

## Synergistic Effect of Selenium Addition and Water Stress on *Melilotus officinalis* L. Mineral Content

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### Abstract

The objective of this study was to examine the combined effects of selenium (Se) enrichment and water stress on the accumulation of available macro- and micronutrients in *Melilotus officinalis* L. aerial parts. Plants of *M. officinalis* were subjected to three levels of Se addition (0, 1 and 3 mg Se L<sup>-1</sup> water) and to two water treatments: a) full irrigation and b) limited irrigation (water stress). The above ground biomass (stems and leaves) was analyzed for Se, potassium (K), sodium (Na), magnesium (Mg), iron (Fe), copper (Cu), calcium (Ca), manganese (Mn) and zinc (Zn). Se addition differentially affected the K, Mg and Ca content of *M. officinalis* aerial parts, while it led to the reduction of the micronutrients Cu, Fe and Mn. Water stress resulted in the increase of K, Na, Mg, Ca and Cu, and to the decrease of the Fe, Zn and Mn content. An interaction between selenium addition and water treatment was more notable for Ca and Mg, which decreased under water stress at low Se level and for Zn and Cu, which increased under water stress at high Se level. According to our findings, Se-induced increased accumulation of some inorganic ions in the aerial parts of this species under water stress conditions could serve as a means to alleviate the adverse impact of water deficit on important metabolic processes, enhancing *M. officinalis* tolerance to water stress.

**Keywords:** limited irrigation, macro-nutrients, micro-nutrients, sodium selenate, yellow sweetclover

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### Introduction

Water comprises one of the most important determinants of plant production and distribution on earth. Water deficit is considered as a crucial factor that decreases the crop yield around the world (Valliyodan and Nguyen, 2006), suppressing plant growth and development due to reduced water absorption and nutrient intake (Gunes *et al.*, 2007; Ciriaco da Silva *et al.*, 2011). One of the most important effects of water deficit is on transport of nutrients to the root and on root growth and extension (Fageria *et al.*, 2002; Samarah *et al.*, 2004). Decreased water availability affects the absorbance of nutrients from plant tissues (Mengel and Kirkby, 2001; Amtmann and Blatt, 2009). Water stress is generally regarded to reduce nutrient uptake by roots and their translocation to the shoots due to its negative effect on transpiration rate, active transport and membrane permeability (Marschner, 1995; Alam, 1999; Baligar *et al.*, 2001).

Nevertheless, numerous studies have shown that many species accumulate inorganic ions as a means to adapt to water stress (Patakas *et al.*, 2002; Zhu *et al.*, 2005), increasing in this way their drought resistance.

Selenium (Se) is an essential trace element for livestock (Mayland, 1994; Gupta and Gupta, 2000), as it is necessary for growth and fertility in animals (Hatch, 1982; Rogers, 1990). Although selenium is not considered as an essential element for plant metabolism (Saggoo *et al.*, 2004) and nutrition of higher plants (Terry *et al.*, 2000), there is evidence that at low concentrations it can modify the uptake and accumulation of essential minerals important for plant metabolism (Pazurkiewicz-Kocot *et al.*, 2003), increase plant resilience to oxidative stress, stimulate plant growth and delay plants senescence (Hartikainen *et al.*, 2000; Xue *et al.*, 2001; Simojoki *et al.*, 2003; Hajiboland and Amjad, 2007). Thus, Se at lower levels

Table 1. Properties of farm soil used in the experimental pots

pH	Organic matter %	N total %	P mg kg <sup>-1</sup>	K mg kg <sup>-1</sup>	Na mg kg <sup>-1</sup>	CEC meq/100g	CaCO <sub>3</sub> %	Sand %	Silt %	Clay %
8.5	0.5	0.4	5.0	52	92	5.5	7.5	51	39	10

could probably contribute to increase drought resistance in plants. However, in higher levels Se can be toxic (Terry *et al.*, 2000).

