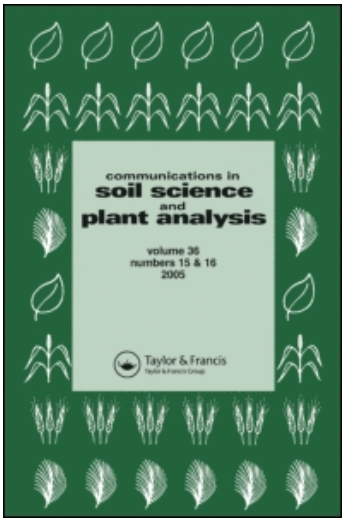

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Problem of Applying Sodium Selenate to Increase Selenium Concentration in Grassland Plants in Southern Belgium

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In a survey of grasslands, mean selenium (Se) concentration in Holcus lanatus *was 83 μg kg^{-1} (less than 100 μg kg^{-1}, the minimal concentration protecting mammals from deficiency disorders). Despite rather high levels of soil extractable Se, plant Se availability was supposed to be low because of high soil humus concentration. A pot experiment with common grassland species showed contrasting responses to selenate addition (9 g Se ha^{-1} yr^{-1}).* Lolium perenne *leaves reached 470 μg kg^{-1}, and* Trifolium pratense *reached 292 μg kg^{-1}. The controls were less than 100 μg kg^{-1}. Leaves of others species showed greater values both in control and treated series and no significant difference. In a second pot experiment,* Melilotus albus*, a supposed secondary accumulator, and* Lolium perenne *as a control were submitted to moderate increased selenate additions (up to 45 g Se ha^{-1} yr^{-1}). The results confirmed that* Melilotus albus *was a better accumulator with a leaf concentration that could reach the toxicity level of 2 mg kg^{-1}.*

Keywords Belgium, fertilizer, grassland, plants, selenium, soil

Introduction

Soil selenium (Se) concentration is highly variable, and according to data reviewed by Gupta and Gupta (2000), ranges from less than 0.1 mg Se kg^{-1} in Finland podzols to as much as 80 mg Se kg^{-1} in seleniferous soils of western United States. Selenium is an essential trace mineral for humans, animals, algae, and plants endemic to seleniferous soils (Gupta and Gupta 2000; Rayman 2004; Tapiero, Townsend, and Tew 2003; Terry et al. 2000; Whanger 2004). The essentiality of Se for vascular plants growing on non-seleniferous soils was still recently a matter of debate, despite the fact that it has been shown to activate the antioxidative system of plants (Hartikainen et al. 1997; Hartikainen, Xue, and Piironen 2000; Cartes, Gianfreda, and Mora 2005; Hartikainen 2005). Selenium mainly reaches food chains and animals through plants, after they absorb it from the soil. Selenium proceeds mainly through sulfur (S) metabolic pathways; in particular, selenate follows sulfate metabolism (Terry et al. 2000). Regions with chronic Se deficiency

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in animal or human populations are generally characterized by total soil Se concentrations less than 0.6 mg kg^{-1} (Gupta and Gupta 2000). On these soils, most crop and forage plants contain less than 100 μg Se kg^{-1}, which is the minimal concentration that protects mammals from deficiency disorders. The recommended Se concentration for forages fed to dairy cattle in the United States ranges between 100 and 300 μg kg^{-1}, whereas the toxicity threshold level is close to 2 mg kg^{-1} (Buchanan-Smith et al. 2001).

