

Effects of foliar application of two forms of selenium on anatomic features of *Thymus daenensis* Celak

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Abstract: To investigate selenium effects on anatomic features of *Thymus daenensis*, three concentrations of bulk and nanoparticles of the element (2, 4 and 8 ppm) along with distilled water, as a control, were sprayed on foliar of young seedlings of the plant species for six times with 2 weeks intervals. Transverse sections of stems, roots and leaves of the treated plants were manually produced and stained by Carmine and Methylene blue. Average of 5 readings was recorded for each treatment on several anatomic traits. Statistical analysis of the data revealed that the effects of selenium treatments were significantly different, at 1% of probability, on all of the studied anatomic characters. The first level of nanoparticles showed the most positive effects on several root characteristics. The highest level of selenium nanoparticles (8 ppm) showed the most positive effects on stem diameter and stem vascular cylinder diameter. The last two levels of nanoparticles had similar and the largest effect on stem epidermis thickness. The lowest level of bulk selenium (2 ppm) showed the most increment on leaf characteristics. Application of a low concentration of selenium by foliar spraying on young plants of the species is suggested for more vegetative growth and higher photosynthetic efficiency.

1. Introduction

Thymus daenensis Celak related to Lamiaceae family is endemic to western part of Iran, known as a medicinal herb, with high concentration of thymol, and high antimicrobial activities on human pathogenic strains (Zarshenas and Krenn, 2015). The species is regarded as a valuable spice

plant, growing mainly on Zagros mountain chain of the country. It is a perennial shrub which was traditionally used as a tonic or herbal medicine to treat gastrointestinal disorders, headache and cold.

Numerous studies indicated that environmental factors such as nutrient deficiency, highly affect biological properties of medicinal plants, including their anatomic features. On the other hand, anatomic studies have found a vast application domain, such as identifying fragmentary plant organs as well as providing characters of taxonomic significance, in medicinal plants. This technique could be used as a proper tool to identify the exact effects of environmental factors such as fertilizers and chemical substances on plant ultrastructure. In other words, the anatomical features are supposed to be good indicators of environmental fluctuation effects. Also in areas with high levels of toxic elements, a sensitive plant species such as *Thymus daenensis* have potential to be used as indicator of the environmental conditions.

Selenium is rarely found in its elemental form in our living environment, it is found in several other forms such as selenide, selenite and selenate oxidation status in soil and water (Gomez-Ariza *et al.*, 1998). However, a suitable level of selenium plays a significant role in plants, animal and human health in direct or indirect ways. So that in selenium deficient areas there may be a health risk for plants, animal and human population.

Selenium is identified as affecting plant growth by causing physiological, morphological and anatomical alterations in plant tissues. Some researches indicated that alterations depend on the concentration of the applied nanoparticle treatment (Tymoszuk and Kulus, 2020). In fact, as a part of selenium role in plant, antioxidant enzymes are activated, minerals are balanced, chlorophyll is also increased, and the plant would be more resistant to various stresses (Raina *et al.*, 2021). Selenium also increases plant resistance against oxidative stress, which is caused by free oxygen radicals. It delays senescence of higher plants, increases their tolerance to UV-induced oxidative stress, and promotes seedling growth. It is also shown that selenium regulates water status of plants under drought conditions (Kuznetsov *et al.*, 2003). Although utilizing proper concentration of selenium have positive effects such as increasing crop yield, but adverse effects of some nanoparticles have also been reported on growth characteristics of some plant

species (Singla *et al.*, 2019; Gao *et al.*, 2023). Plants can absorb chemical substances in nanoparticle form, better than other forms. Achievements of nanofertilizers are mainly due to very small particles that are easily absorbed by plant root system. It is also due to their large surface area that increases the plant sorption effectiveness by three folds (El-Saadony *et al.*, 2021).

