Effects of Exogenous Melatonin Supplementations on Some Elemental Contents in Anatolian Black Pine (*Pinus nigra* J.F. Arnold. subsp. *pallasiana* (Lamb.) Holmboe) Seedling Tissues

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A multi-directional relationship may be conceivable between elements and melatonin in sessile organisms. Melatonin is an important hormone that helps regulate metabolism. This study investigated how different doses (0 μM/control, 250 μM, 500 μM, 1000 μM, and 1500 μM) of exogenous melatonin supplementations (EMS) affected the elemental contents in Anatolian black pine (*Pinus nigra* Arnold. ssp. *pallasiana* (Lamb.) Holmboe) seedling tissues (root, stem, and needle). Two different application forms (root-dipping and needle-spraying) were selected in the study. In the samples of seedling tissues, sodium (Na), potassium (K), calcium (Ca), iron (Fe), aluminum (Al), magnesium (Mg)/ppm; chrome (Cr), cobalt (Co), copper (Cu), manganese (Mn), nickel (Ni), phosphorus (P), selenium (Se), silicium (Si), silver (Ag), sulfur (S), zinc (Zn), and molybdenum (Mo)/ppb were analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES). Of the 18 elements examined, there was a statistically significant difference ($p<0.05$) between all seedling tissues and different doses of EMS. The results show that EMS may have the regulatory effect on seedling tissue element metabolism.

Keywords: Mineral element interaction; Exogenous melatonin; Root-dipping; Needle spraying; Anatolian black pine tissues

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INTRODUCTION

According to the most recent data, 22.7 million ha of forested land covers approximately 29% of Turkey’s territory. Productive forest areas account for approximately 58% of total forest areas, while non-productive forest areas account for approximately 42%. Coniferous forests cover approximately 47% (10.7 million ha) of the country’s whole forest area (Anonymous 2019). Although Turkey has a great forest potential in terms of ecology, only 29% of its land has characteristics of forest due to anthropogenic and environmental effects (Atalay 2002). Black pine occupies a wide geographical distribution in the world. It is naturally distributed on three continents—Asia, Africa, and Europe—with five species (Mirov 1967). These are subsp. *nigra*, subsp. *salzmannii*, subsp. *dalmatic*a, subsp. *laricio*, and subsp. *pallasiana* (Anonymous 2013). It is one of these five subspecies, with a range that includes the Balkans, Anatolia, Crimea, the Southern Carpathian Mountains, Cyprus, and Syria (Richardson 1998). *Pinus nigra* J.F. Arnold. subsp. *pallasiana* (Lamb.) Holmboe is known also as Anatolian Black Pine. It is one among the most common and economically important native primary tree species in Turkey (Atalay and Efe 2012). This subspecies has the third largest expansion area (4.3

million ha) after oak (*Quercus* sp.) and red pine (*Pinus brutia* Ten.) among forest tree species in Turkey (Anonymous 2019). Anatolian black pine grows naturally throughout most of Turkey, with the exception of the Eastern Black Sea, Eastern, and South-eastern ecological regions (Atalay 2002; Öner and Eren 2008). Anatolian black pine is found in both pure and mixed forests in Turkey's coastal mountains, and it can also be found in steppe. Its naturally propagation within the vertical direction is between 700 and 2100 m (Mataracı 2002). Every year, approximately 100 million black pine seedlings are grown in Turkish forest nurseries to meet the demand for seedlings. It is the primary species for seedling propagation in the rate of approximately 22% afforestation carried out in Turkey (Anonymous 2019).

Reforestation is critical around the world, and Turkey has encouraged the planting of tree species in recent years. However, many trees die after outplanting, and failure rates rise within the first five years following establishment. Poor output in such cases may be due to low standards of careless planting or a scarcity of quality seed selection (Radoglou 1999). The quality of seedlings is used to assess the afforestation value and adaptation capacity of seedlings (Şimşek 1987; Avanoğlu *et al.* 2005). Even seedlings grown under optimum cultural practices and carefully handled are known to go into shock following transplanting. It is unlikely that transplant shock can be entirely eliminated. The damage to the root system during lifting and handling procedures may also be a significant factor. Plants are exposed to drying conditions at several stages during the process of lifting, handling, and transportation before planting (McCreary and Lippitt 2000). The root system of the seedlings accustomed to the afforestation of arid and semi-arid areas should be developed, and the stem / root ratio should be in favor of the root (Öner and Eren 2008). Considering that nutrient intake affects the growth of seedlings, a better understanding of the nutritional amount of elements to produce drought-resistant seedlings is needed. Planting quality is decided by characteristics of seedlings and might be assessed after planting, by detecting the elemental nutrient value of plants (Breoadley *et al.* 2012). Therefore, there is a need for natural substances to extend the afforestation value of seedlings and adaptation ability.