It has been established that the human population in Belgium is deficient (Robberecht and Deelstra 1994; Rayman 2000, 2004; Combs 2001), most likely due to low Se concentration in agricultural products. According to Sillanpää and Jansson (1992), soil extractable Se concentrations in Belgium (10 μg Se kg^{-1}) are median among the 30 studied countries (international mean: 16 μg Se kg^{-1}) and close to those of Finland (11 μg Se kg^{-1}), one of the countries with the lowest Se soil concentrations in agricultural production. In reference cereal crops, Se concentrations in Belgium (33 μg Se kg^{-1}) were found to be well below international values (mean 108 μg Se kg^{-1}) but about four times greater than those found in Finland (7.5 μg Se kg^{-1}). Data collection mainly covered northern Belgium (Sillanpää and Jansson 1992). More recent data (de Behr et al. 2003; Cabaraux et al. 2005) gathered in some grasslands of southern Belgium confirmed that plant production Se concentration was less than the 100 μg kg^{-1} threshold limit for animal production. Attempts at food chain enrichment were undertaken using Se-supplemented fertilizers. Selenium applications to winter barley and pastures used in suckler cow (*Bos taurus taurus*) production increased the Se concentrations in feedstuffs to 200 μg kg^{-1}, which meets the recommended Se range; consumption of Se-enriched feedstuffs also improved animal health (Dufrasne et al. 2004). This practice of adding Se to fertilizers was performed in Finland since 1984 and resulted in a general increase of Se content in agricultural productions (Jukola et al. 1996).

Before recommending systematic Se addition to fertilizers in Belgium, three questions have to be solved to guarantee safety in the food chain and in the environment. First, only a limited fraction of the applied Se to grassland is absorbed (e.g., 23 to 52% in de Behr et al. 2003). In Finland, Se leaching from fields is a concern (Govasmark et al. 2008). A concentration of 1 μg Se L^{-1} could be reached in the leaching water, assuming 50% dissolution of 10 g Se ha^{-1} in an annual 0.5-m leaching water flux. Such a Se concentration is close to the 2 μg Se L^{-1} considered toxic for aquatic organisms (Crane et al. 1992). In contrast, if Se soil immobilization is effective, it is of interest to know the conditions and the risk of a further release. Second, Se fertilization could not be a reliable method to consistently increase plant Se levels when floristic composition is poorly controlled. Indeed, in a pot experiment with a grassland soil from southern Belgium, the addition of 9 g Se ha^{-1}, as sodium selenate, with standard fertilizer application resulted in the accumulation of Se in treated plant species up to 0.3 mg kg^{-1}, and tissue Se concentrations were consistently less than the 2 mg kg^{-1} toxicity threshold level (Hambuckers et al. 2008). However, several tested plant species (adventices) appeared to be poor Se accumulators, with the exception of *Sinapis arvensis* and *Melilotus albus*, which are probably secondary accumulators. Those kinds of species grow on soil with low to medium Se levels and can accumulate up to 1000 mg Se kg^{-1} when Se is available (Brown and Shrift 1982; Terry et al. 2000); they also accumulate more Se than nonaccumulators even when grown on low-Se soils. The tolerable Se concentration range from sufficiency to toxicity is quite narrow (Terry et al. 2000). For mammals, the toxic dose is only 10 times more than the recommended requirements (Yang and Zhou 1994; Wilber 2000; Buchanan-Smith et al. 2001). Finally, there is a lack of knowledge on Se status and fate of Se in Belgian agricultural soils, especially in the southern part of the country.

The present study addresses the problem of Se fertilization reliability, considering accumulation variability in grassland plants and the question of Se soil status in southern

Belgium. We present data on exchangeable Se in soil and Se concentration in plant samples collected in grasslands from various ecoregions of eastern Belgium. We also report results on the ability of meadow plants to accumulate Se from a native soil amended or not with sodium selenate fertilizers following standard treatment, and finally we examine the consequence of amending a nonaccumulator plant and a supposed secondary accumulator plant with an increasing amount of sodium selenate.

Material and methods

Twenty-five grasslands were surveyed on 17 and 18 April 2008 for Se in upper soil horizon (organomineral horizon) and in plant tissues. At each site, three soil samples spaced along 5 m were collected from the first 25 cm using a 3-cm-diameter core sampler. Aerial parts of *Holcus lanatus* grass species were also collected in the vicinity of soil samples. To get a representative picture of Se status in southern Belgium grasslands, sites were chosen across most of the ecoregions according to Delvaux and Galoux (1962) and Onclincx et al. (1987).