Selenium used to be considered as a toxic element for human being. Whereas, its role has been changed during the past 40 years. It contributes to healthy growth, as well as reducing the toxicity of elements such as mercury. Maximum limit of selenium intake for human being is suggested to be 400 µg/day, over which negative effects of the element are expected (Arthur, 1991). However, daily selenium intake of 50-200 µg/day is recommended to overcome deficient effects of the element (Navarro-Alarcon and Lopez-Martinez, 2000). Although selenium may be available at toxic level in some areas, which is harmful for grazing livestock, but as a result of soil acidity, it is not available enough in some areas, resulting to low selenium intake in human at the final food chain. In other words, suitable level of selenium supplementation seems to be beneficial for plants, animals and human being. In areas with selenium deficit in soil, fertilizers are recommended to be supplemented with some selenium forms such as sodium selenite.

This research was conducted mainly to investigate the possible effects of exogenous application of bulk and nanoparticles of selenium on anatomic structure of *Thymus daenensis*, as well as finding a safe dosage of the element to optimize foliar application of selenium for the species.

2. Materials and Methods

Seed germination and plant establishment

A trial was performed at Research Institute of Forests and Rangelands of Iran, to study influence of selenium on growth and anatomic structures of a medicinal plant species named *Thymus daenensis* Celak. Seeds of the species were first washed with tap water and soaked in distilled water for two days. Then they were first scattered on wet filter paper and kept at 4°C for 72 hours. Then they were sown in planting trays. The new grown seedlings were transplanted to plastic pots containing peat moss growing media and kept in a greenhouse.

Treatment application

By adaptation of the seedlings to the greenhouse conditions, the pots were arranged in five replications with three pots per each plot. Exogenous application of selenium treatments was conducted on the plantlets, as follows: three concentrations of selenium nanoparticles (2, 4, and 8 ppm of NanoSe), and sodium selenate as the bulk form (2, 4, and 8 ppm of SoSe), along with distilled water as a control for the experiment, were applied on the plantlets by 6 times foliar spraying with intervals of two weeks, starting at four leaf stage of the plantlets based on a completely randomized design, with five replications. The concentrations of selenium treatments were determined based on a preliminary experiment in the greenhouse.

Microscopic sample preparation

Three days after the last application of the treatments, several leaf, stem and root samples were randomly taken from each plot and fixed in a mixture of ethanol 96% and glycerin (1:1). Transverse sections of the fixed samples of stems, roots and leaves were performed manually to produce slices in about 20 µm thickness and washed by distilled water. To distain the samples, they were soaked in 10% sodium hypochlorite solution for 10 minutes and washed thoroughly by distilled water. For staining the sections, a combination of Carmine (for 20 min) and Methylene Blue (for 30 sec.) were used to make the tissues with wooden skeleton, in green, and tissues with cellulosic skeleton in pink (Sotoodehnia-Korani et al., 2020). Then the sections were washed by distilled water again. The process was completed with the samples assembled on glass slides. The slides were observed under an Olympus microscope coupled with digital camera. Five readings and measurements were recorded per each experimental unit. Several anatomic characteristics were recorded as indicated in Table 1.

Statistical analysis

The mean values of the data recorded on the anatomic features of the experimental units, affected by the seven different selenium treatments, were subjected to analysis of variance, after testing the homogeneity of their error variance using SAS software. In other words, regarding five replications and seven treatments of the experiment, 35 mean values were analyzed for each anatomic characteristic, based on a completely randomized

Table 1 - Results of analysis of variation on the data recorded on the effects of seven different selenium treatments on anatomic characteristics of roots, stems and leaves of *Thymus daenensis*

Source of variation	Treatment ^z	Error ^y
Traits	(DF=6)	(DF=28)
Root diameter	17078.5 **	479.6
Root vascular cylinder diameter	7580.9 **	453.9
Root epidermis thickness	748.6 **	119.4
Stem diameter	36951.9 **	5871.4
Stem vascular cylinder diameter	4679.7 **	681.2
Stem epidermis thickness	3138.7 **	305.3
Stem xylem diameter	2.8 **	0.07
Leaf thickness	8944.2 **	666.6
Leaf upper cortex thickness	58.0 **	8.8
Leaf main vascular thickness	1372.0 **	56.0
Leaf lower cortex thickness	63.1 **	11.8
Leaf spongy mesophyll thickness	141.5 **	5.8
Leaf palisade mesophyll thickness	104.1 **	6.7
Leaf upper epidermis thickness	2.5 **	0.5
Leaf lower epidermis thickness	1.2 **	0.2
Leaf number of vascular bundles	3.0 **	8.8

(z) **= Significant at 1% level of probability.

