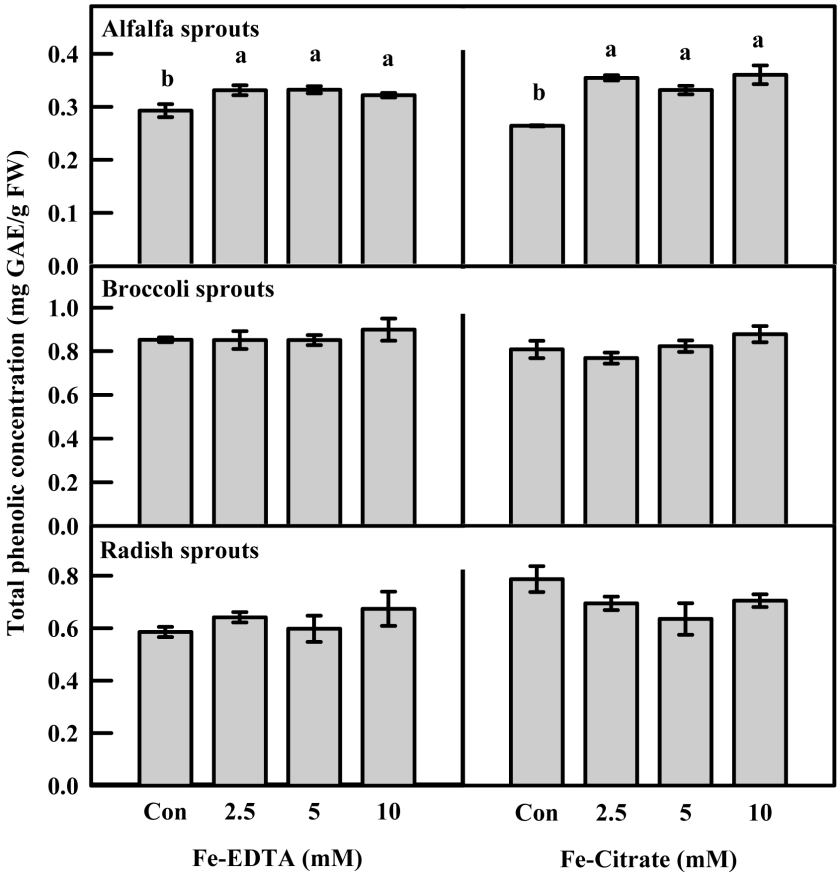

Fig. 3. Total phenolic concentrations in the sprouts of alfalfa, broccoli, and radish, measured 5 days after sprouting. The vertical bars indicated standard errors. Different letters in bars indicate significant difference at $p < 0.05$.

Growth Characteristics

The iron-chelate soaking treatments did not adversely affect dry matter accumulation in any of the three sprout species (Table 2), with no significant difference in dry weight between treatments and controls in terms of iron chelate and/or iron concentration. The iron soaking treatments also had little effect on fresh weights; although there were some significant differences between treatments and controls,

Table 2. Growth characteristics of sprouts of alfalfa, broccoli, and radish soaked with Fe(III)-EDTA or Fe(III)-Citrate, measured 5 days after sprouting.

Iron chelate (A)	Concentration (mM) (B)	Alfalfa		Broccoli		Radish	
		Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)
Fe(III)-EDTA	2.5	17.98 ab	1.35	28.18 b	3.02	156.85	13.93
	5	17.82 ab	1.33	30.70 ab	2.90	145.07	12.60
	10	17.18 bc	1.30	33.62 ab	3.20	136.13	12.93
Fe(III)-Citrate	2.5	16.56 c	1.28	35.82 a	2.97	115.83	13.02
	5	18.62 a	1.36	31.03 ab	2.95	118.15	12.81
	10	16.94 bc	1.27	29.69 b	2.89	119.09	13.45
Control		16.82 bc	1.27	32.29 ab	2.86	119.74	12.59
A		NS	NS	NS	NS	***	NS
B		*	NS	NS	NS	NS	NS
A × B		*	NS	*	NS	NS	NS

NS,*,*** Nonsignificant and significant at p = 0.05 and 0.001, respectively.

there was no pattern of increasing effect with increasing iron concentration.