The experiments of Se accumulation in plant species were carried out mainly following Hambuckers et al. (2008). Seeds were obtained from European botanical gardens or plantlets were collected in the wild. During March 2007, the seeds were sowed in 10-cm-diameter containers. After germination, seedlings or plantlets of each species were transferred to ten 30-cm-diameter × 26-cm-high polyvinylchloride (PVC) containers filled with a meadow cambisol of the Entre-Vesdre-et-Meuse district (Baelen locality). Containers were installed outdoors in a trench to minimize solar lateral heating, and it was verified at the end of the experiment that no root had grown out of the containers through the drain holes. The culture site was a meadow in the area of Liège (Belgium) at 150 m above sea level. Mean annual rainfall was 848 mm, and average temperature was 8.9 °C (temperate climate with mixed oceanic and continental influences). To examine Se accumulation under standard Se treatment, we grew cultivated species (*Lolium perenne* Elgon, *Lolium perenne* Ritz, *Trifolium pratense*) or frequently encountered adventitious species (*Melilotus albus*, *Plantago major, Plantago lanceolata, Rumex acetosa, Rumex conglomerata, Knautia arvensis, Sanguisorba officinalis*). Standard Se treatment group plants received a total of 9 g Se ha^{-1}, applied as sodium selenate, split over three separate monthly applications. The first application was made with a 15 nitrogen (N)–9 phosphorus pentoxide (P_2O_5)–18 potassium oxide (K_2O) + 8 magnesium oxide (MgO) fertilizer (+ 8 mg Se kg^{-1} for the treatment groups) at a rate of 375 kg ha^{-1} on 6 June 2007. The second and third applications took place on 1 July and 1 August 2007, with a 25 N + 5 MgO fertilizer (+ 10 mg Se kg^{-1} for the treatment groups) at rates of 300 kg ha^{-1}. Aerial plant parts were collected on 20 and 21 August 2007. To test Se accumulation with various amount of Se added to soil, two species (*Melilotus albus* and *Lolium perenne* Elgon) were processed as before with applications of fertilizer without Se. The containers also received 100 ml of sodium selenate (Na_2SeO_4) solutions on five occasions (6 June, 15 June, 1 July, 16 July, and 1 August 2007) to reach 0 to 45 g Se ha^{-1} in the treatment series or 100 mL of deionized water in the control series.

Harvested plant material or soil samples were dried for 1 month in paper bags in a herbarium drying room (40 °C, 20% relative humidity). Plant parts and soil samples were dried to constant weight (60 °C) before subsampling for analysis. Soil exchangeable cations (CEC), pH, and humus concentration were estimated following standardized procedures. Acetic acid / ammonium acetate / ethylenediaminetetraacetic acid (EDTA)–extractable Se (SeAAAc) was prepared following Lakanen and Erviö (1971). A 25-g soil sample was suspended and shaken for 1 h in 250 mL of the extraction solution in which