(y) Seven treatments and five replications, resulted in 35 data values for each trait.

design. Duncan multiple range test was used to classify the treatment means on the studied characteristics.

3. Results and Discussion

The statistical analysis of the data revealed that the selenium treatments had significant effects on all of the studied anatomic characteristics at 1% of probability (Table 1). Classified means of the studied characteristics are presented in Table 2.

The lowest level of nanoparticles (2 ppm of NanoSe) promoted root growth by increasing root diameter (595 µm), root epidermis (75.5 µm) and root vascular cylinder diameter (38 µm) (Table 2). Therefore, addition of 2 ppm NanoSe promoted the root growth and its vascular cylinder increment. This is in accordance with the results of Domokos-Szabolcsy et al. (2012). Root sections formed concentric cylinders and deformation was not observed by the studied treatment levels. Meanwhile, root diameter was negatively affected by the treatments, so that the smallest diameter

Table 2 - Classification of the anatomic characteristics of *Thymus daenensis*, affected by seven different concentrations of sodium selenate as the bulk selenium (SoSe), and nanoparticles of selenium (NanoSe)

Treatments ^z	Control ^y	2 mg/L of SoSe	2 mg/L of NanoSe	4 mg/L of SoSe	4 mg/L of NanoSe	8 mg/L of SoSe	8 mg/L of NanoSe
Traits							
Root diameter (µm)	520.2 b	480.0 c	595.0 a	518.7 b	450.1 d	465.4 cd	415.8 e
Root vascular cylinder diameter (µm)	286.6 de	326.4 b	381.6 a	316.5 bc	295.8 cd	275.7 de	263.8 e
Root epidermis thickness (µm)	36.3 c	52.2 b	75.5 a	60.3 b	65.3 ab	51.8 b	56.8 b
Stem diameter (µm)	734.0 b	618.6 c	662.3 bc	685.2 bc	747.2 b	711.3 bc	888.9 a
Stem vascular cylinder diameter (µm)	323.2 ab	258.7 c	294.4 b	321.9 ab	290.0 bc	304.0 b	355.4 a
Stem epidermis thickness (µm)	112.8 bc	91.8 cd	99.3 cd	75.8 d	148.3 a	87.8 d	127.0 ab
Stem xylem diameter (µm)	8.1 a	6.1 d	7.7 b	6.4 cd	6.7 c	6.8 c	7.6 b
Leaf thickness (µm)	259.8 c	260.5 c	293.0 bc	268.1 c	319.5 b	327.7 b	373.1 a
Leaf upper cortex thickness (µm)	30.7 a	22.4 c	23.1 bc	23.6 bc	29.5 a	26.8 ab	23.1 bc
Leaf main vascular thickness (µm)	138.7 d	170.4 b	155.1 c	185.5 a	147.7 cd	174.6 b	171.0 b
Leaf lower cortex thickness (µm)	27.8 b	32.5 a	24.2 bc	24.2 bc	22.3 c	25.6 bc	22.7 c
Leaf spongy mesophyll thickness (µm)	100.6 c	108.9 a	106.1 ab	103.7 bc	94.2 d	97.0 d	106.0 ab
Leaf palisade mesophyll thickness (µm)	90.6 c	98.1 a	92.5 bc	87.5 de	89.3 cd	84.8 e	95.3 ab
Leaf upper epidermis thickness (µm)	6.0 ab	6.1 a	5.0 bc	5.6 ab	5.0 bc	4.2 c	6.0 ab
Leaf lower epidermis thickness (µm)	4.2 bc	4.9 a	3.7 c	4.4 ab	4.1 bc	3.5 d	4.5 ab
Leaf number of vascular bundles	5.0 a	5.0 a	4.0 b	5.0 a	3.0 c	5.0 a	4.2 ab

^(z) Mean of each trait and treatment, resulted from 5 data values obtained by five replicates.