Se uptake by plants is governed by soil and plant factors. Among them the most important is its form and concentration in the soil (Wu *et al.*, 1994). Moreover, soil moisture affects soil Se availability for plant uptake (Banuelos and Meek, 1989). Several studies on soils enriched with Se (Wu *et al.*, 1993; 1996) found a strong relationship between water supply and Se accumulation by plant tissues. According to Kuznetsov *et al.* (2003), Se can stimulate more efficient water absorption through roots, and thereby decrease water loss from plant tissues.

Similarly to other heavy metals, Se can modify the uptake and accumulation of macro and micro-nutrients which are important for plant metabolism (Kopsell *et al.*, 2000; Pazurkiewicz-Kocot *et al.*, 2003; Tennant and Wu, 2000). Kostopoulou *et al.* (2015) reported various changes in macro-nutrient concentrations of Se enriched plants, while the micro-nutrient content decreased significantly. Generally, Se in high concentrations may compete with S and P (Tennant and Wu, 2000) but the effects on the uptake of other nutrients in plants are not very clear (Feng *et al.*, 2009).

Legume species are considered to accumulate greater amounts of Se than grasses (Mackowiak and Amacher, 2003). However, the ability of forage legumes to accumulate Se has been studied only in limited number of species, such as *Trifolium repens* and *Medicago sativa* (Terry *et al.*, 2000; Mackowiak and Amacher, 2003). *Melilotus officinalis* L. (yellow sweetclover) is a biennial legume species, considered as palatable forage of high quality both for livestock and wildlife (Mueggler and Stewart, 1980). This species has the ability to accumulate more than 100 mg Se kg<sup>-1</sup> DM in its tissues when irrigated with sodium selenate solution (Kostopoulou *et al.*, 2010). Therefore, it could be used either as a dietary supplement, in mixture with non-accumulator species, for livestock feed deficient in Se or for restoration of grasslands in seleniferous soils (Kostopoulou *et al.*, 2015).

Limited number of studies has evaluated the combined effects of Se concentration and water deficit conditions on the mineral content of plant species. Thus, the objective of this study was to examine if the synergism of Se concentration and water stress enhanced the uptake of available macro- and micro-nutrients by *Melilotus officinalis* tissues.

## Materials and Methods

### Description of the study site

The study was conducted at the farm of Aristotle University of Thessaloniki, 14 km south of the city of Thessaloniki (40°32' N, 22°59' E), at an elevation of about 5 m a.s.l. in spring 2002. The climate of the area, according to the bioclimatogram of Emberger (1942), could be characterized as Mediterranean semi-arid with cold winters. For the period 1978-2008, the mean annual temperature of the site was 15.5 °C and the mean annual rainfall 446 mm.

### Biological material

In February, seeds (commercial seedlot of Spanish origin) of yellow sweetclover (*M. officinalis* L.) were sown in each of sixty pots. All pots were 25 liters in volume and were filled with a 5:4:1:1 (on a volume basis) peat:soil:manure:sand mixture. The mixture comprised of white peat medium bedding, enriched with nutrients and trace elements (Klassmann TS1, Klassmann-Deilmann GmbH, Geeste, Germany), grassland soil (0-20 cm), classified as Typic Xerorthent (USDA-NRCS 1996), collected the same year from the farm of the Aristotle University of Thessaloniki, used as a managed grassland, farmyard manure and sand. Selenium concentration in the substrate was less than 1 mg kg<sup>-1</sup> dw. Soil properties are presented in Table 1.