pH was 4.65, ammonium acetate and acetic acid were 0.5 M, and Na_2EDTA was 0.02 M. Soil suspension was then filtered, and Se in the filtrate was analyzed. Selenium analyses of the survey were carried on with an Agilent series 4500 inductively coupled plasma–mass spectroscopy (ICP-MS) apparatus (Diegem, Belgium). Plant material was processed following conditions of the ICP-MS method of Huang and Gulson (2002), validated by the authors according to a standardized procedure of the Australian Government Analytical Laboratories. Two hundred–mg subsamples were left overnight covered with 4 mL distilled nitric acid (HNO_3) in Duran 50-ml open vessels followed by 1.5 h at 95 °C (water bath). Milli-Q water was added to each tube to bring it up to 40 mL; the tubes were sonicated for 1 h. Then the supernatant was filtrated, and 0.4 mL of internal standards (In + Rb) was added for ICP-MS analysis. Soil extracts were analyzed following the ISO 17294-2 method for water. Detection limits were 2 μg Se kg^{-1}. For plant samples collected in accumulation experiments (standard Se treatment and increasing Se addition), Se was determined by the high-pressure liquid chromatography (HPLC)–fluorescence method of Hawkes and Kutnink (1996), validated by the authors with respect to 23 biological reference materials spanning a 1800-fold range of Se concentrations. Four hundred–mg samples were hot-ashed with a mixture of concentrated HNO_3 and perchloric acid ($HClO_4$; 5/1, v/v) in open vessels. Selenium(VI) in the form of oxyanions was reduced to Se(IV) by the addition of 1 ml 4 M HCl and heating to 160 °C. Derivation with 2,3-diaminonaphthalene was performed after addition of glycine and Na_4EDTA at a pH adjusted to 1.75. The fluorophore was extracted in cyclohexane and injected in a Gilson HPLC system (Pump 307, Autoinjector 234; Middleton, Wisc.) equipped with a Lichrosorb Merck 10-μm column, 25 cm × 0.4 cm i.d. (Darmstadt, Germany). The mobile phase was 90% cyclohexane / 10% ethyl acetate. Fluorescence was measured using an excitation wavelength of 378 nm and an emission wavelength of 530 nm with a Perkin-Elmer LS30 detector (Waltham, Mass.). Detection limit was 0.2 μg kg^{-1}.

Plant Se concentrations were normalized by using square root values. They were compared according to a general linear model (SAS 1999) using Se treatment, species, and treatment × species as factors of variation. The means were compared between them by the Student's t-test.

Results

Results of the survey in the ecoregions of southern Belgium are summarized in Table 1 (soil properties and *Holcus lanatus* Se concentrations). Correlations between plant Se concentrations and soil properties are not significant (Table 2). Ecoregions have contrasted climates because of differences in altitude, distance from sea, or sun exposure. As an example, annual rainfall ranges from 700 to 1200 mm. Most of the soil belongs to the cambisol division but is derived from various geological bedrocks. The sampling did not take the whole range of condition variability (only a limited sampling of each meadow, 20 ecoregions sampled over 26, only one meadow per ecoregion) but nevertheless should give a representative profile of the Se status of soil and plants in the country. In addition, the sites should constitute a valuable reference network; indeed, it was ascertained that all but perhaps one did not receive mineral fertilizers in at least the past 3 years because the meadows are part of a biodiversity conservation network. Even if Se was added with fertilizer as selenate 3 years ago, it is known that its effect would be reduced because residual effect of selenate addition in the subsequent years is poor (Sager and Hoesch 2006).

Comparisons of accumulation by leaves of plant species cultivated with or without Se addition are given in Table 3. Sample numbers were too small to perform statistical analysis with stem and flowers results, so these analyses are not presented. Factor (plant species, Se

Table 1
Means, standard deviations (SD), maxima (max), and minima (min) of soil properties of the 24 surveyed grasslands of southern Belgium

Parameter	Humus (%)	Conductivity ($\mu S\ cm^{-1}$)	pH_{KCl}	CEC ($Cmol\ kg^{-1}$)	Se_{AAAc} ($\mu g\ kg^{-1}$)	Se_{plant} ($\mu g\ kg^{-1}$)
Means	5.54	65.2	5.03	29.4	36.9	82.7
SD	1.35	38.2	0.93	7.2	14.0	55.0
Min	2.47	21.4	3.89	18.6	17.5	1.3
Max	7.88	160.6	7.46	47.0	79.8	196.8

Table 2
Linear regression of Se concentration in *Holcus lanatus* as a function of soil properties of the southern Belgium surveyed grasslands

Parameter	P > F
Humus	0.2237
Conductivity	0.7350
pH_{KCl}	0.4865
CEC	0.9819
Se_{AAAC}	0.3567

treatment) and interaction effects were all significant ($P < 0.05$), emphasizing differences between species Se concentrations and between plants treated or not with Se. However, comparisons of means were only significant for two species, *Lolium perenne* Elgon and *Trifolium pratense*.