Discussion

We investigated the efficacy of soaking seeds in an iron-chelate solution as a method for iron supplementation in three species of edible sprouts. Our results demonstrated that such a treatment could be effective in increasing the concentration of minerals such as iron in sprouts. However, the efficacy of the treatment for iron supplementation varied by species. Alfalfa seeds subjected to a high-iron soaking treatment produced sprouts with significantly higher iron concentrations than the controls, but the observed increase in iron concentrations was not significant in broccoli and radish sprouts (Fig. 2). Scarification of broccoli and radish seeds (but not alfalfa seeds) was required to obtain acceptable R^2 values for their imbibition curves, suggesting relatively low permeability of the seed coats of those species. Scarification has been used to improve seed germination rates (Hutchison and Ashton, 1979) by cracking the cuticle layer of the seed coat so that water can more easily penetrate (Ma et al., 2004).

Leakage of a variety of substances, including minerals, from seeds is a common phenomenon during imbibition (Duke and Kakefuda, 1981; Larson, 1968; Simon and Raja Harun, 1972). Among the essential minerals, K, Mg, Cl, Ca, and Mn are the minerals most subject to leakage in various types of seeds (Loomis and Smith, 1980; Lott et al., 1991). Consistent with this, imbibing seeds with iron-chelate solution induced a significant decrease in the concentrations of several minerals, such as Ca, Mg, Na and/or Mn, in all three species of sprouts tested in this study. The mineral concentrations of alfalfa sprouts derived from seeds soaked in iron-chelate solution for 7 h (Table 1) were much lower than those of sprouts derived from seeds subjected to a 2-h soaking (data not shown), suggesting the role of imbibition in the loss of minerals. Meanwhile type of iron chelate affected the concentration of several minerals of the sprouts although there was no significant effect in iron concentration of the sprouts, which was consistent with the result of the previous study using tomato plants (Kołota et al., 2013).

Typically, plants exposed to high concentration of metals increase their production of antioxidants, including phenolic compounds, to maintain intracellular redox homeostasis and prevent damage by toxic reactive oxygen species (Bailly, 2004; Kranner and Colville, 2011). There are few reports of this phenomenon in seeds; however, in our study, the increased iron concentrations in alfalfa sprouts as a result of the treatment of seeds with iron-chelate solution induced significantly higher concentrations of phenolic compounds (Figs. 2 and 3). Our study thus suggested that excess iron in seeds might elicit production of antioxidants such as phenolic compounds. However, additional research is needed to elucidate the exact mechanism for this, as well as the duration of the effect.

Iron and phenolic compounds are both very important nutrients for humans. Since iron deficiency is a major cause of disease in humans, many studies regarding iron supplementation and homeostasis have been conducted (Murgia et al., 2011). Furthermore, several thousand phytochemicals present in plant-based foods like fruits and vegetables, including many phenolic compounds, have been found by research to have beneficial effects on health (Rajashekar et al., 2009;

Treutter, 2010). Because of these health benefits, the positive relationship between the concentrations of iron and phenolic compounds in alfalfa sprouts is advantageous. It is also convenient that total phenolic concentrations in broccoli and radish sprouts did not decrease in response to the iron-chelate treatments. However, further studies are needed to better understand the interaction between iron and phenolic compounds.

Excessive iron in plants inhibits photosynthesis and induces oxidative stress that results in severe cellular damage (Kampfenkel et al., 1995). However, the increased iron concentrations in the sprouts in this study did not significantly inhibit growth (Table 2) and did not affect germination rate (data not shown). This suggested that the iron concentrations used in this study were too low to cause negative effects on growth, although it was also possible that iron toxicity might have manifested later than the age assessed in this study.

In conclusion, soaking seeds in high-iron-chelate solutions led to iron supplementation of the resulting sprouts, particularly for alfalfa, and had positive or neutral effects on total phenolic concentrations, suggesting that iron-chelate treatment of seeds might be an effective strategy for increasing the mineral content and the nutritional value of the sprouts of some plant species.

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