### Experimental design

After seedling emergence (March 2002), the pots were thinned to a total of 10 seedlings per pot. Then the pots were transferred under a permanent rain shelter, a wooden construction of 2.5 m height, with a transparent plastic cover on top. The rain shelter was designed in such a way as to avoid rainfall reaching the pots, while keeping the atmospheric conditions unchanged. The pots were randomly divided in three groups of twenty pots each. The first group was irrigated only with tap water (0 mg Se L<sup>-1</sup> water); the second group was irrigated with a 1 mg Se L<sup>-1</sup> tap water solution and the third group received a 3 mg Se L<sup>-1</sup> tap water solution, both resulting from aqueous Na<sub>2</sub>SeO<sub>4</sub> (sodium selenate) dilution. Each of these groups was also divided into two subgroups of ten pots each: one subgroup (ten pots) was irrigated frequently (Full Irrigation) so that the soil in the pots was always near field capacity, as determined by tensiometers. The other subgroup (ten pots) received 50% less water than the fully irrigated plants (Limited Irrigation - Water Stress). Consequently, six treatments were established: a) No Se - Full Irrigation, b) No Se - Limited Irrigation, c) 1 mg Se L<sup>-1</sup> water - Full Irrigation, d) 1 mg Se L<sup>-1</sup> water - Limited Irrigation, e) 3 mg Se L<sup>-1</sup> water - Full Irrigation and f) 3 mg Se L<sup>-1</sup> water - Limited Irrigation. The total duration of the experiment was 45 days.

### Sampling and chemical analysis

By the end of the experimental period the herbage biomass from all treatments was cut to ground level. The above ground biomass (stems and leaves) was rinsed with deionized water, then dried for 48 h at 60 °C and ground through to ≤1 mm screen. Selenium concentration was determined colorimetrically after digestion using a combination of HNO<sub>3</sub>-HClO<sub>4</sub> (Holtzklaw *et al.*, 1987). A sample of 1 g from each tissue material was dry-ashed in a HCl 2N solution at 500 °C for 16 h. Potassium and Na concentration was determined using a flame photometer (PFP7, Jenway, Essex, England), while Mg, Fe, Cu, Ca, Mn and Zn concentration was determined with an atomic absorption spectrophotometer (AA-6300, Shimadzu Corporation, Tokyo, Japan).

Table 2. The effect of selenium treatment (in mg Se L<sup>-1</sup> irrigation water), water treatment and their interaction on selenium and macro- and micro-mineral content (in mg kg<sup>-1</sup> dry weight) of *Melilotus officinalis* aerial parts. Data represent means (n=6 for selenium treatments and n=9 for irrigation treatments) ± standard error. Different letters in each column indicate significant differences (p<0.05)

	Se	K	Na	Mg	Ca	Cu	Fe	Zn	Mn
<b>Selenium treatment</b>									
0	9±1 <sup>a</sup>	48896±1855 <sup>a</sup>	638±35	7647±362 <sup>a</sup>	29119±1222 <sup>a</sup>	20.1±0.3 <sup>a</sup>	1968±329 <sup>a</sup>	109±7	173±13 <sup>a</sup>
1	136±4 <sup>b</sup>	48846±167 <sup>a</sup>	656±20	8115±204 <sup>b</sup>	30465±258 <sup>b</sup>	17.0±0.3 <sup>b</sup>	1081±104 <sup>b</sup>	97±3	128±5 <sup>b</sup>
3	186±4 <sup>c</sup>	52848±644 <sup>b</sup>	688±13	7440±35 <sup>c</sup>	26528±758 <sup>c</sup>	17.5±0.6 <sup>b</sup>	812±86 <sup>c</sup>	95±5	103±5 <sup>c</sup>
Significance	***	***	ns	***	***	**	***	ns	***
<b>Irrigation treatment</b>									
Full	110±26	48222±967	617±15	7619±252	27410±926	17.9±0.6	1667±263	103±6	147±14
Limited	111±26	52171±766	705±12	7850±159	29998±529	18.5±0.6	907±91	98±3	122±7
Significance	ns	***	***	***	***	**	***	**	***
<b>Se x irrigation treatment interaction</b>									
Significance	**	***	*	***	***	***	***	***	**