^(y) Means with similar letters in the same raw are not significantly different at 5% level of probability.

belonged to the highest level (8 ppm) of both NanoSe and SoSe (Table 2). Therefore, it may be concluded that the application of a proper quantity of selenium, in either form of NanoSe or SoSe, would improve organogenesis and root growth of *Thymus daenensis*. Zsiros *et al.* (2019) had the same conclusion on tobacco. However, high concentrations of the element would not be recommended. Zayed *et al.* (1998) concluded that plants accumulate more selenium in their shoots and leaves than in their root tissues. Regarding their conclusion roots of the plant species studied in this research could have been less affected by the treatments than its stems and leaves. Regarding the effects of the treatments on appearance of the root transverse sections, all the treatments had similar effects, so that all of the sections of the different treatments looked circular (Fig. 1).

Transverse sections of the stems were not completely quadrangular for the studied treatments (Fig. 2). Meanwhile, there were several fluctuations due to the treatments effects. Two to four layers of collenchyma cells were located at the corners of the

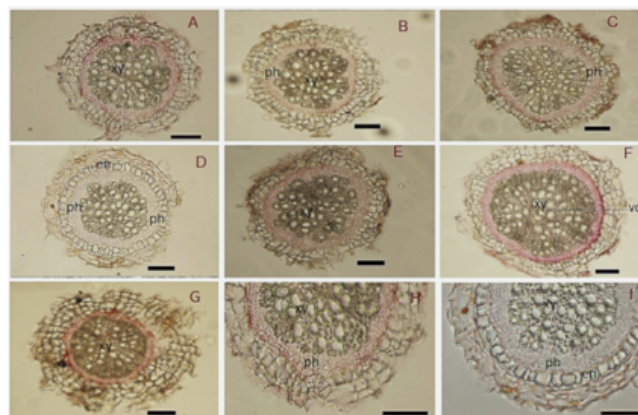


Fig. 1 - Transvers sections of roots of *Thymus daenensis* affected by seven different selenium treatments. A) 8 ppm of sodium selenate (SoSe), B) 4 ppm of SoSe, C) 2 ppm of SoSe, D) 8 ppm of nanoparticles of selenium (NanoSe), E) 4 ppm of NanoSe, F) 2 ppm of NanoSe, G) control, H) 8 ppm of SoSe, I) 8 ppm of NanoSe, e= epiderm, en= endodermis, ph= phloem, vc= vascular cylinder, xy= xylem, Bars = 50 µm.

stems. Five to six layers of roundish parenchyma cells formed the cortex of the stems. Two opposite sides of the quadrangular stems were convex (Fig. 2A),

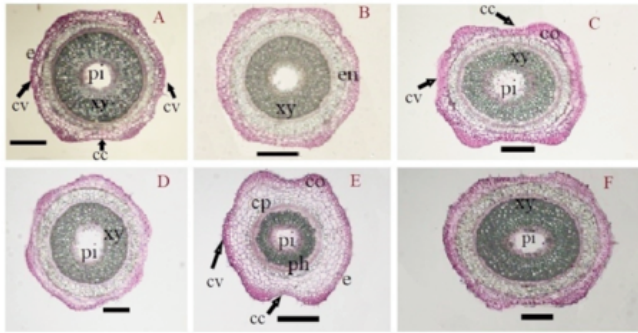


Fig. 2 - Transvers sections of the stems of *Thymus daenensis* affected by seven different selenium treatments. A) Control, B) 4 ppm of sodium selenate (SoSe), C) 2 ppm of SoSe, D) 8 ppm of nanoparticles of selenium (NanoSe), E) 4 ppm of NanoSe, F) 2 ppm of NanoSe, cc= concave, co= collenchyma, cp= cortex parenchyma, cv= convex, e= epiderm, en= endodermis, pi= pith, ph= phloem, xy= xylem, Bars = 100 μ m.