The results of Se accumulation in parts of the two plant species treated with increasing amount of Se are given in Figure 1. For each plant part (stem, leaf and flower), linear regression between Se added to the soil and plant part Se concentration was significant ($P < 0.05$).

Discussion

Levels of Se in plants (*Holcus lanatus*) and in soil (Se_{AAAc}) from the survey revealed two important features about Se distribution in the studied area. First, Se mean level in collected plants was actually less than 100 $\mu g\ kg^{-1}$ in the area of interest. Some values were close to 200 μg Se kg^{-1}, but one has to consider that sampling was done early in the growing season when mineral concentrations of plant parts are the greatest (Fitter and Hay 2002); that plant samples were only leaves, which are richer in Se than stems, flowers, or fruits; and that most of the sampled meadows were extensively managed or even unexploited, which would favor greater soil mineral availability. Therefore, these data indicate potential Se deficiency in meadow production of eastern Belgium. Second, Se_{AAAc} in the soil sampled were rather high considering the values of 16 μg Se L^{-1} obtained for culture soils in Belgium by Sillanpää and Jansson (1992), but these soils also had

Table 3
Comparisons of means (P > |t|) and variance analysis (P > F) and of leaf Se concentration of plant species cultivated with or without sodium selenate addition (9 g Se ha^{-1} yr^{-1}) on a grassland cambisol of southern Belgium (Baelen locality)

Variety	Control ($\mu g\ kg^{-1}$)	Se($\mu g\ kg^{-1}$)	P > |t|
Lolium perenne Elgon	55	470	0.0010
Lolium perenne Ritz	269	481	0.1008
Trifolium pratense	90	292	0.0321
Rumex acetosa	463	439	0.9881
Plantago major	631	356	0.1012
Plantago lanceolata	288	390	0.4218
Sanguisorba officinalis	605	781	0.2336
Knautia arvensis	123	285	0.2269
			P > F
Treatment	315	437	0.0028
Species			< 0.0001
Species × treatment			0.0396
Model			< 0.0001

lower humus concentration (about 1%) according to agricultural use. According to Se plant concentrations recorded in this study, it is to be expected that Se availability should be dramatically low in the soil of eastern Belgium. Nevertheless, high Se_{AAAc} means that all tested soils had significant exchangeable Se pool but organic forms are unavailable for plants. Indeed, Sillanpää and Jansson (1992) observed that Se_{AAAc} predictive value of Se plant concentration increased when corrected for humus soil concentration.

Mean values of accumulation in leaves of plant species cultivated with or without Se addition were high. Indeed, Se concentration largely exceeded the expected values (i.e., less than 100 $\mu g\ kg^{-1}$ in control plants or around 200–300 $\mu g\ kg^{-1}$ in Se-treated plants) obtained by Hambuckers et al. (2008). In the comparisons of means, both species displayed significant differences between control and Se-treated plants (*i.e., Lolium perenne* Elgon and *Trifolium pratense*); Se concentrations were, however, close to expected values. When comparing the cultural conditions of the present study (2007) with those of Hambuckers et al. (2008) in 2005, we noted that weather was very different. In 2005, the growing season was characterized by several periods of poor conditions (i.e., low insulation and heavy rain), then water deficit for 1 month, followed at the end of June by 5 days with temperature greater than 25 °C and 2 days with temperature greater than 30 °C. In contrast, 2007 was more favorable for plant growth with the first month warm and rainy followed by normal precipitation and temperature for the remaining period. As a rule, Se leaching and plant absorption strongly depend on water because they rely on the solubilization of selenate or of selenite (Havlin et al. 1999). One can suppose that the weather would be also more propitious for Se availability to plants in 2007 (less leaching, better absorption) than in 2005. This hypothesis could explain the higher Se level even in the controls. High Se_{AAAc} value could additionally explain the greater than expected Se plant concentrations. It is possible that some weather conditions favor Se plant availability by promoting microbial release of organic Se into soluble mineral forms. For example, Laporte, Duchesne, and