†: P<0.05; \*\*: P<0.01; \*\*\*: P<0.001; ns: not significant

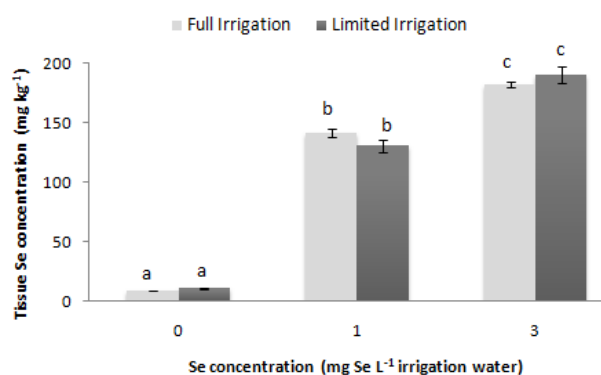


Fig. 1. Mean values (n=3) ± S.E. of selenium concentration in the aerial parts of *Melilotus officinalis* seedlings. Plants were subjected to three different Se concentrations (0, 1 and 3 mg Se L<sup>-1</sup> irrigation water) and two water regimes (full and limited irrigation). Different letters in each column indicate significant differences (p<0.05)

#### Statistical analysis

A split-plot experimental design was followed with ten replications for each treatment. Statistical analysis of the data was performed using ANOVA with the help of the SPSS® statistical software v. 22.0 (SPSS Inc., Chicago, IL, USA). Tukey test at 0.05 level of significance was used to determine differences among means.

#### Results

The accumulation of Se, K, Mg, Ca, Cu, Fe and Mn in the aerial parts (stems and leaves) of *M. officinalis* seedlings (across the irrigation level) was significantly affected by the addition of sodium selenate in the soil (Table 2), while the accumulation of Na and Zn was not affected (P≥0.05). In particular, the addition of 1 mg Se L<sup>-1</sup> and 3 mg Se L<sup>-1</sup> significantly increased the accumulation of Se about 15-fold and 20-fold respectively (Fig. 1). Similarly, the level of K was significantly increased but only with the addition of 3 mg Se L<sup>-1</sup>. Moreover, the addition of 1 mg Se L<sup>-1</sup> significantly increased the accumulation of Mg about 6% and Ca about 5%, while the addition of 3 mg Se L<sup>-1</sup> significantly

reduced the level of Mg and Ca about 3% and 9% respectively (Table 2, Fig. 2). As far as the micronutrients were concerned, the accumulation of Cu was significantly higher in the control treatment (no Se), while it did not significantly differ between the levels of sodium selenate (Table 2). Conversely, the content of Fe and Mn was gradually reduced with the addition of 1 mg Se L<sup>-1</sup> and 3 mg Se L<sup>-1</sup>.

The water treatment significantly affected the macro and micro-nutrient content (across the sodium selenate levels) but not the content of Se in the aerial parts of *M. officinalis* seedlings (Table 2). Specifically, the content of K, Na, Mg, Ca and Cu was significantly higher about 8%, 14%, 3%, 9% and 3% respectively under limiting irrigation compared to full one (Table 2). Inversely, the content of Fe, Zn and Mn was significantly lower about 46%, 5% and 17% respectively.

A significant interaction between the water treatment and the level of selenium addition was observed for the accumulation of all macro and micro-nutrients (Table 2), indicating that the effect of the irrigation treatment was not consistent in the level of sodium selenate. Thus, the concentration of Se did not significantly differ between full and limited irrigation without sodium selenate. On the contrary, the limiting irrigation with sodium selenate solution of 1 mg Se L<sup>-1</sup> decreased and of 3 mg Se L<sup>-1</sup> increased the concentration of Se in the aerial parts of the plants respectively (Fig. 1) in comparison with the full irrigation. The concentration of K and Na was significantly higher under water stress compared to full irrigation with and without the addition of sodium selenate (Fig. 2). The concentration of Mg and Ca was significantly lower under limited irrigation with the solution of 1 mg Se L<sup>-1</sup> compared to full irrigation, whereas the opposite trend was observed under limited irrigation with the solution of 3 mg Se L<sup>-1</sup> and without sodium selenate.