whereas, the other two sides were concave. The mentioned cavity was deeper in the selenium treatments (Fig. 2C and 2E). Epidermis, exodermis and endodermis were all single layer. Epidermis with one layer of cells covered by cuticle and various trichomes, were similar for the studied treatments. Within the range of studied concentrations of selenium nanoparticles (NanoSe), the largest stem xylem diameter was obtained by control of the experiment (no selenium in both forms). The highest level of the nanoparticles showed the most effects on stem diameter (888.9 μ m) (Table 2) and stem

vascular cylinder diameter (355.4 μ m). Four ppm concentration of selenium nanoparticles had the largest effect on stem epidermis thickness (148.3 μ m) (Table 2). According to Aly *et al.* (2023) there is a significant correlation between nutrients content, growth parameters and nanoparticles concentrations. They came to this conclusion that some nanoparticles were more efficient by low concentrations, whereas, others were more efficient by higher concentrations. We came to this conclusion that even in a single nanofertilizer, some part of the plant species, lower concentration of selenium was more effective (leaf mesophyll) (Table 2), whereas, in other parts of the same plant, higher concentration of the element was more effective (stem diameter and leaf thickness). This study confirmed that an appropriate concentration of NanoSe enhanced the vascular cylinder and epidermis of both roots and stems of the plant species.

Two ppm of bulk selenium showed the largest increments on leaf characteristics such as leaf upper and lower epidermis thickness, leaf spongy and palisade mesophyll thickness (Table 2). Leaf main vascular thickness was mainly affected by 4 ppm concentration of bulk selenium treatment (Fig. 3). Whereas, the highest level of nanoparticles (8 ppm) showed the same trend but weaker effects on majority of the mentioned leaf characters (Table 2). Epidermis of the leaves, along with cuticle, provide protection for plants, thickening of epidermis on both upper and lower epidermis of the leaves

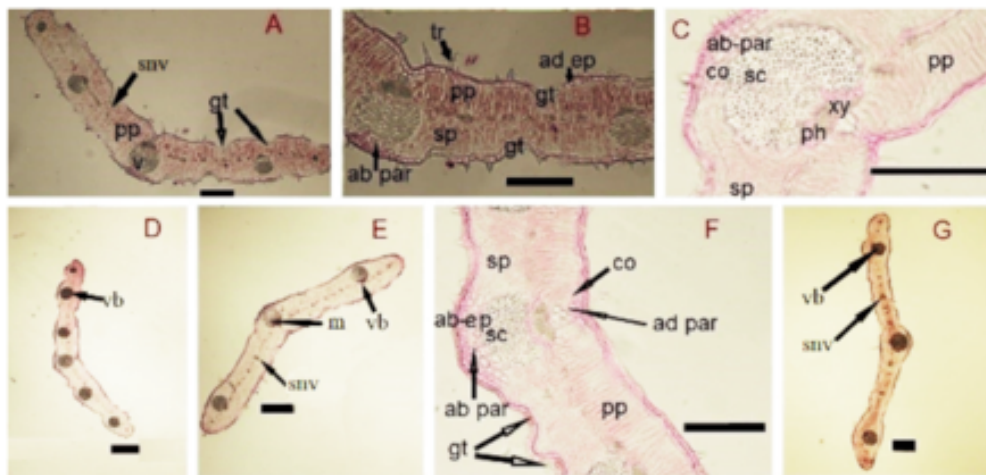


Fig. 3 - Transvers sections of the leaves of *Thymus daenensis* affected by seven different selenium treatments. A, B and C) 8 ppm of sodium selenite (SoSe), D) 2 ppm of SoSe, E) 4 ppm of NanoSe, F) 2 ppm of SoSe, G) control, ab-ep= abaxial epidermis, ab-par= abaxial parenchyma, ad-ep= adaxial epidermis, ad-par= adaxial parenchyma, co= collenchyma, gt= glandular trichome, m: main middle vein, ph: phloem, pp= palisade parenchyma, sc= sclerenchyma, sp= spongy parenchyma, snv= small netted vein, tr= trichome, vb= vascular bundle, xy= xylem, Bars = 100 μ m.