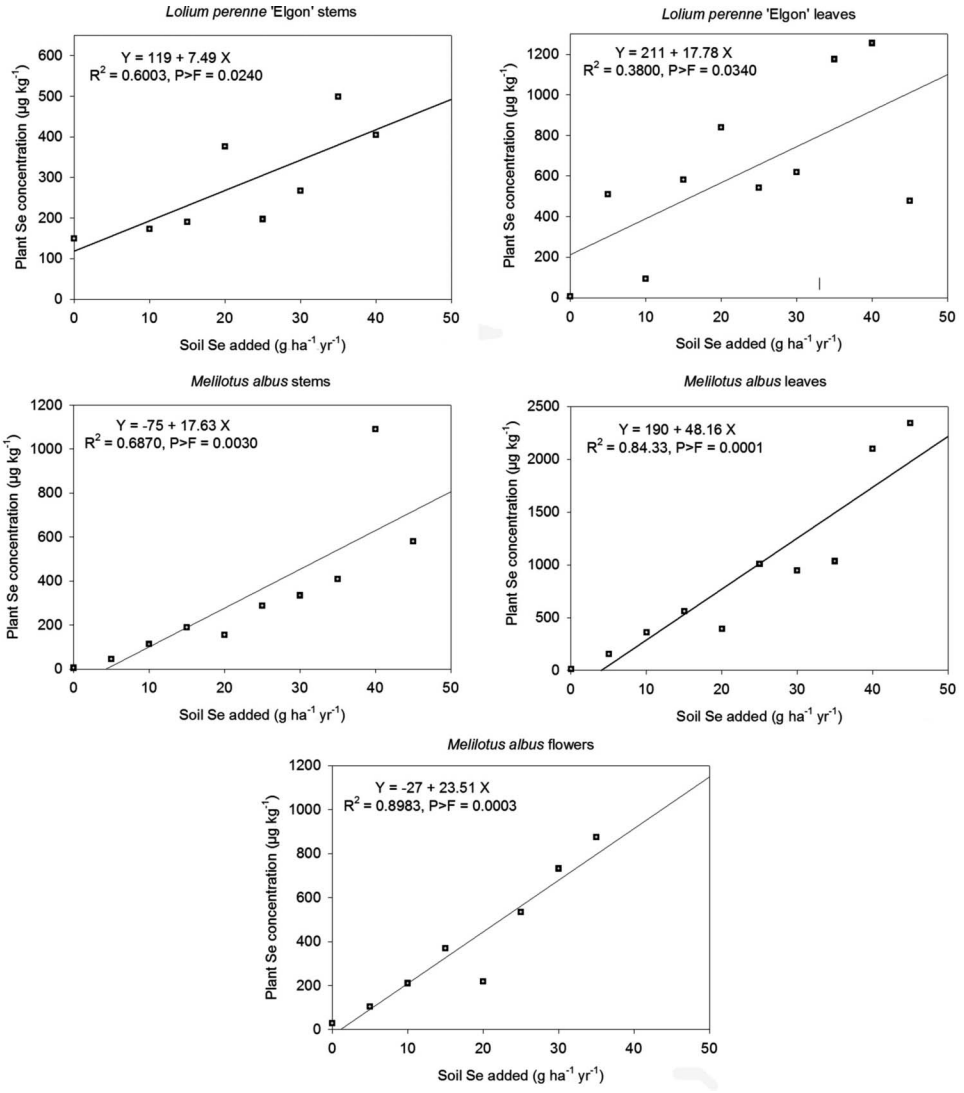

Figure 1. Relationships between Se concentrations in plant parts of *Lolium perenne* 'Elgon' and *Melilotus albus* cultivated on a grassland cambisol of Southern Belgium (Baelen locality) and Se addition as sodium selenate.