Regarding the micro-nutrients, the concentration of Cu did not significantly differ between full and limiting irrigation without sodium selenate and with 1 mg Se L<sup>-1</sup>. However, the concentration of Cu was significantly higher under limited irrigation with 3 mg Se L<sup>-1</sup>. Generally, the concentration of Fe, Zn and Mn was reduced under limited irrigation with and without sodium selenate. The only exception was the concentration of Zn under limiting irrigation with solution of 3 mg Se L<sup>-1</sup> which was significantly higher in comparison with that under full irrigation.

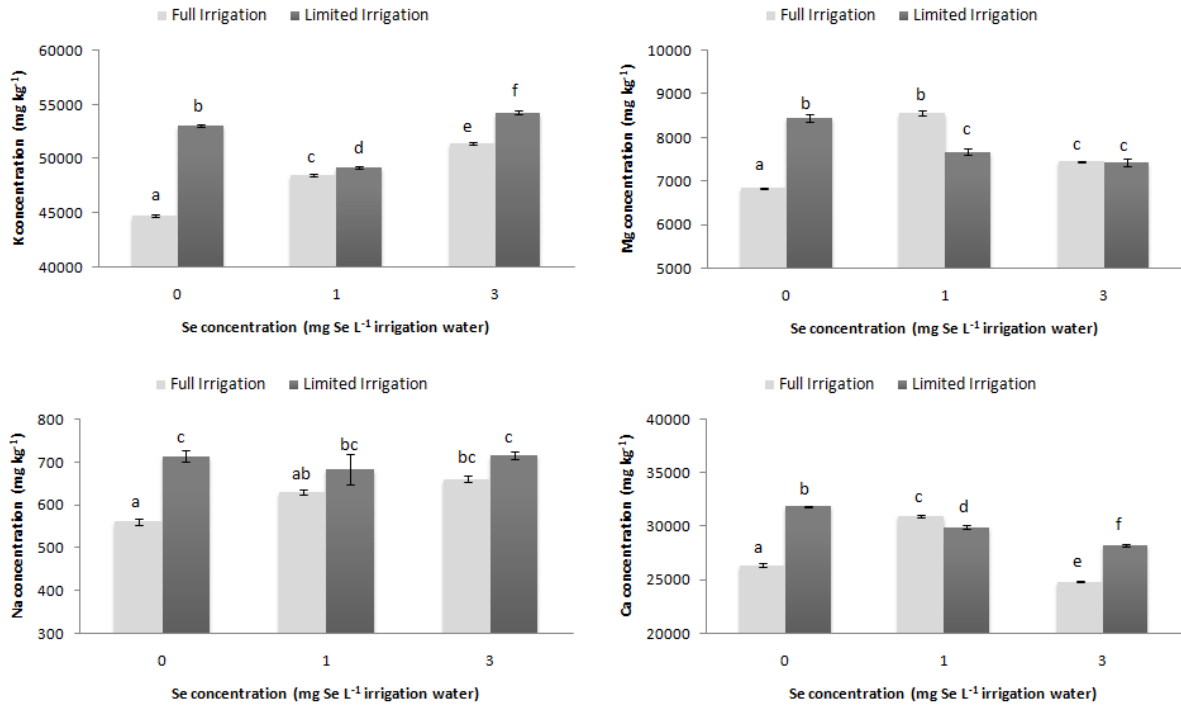


Fig. 2. Mean values (n=3) ± S.E. of macro-mineral content in the aerial parts of *Melilotus officinalis* seedlings. Plants were subjected to three different Se concentrations (0, 1 and 3 mg Se L<sup>-1</sup> irrigation water) and two water regimes (full and limited irrigation). Different letters in each column indicate significant differences (p < 0.05)