generated by selenate sodium (SoSe) may alleviate the negative and diverse effects of biotic and abiotic stresses. In fact, both forms of selenium, increased leaves upper and lower epidermis thickness, thereby leaf heat load is reduced, causing leaf internal temperature and resultantly transpiration rate to be reduced. Application of nanoparticles of other elements such as silicates in K_2SiO_3 and $CaSiO_3$ forms also showed positive results on banana, by higher stomatal density and smaller stomatal size (Asmar *et al.*, 2015). Both upper and lower epidermises may act as a barrier to excessive transpiration, therefore, the thicker epidermis observed on selenium containing treatments, can better protect the plant leaves against water loss as well as protecting the plant tissues against biotic and abiotic stresses. Abbas-Azimi *et al.* (2020) also came to this conclusion that some plant species change their anatomic features to combat dehydration. Application of either form of selenium has thicken both mesophyll tissue layers (spongy and palisade parenchyma). It can be noticed that the leaves under either form of selenium treatments, have wider palisade and spongy mesophyll compared to untreated leaves. Since mesophyll tissues are the main container of chloroplasts, thickness increment of the mesophyll layers would lead to increment of chlorophyll in the plant tissues, which in turn leads to improvement of photosynthetic efficiency. Smrkolj *et al.* (2006) also showed that selenium induced higher respiratory potential in the leaves of foliar treated potato plants.

NanoSe also negatively affected the number of vascular bundles, compared to the control of the experiment. Whereas, the three levels of bulk selenium had the same effect as that of the control. It means selenate sodium (SoSe) treatment does not have significant effect on the number of vascular bundles. It should be noticed that according to Yang *et al.* (2022), selenium content is higher in actively plant tissues and younger leaves, but higher buildup of the element occurs in aging leaves. Therefore, selenium toxic effects may be seen in older leaves, whereas its deficient symptoms appear on new leaves. Therefore, the exact toxic effects of the used dosage of the element could be examined on the older leaves of the plants under study. However, this study suggested that the leaf anatomic characteristics of *Thymus daenensis* are highly affected by different concentrations of both forms of selenium.

Different plant species respond differently to

selenium fertilization. Plants are able to transform selenite into organic selenium by foliar spraying or fertilization. Meanwhile, plant species have different capabilities to absorb and maintain selenium in their tissues. Most plant species contain 1-2 mg/kg of selenium, but there are some species with ability to accumulate a larger amount of the element (Juhaszne Toth and Csapo, 2018). For instance, *Brassicaceae* and *Fabaceae* families are able to accumulate much more selenium in a kilogram of dry matter than other plant species (Ellis and Salt, 2003). These species may be used for soil purification purposes in toxic soils.

In this study, the highest level of selenium did not favor several studied characteristics such as root diameter, root vascular cylinder diameter, root epidermis thickness and leaf lower and upper cortex thickness; accordingly, the species under study seems to be sensitive to the large quantities of selenium.

According to Tymoszuk and Kulus (2020), treating plants with nanoparticles, particularly high level of the particles, may even cause genetic variation, depending on their size, type and concentration. Therefore, using the right concentration of the element is the first step to be taken. This was done in this research by a preliminary experiment to select the appropriate concentrations as the treatments of the experiment.

The interaction between plant cells and nanoparticles may also cause positive or negative morpho-physiological alterations, depending on several factors such as size, shape, concentration, surface covering and mode of nanoparticle application as well as plant age, genotype, and developmental phase (Rajput *et al.*, 2021).

However, utilizing appropriate form and concentration of selenium by foliar spraying results in anatomical and structural changes of *Thymus daenensis*, that seems beneficial to the species. Moderate concentrations of selenium acts highly positive in relation to different anatomic characteristics of the foliar parts of the plant species. Root and stem epidermis thickness and vascular cylinder diameter, are favored by foliar addition of selenium in moderate concentrations of either nanoparticle or bulk forms. Spongy and palisade mesophyll thickness of leaves of the plant species are also favored by the foliar spraying of selenium especially when 2 ppm of nanoparticles of the element or 8 ppm of bulk selenium is used. The lower

level of nanoparticles and higher examined level of bulk selenium also showed their positive effects on increasing upper and lower leaf epidermis thickness as well as the number of leaf vascular bundles. Therefore, foliar spraying on the young plants of the species by a moderate concentration of selenium, within the range of studied concentrations of the element is suggested for more vegetative growth and higher photosynthetic efficiency.

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