Wetzel (2002) observed on a grassland soil that soil surface carbon dioxide (CO_2) efflux (this measure integrating microbial and root respiration) decreased when reducing irrigation frequency. Moreover, fertilizers were applied in 2007 as granules, whereas they were applied as water suspensions in 2005 by Hambuckers et al. (2008). Selenate mainly follows the sulfate metabolic pathway in the plant (Terry et al. 2000), and for this reason Se absorption should increase as a function of N absorption rate like sulphate (Leustek et al. 2000). Applying N fertilizers in the form of granules probably increased soil heterogeneity more than those in the form of water suspension. Fertilizer application in granules probably resulted in increased Se absorption heterogeneity. Such an effect could happen by

means of any N fertilizers by increasing mobilization of native Se and could be reinforced when plant density in containers is low, as was the case in our experiment for several of the cultivated species. It could explain the greater than expected concentrations in Se-treated and control plants and the absence of significant difference between them. Strong variability of Se accumulation by plant was previously observed (de Behr et al. 2003). Shoots of *L. perenne* collected from a meadow fertilized with selenate had weaker concentration (76 μg Se kg^{-1}) than control samples (239 μg Se kg^{-1}), with the difference being nonsignificant. At the meadow scale, variability was also high. The Se concentration of hay samples collected in treated meadow (274 μg Se kg^{-1}) was significantly different from that in the control (131 μg Se kg^{-1}), but standard deviations were considerable (more than 100% of means).

When considering Se accumulation in plant parts with increasing Se addition to soil, one can note that variability of Se concentration was strong (low R^2 in Figure 1): for example, in *Lolium perenne* leaves, 94 μg Se kg^{-1} with 10 g Se ha^{-1} or 478 μg Se kg^{-1} with 45 g Se ha^{-1}; in *Melilotus albus* stems, 1,091 μg Se kg^{-1} with 40 g Se ha^{-1}, and so on. Moreover, *Melilotus albus* was a better accumulator than *Lolium perenne,* particularly in leaves (in Figure 1, the slope was more than the double for *Melilotus albus* compared with *Lolium perenne*). Otherwise, in *Melilotus albus* leaves, Se concentration exceeded the toxicity threshold of 2 mg kg^{-1} when the plant was treated with 40 g Se ha^{-1}, that is, five times less than the recommended soil Se addition. The fact that this plant species was a better accumulator than *Lolium perenne* reinforced the idea that it could be a secondary accumulator. The other reasons were that two members of the *Melilotus* genera are notorious secondary accumulators (Wu, Guo, and Bañuelos 1997; Guo and Wu 1998) and that many members of Leguminosae plant family produce numerous nonprotein amino acids, including sulfuro-amino acids (Seigler 1995), which constitute potential Se accumulation sites by substitution with sulfur. Because secondary accumulators concentrate more than the other plant species even without Se addition, fortuitous spreading of such species in meadow would increase Se concentration and Se variability in plant production.

Conclusions

Selenium meadow plant concentration is apparently too low in eastern Belgium to satisfy Se animal requirements, but the soil contains a significant Se exchangeable pool, probably in an organic form of low availability for plants. Treating meadow plants with 9 g ha^{-1} sodium selenate increased Se concentrations in the plant production, but it seems that the risk of exceeding the 200–300 μg kg^{-1} recommended value is significant. This would happen as a result of soil heterogeneity due to fertilizer application or from favorable weather conditions; both those factors promoted the mobilization of native soil Se. Despite a possible increased availability of Se when fertilizer is applied, plant Se at a meadow scale is not expected to reach levels exceeding the recommended value with the proposed dose. In contrast, if weather conditions significantly influence Se availability, Se plant concentration could vary yearly because of variation in the mobilization of the selenate pool or in the release of organic Se into plant-available forms. This conclusion reinforces the idea that it would be useful to study Se speciation and turnover, in particular with organic carbon (C) in those soils, to know the conditions of the release-available forms for plants. In addition, the fortuitous growing population of supposed secondary accumulators could increase yearly plant Se variations because these species accumulate more Se than the other ones, even on soils with low Se availability.

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