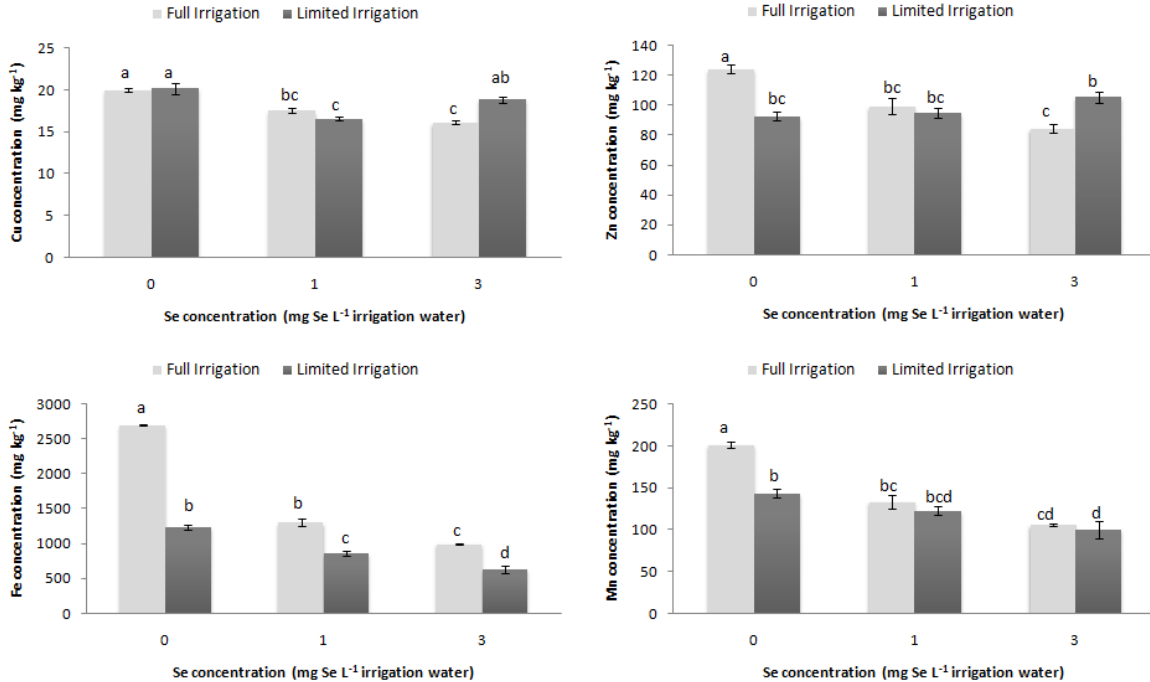


Fig. 3. Mean values (n=3) ± S.E. of micro-mineral content in the aerial parts of *Melilotus officinalis* seedlings. Plants were subjected to three different Se concentrations (0, 1 and 3 mg Se L<sup>-1</sup> irrigation water) and two water regimes (full and limited irrigation). Different letters in each column indicate significant differences (p < 0.05)

## Discussion

### *Effect of selenium addition*

A relatively high amount of Se in tissues of *M. officinalis* was observed as the selenate addition increased in the treatments. Similarly, Kopsell *et al.* (2000) found that Se increased linearly in response to increasing sodium selenate concentrations in *Brassica oleracea* leaves. According to Li *et al.* (2008) selenate is easily taken up by plant roots and since it is extremely mobile in xylem transport, it is distributed quickly to the plant shoots.

Adding sodium selenate affected the K content in the aerial parts of *M. officinalis*. Se addition at low level decreased K content, while at the higher Se level, an increment of K was detected. Similar results were also reported for *M. officinalis* (Kostopoulou *et al.*, 2015) and *Brassica oleracea* leaves (Kopsell *et al.*, 2000), suggesting a possible involvement of this element in the tolerance mechanism of Se (Kostopoulou *et al.*, 2015). On the contrary, Hawrylak-Novak (2008) reported that at low Se concentration in the nutrient solution, K content in the aerial parts of maize plants increased, while at high Se concentration the opposite effect was found. On the other hand, Wu and Huang (1992) found that tissue K concentration of *Trifolium repens* and *Festuca arundinacea* was not affected by Se.

The addition of sodium selenate did not affect the level of Na in the aerial parts of *M. officinalis*. Kostopoulou *et al.* (2015) found that Na concentration was increased in the shoots, but there was no increase in the leaves, after the addition of sodium selenate. The Ca and Mg concentrations were affected by sodium selenate addition, with an increase at the low and a decrease at the high level. Feng *et al.* (2009) have found contrasting results. According to their research, at low Se content Ca and Mg concentration in *Pteris vittata* decreased, whereas at high Se it increased. On the other hand, Kopsell *et al.* (2000) found that Ca concentration in *B. oleracea* plants was not affected by Se addition, while Hawrylak-Novak (2008) found that its content in shoots of maize plants increased after Se addition. It is obvious from this wide variability of the results regarding the effect of selenium on plant mineral content that the effects of Se are species-dependent. In addition, selenium chemical form, its concentration in the nutrient solution and the plant's developmental stage in each experiment also play a significant role on the effect of Se on plants (Hartikainen *et al.*, 2000; Xue *et al.*, 2001; Hawrylak-Novak, 2008). Unfortunately there were scant studies about the effect of Se on the uptake of macro- and micro- elements in plants (Feng *et al.*, 2009) and for this reason their results are not always definite (Hawrylak-Novak, 2008).

Sodium selenate uptake by *M. officinalis* tissues reduced the micronutrient content in aerial parts of the plant. This result is in agreement with the general assumption that absorption of elements such as Mn, Zn, Cu and Fe is inhibited by increasing Se levels (Kabata-Pendias and Pendias, 2001; Fargašová *et al.*, 2006). Moreover, similar results have been found in a previous experiment using *M. officinalis* (Kostopoulou *et al.*, 2015). However, contrasting results have been reported in other plant species. According to Wu and Huang (1992) Mn, Fe and Zn increased under Se treatment in *Trifolium repens*, while Cu content was not affected. Total Cu, Mn and Zn content was unaffected by the addition of Se in *Brassica oleracea* (Kopsell *et al.*, 2000).

### *Effect of water treatment*

There was no significant effect of limited irrigation in the concentration of Se. This result is inconsistent with the results of Tennant and Wu (2000) who found an increment of the concentration of Se in *Festuca arundinacea* Schreb under water deficit.

Concerning the macro-nutrients K, Na, Mg and Ca, there was an increase in their concentration under water stress. These results are in agreement with those of Tennant and Wu (2000) for *Festuca arundinacea*. However, Hu and Schmidhalter (2005) have reported a decrease in the above macronutrients under water stress in *Triticum aestivum*. The difference in these results could be associated to several factors, as the experimental conditions, plant species and duration of water stress (Tennant and Wu, 2000). Potassium plays an important role in survival of plants under abiotic stress (Waraich *et al.*, 2011), contributing to osmotic adjustment (Utrillas *et al.*, 1995; Patakas *et al.*, 2002). Leaf K increase could have contributed to the osmotic adjustment of *M. officinalis* plants, acting as a mechanism to maintain turgor pressure and stomatal conductance (Patakas *et al.*, 2002) and, hence, photosynthesis under water stress conditions (Waraich *et al.*, 2011). In compliance with this result, Kostopoulou *et al.* (2010) in their study found that stomatal conductance of *M. officinalis* under similar experimental conditions was not affected by water deficit. On the other hand, little information is available on the effect of water stress on Na and Mg. A decrease in sodium content of *Cynodon dactylon* was also observed under water deficit conditions (Utrillas *et al.*, 1995). On the contrary, plants of *Sorghum bicolor* grown on different irrigation regimes did not differ in the concentration of Na (Asgharipour and Heidari, 2011). Moreover, Karimi and Hasanpour (2014) have found similar results for Mg for *Punica granatum*. Magnesium plays a significant role in reducing the generation of reactive oxygen species, protecting the chloroplasts from photo-oxidative damage under water stress (Waraich *et al.*, 2011). Calcium, on the other hand, has a prominent role in maintaining cell structure (McLaughlin and Wimmer, 1999) and recovery after a period of water stress (Palta, 1990). Increase of Ca under water deficit has been reported by others (Utrillas *et al.*, 1995; Patakas *et al.*, 2002).

Information dealing with the effect of water stress on the concentration of micro-minerals in plant tissues is rather scarce. In the present study, the limited irrigation resulted to a reduced concentration of the micronutrients Fe, Zn and Mn in the aerial parts of *M. officinalis*. Generally, water stress reduces nutrient uptake by roots through the decrease in the diffusion rate of nutrients in the soil towards the absorbing root surface (Pinkerton and Simpson, 1986; Alam, 1999). Additionally, it also reduces the transport from the roots to the shoots due to restricted transpiration rates, impaired active transport and membrane permeability (Viets, 1972; Alam, 1999). According to Hu and Schmidhalter (2005) low soil moisture can induce deficiencies in Mn, Fe and Zn. The only exception was the Cu concentration which was increased, but this was obvious only with the addition of sodium selenate (Fig. 3). Copper is required for lignin synthesis, needed for cell wall strength and prevention of wilting. Increase of Cu content could alleviate the adverse effects of drought by reducing dieback of stems, yellowing of leaves and stunted growth (Waraich *et al.*, 2011).

*Interaction between selenium addition and water treatment*

There was a notable interaction between water treatment and addition of sodium selenate in the soil only for Ca and Mg concentrations that decreased under water stress at the low Se level. However, under the addition of 3 mg Se L<sup>-1</sup>, Ca has followed the same pattern as the other macro-nutrients, while Mg had no significant difference in its concentration. Water treatment could affect the nutrient balance in plants (Romero *et al.*, 2004). Its combination with Se addition at various levels could have contrasting effects on nutrient uptake.

Interaction between water treatment and addition of sodium selenate in the soil was notable only for Zn and Cu, the content of which increased under water stress with the addition of 3 mg Se L<sup>-1</sup>. Accumulation of inorganic ions may contribute to the adaptation of this species to water stress (Kostopoulou *et al.*, 2012). This could be attributed to the defence mechanism of the plants to water deficit and the antioxidant activity of selenium. Selenium has been recently reported to improve drought resistance in several species, mainly by mitigating the water deficit stress damages in plants (Nejat *et al.*, 2009; Valadabadi *et al.*, 2010; Soleimanzadeli, 2012; Proietti *et al.*, 2013; Emam *et al.*, 2014). The protective role of Se under water stress has been attributed to the protection of cells from oxidative damages (Xue *et al.*, 2001; Prioetti *et al.*, 2013; Habibi, 2013; Ibrahim, 2014), to the regulation of water status of plants (Prioetti *et al.*, 2013; Nawaz *et al.*, 2014), to the increase in root activity (growth and uptake) (Prioetti *et al.*, 2013; Nawaz *et al.*, 2014) and to the prevention of chlorophyll degradation under water stress (Seppanen *et al.*, 2003). In addition, the application of selenium enhanced the antioxidant defence in sorghum (Djanaguiraman *et al.*, 2010) by increasing the antioxidant enzyme activities. This antioxidative effect in soybean (Djanaguiraman *et al.*, 2005) was associated with an increase in superoxide dismutase (Cu/ZnSOD) and glutathione peroxidase (GSH-Px) enzymes activity. However, the physiological and molecular mechanisms that underlie the beneficial role of selenium in plants need to be further explored.

**Conclusions**

Limited Se addition differentially affected the K, Mg and Ca content of *M. officinalis* aerial parts, while it led to the reduction of the micronutrients Cu, Fe and Mn. Water stress resulted in the increase of K, Na, Mg, Ca and Cu, and to the decrease of the Fe, Zn and Mn content. An interaction between selenium addition and water treatment was more notable for Ca and Mg, which decreased under water stress at low Se level and for Zn and Cu, which increased under water stress at high Se level. According to our findings, Se-induced increased accumulation of some inorganic ions in the aerial parts of this species under water stress conditions could serve as a means to alleviate the adverse impact of water deficit on important metabolic processes, enhancing *M. officinalis* tolerance to water stress. However, the role of Se on nutrient accumulation and on plants' water stress resistance needs to be further elucidated.

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