Melatonin (N-acetyl-5-methoxytryptamine) was discovered in 1958; it is a highly conserved biomolecule (Lerner *et al.* 1958). The existence of melatonin was discovered in plants in 1996. However, scientific studies on the effects of melatonin on plants have gained speed in the last decade (Arnao and Hernandez-Ruiz 2015). It is a multifunctional signal particle and plant growth regulator that is ubiquitous in various parts of plants (Van-Tassel *et al.* 2001a; Van-Tassel *et al.* 2001b; Debnath *et al.* 2019).

Melatonin has the potential to behave as a metal chelator due to its contents of a 5-methoxyindole group and an N-ethylacetamide group with nitrogen and oxygen atoms. Melatonin's capacity to chelate metals was found to be concentration dependent (Limson *et al.* 1998; Gulcin *et al.* 2002). Clearly, the creation of complexes is influenced by the amount of metal ions, which varies between tissues, as well as melatonin concentrations. Melatonin, in addition to binding Cu^{2+}, appears to protect against copper-mediated free radical damage *via* binding Cu^{+}, according to electrochemical experiments. Melatonin forms compounds with lithium, potassium, sodium, and calcium (Lack *et al.* 2001). Melatonin appears to be an effective metal chelating agent based on these findings. Interestingly, the majority of research revealed that melatonin has the ability to bind metal ions, although a clear mechanism has yet to be discovered (Galijasevic 2017).

Melatonin is synthesized ubiquitously in plant organs (Park *et al.* 2012; Byeon *et al.* 2013; Byeon and Back 2014; Byeon *et al.* 2014; Wang *et al.* 2014; Nawaz *et al.* 2016).

Different amounts of melatonin are found in various plant tissues/organs (Erland *et al*. 2015; Reiter *et al*. 2015). Melatonin is involved in multiple developmental processes in plants. Several studies have noted that melatonin is active in plants and is responsible for stimulating the growth of roots and shoots or preventing the growth of primary roots (Park and Back 2012) and encouraging lateral and adventitious rooting in different species (Murch *et al*. 2001; Hernández-Ruiz *et al*. 2005; Hernández-Ruiz and Arnao 2005), including governing the growth of roots and shoots (Tan *et al*. 2012).

Furthermore, melatonin activates the antioxidant system, scavenging ROS under stress, chelating heavy metal, and alleviating oxidative stress (Zhang *et al.* 2015; Yu *et al*. 2018; Yu *et al*. 2021). Hence, exogenous melatonin could be utilized as a bio-stimulator for the plants. Plant hormones such as melatonin are used in nascent forestry research, just as they are in other types of agriculture (Atik 2013).

The aim of this study is to investigate the interaction of exogenous melatonin supplements with the amounts of the elements. This study examined how different doses (0 µM/control, 250 µM, 500 µM, 1000 µM, and 1500 µM) of exogenous melatonin supplementations (EMS) affected the amount of elements in Anatolian black pine (*Pinus nigra* Arnold. ssp. *pallasiana* (Lamb.) Holmboe) seedling tissues (root, stem, and needle). Two different application forms (root-dipping and needle-spraying) were examined to determine which application form was more effective. Morphological, physiological, and genetic measurements of the seedling quality characteristics will be a different subject and will be studied in the future. This study provides new insights into the health of forest tree seedlings and the use of EMS.

EXPERIMENTAL

Research Area

The field experiment was conducted in 2019 (March to November) at Tosya, a city on the west coast of the Black Sea Region in Turkey and of one of the biggest towns of Kastamonu province (Fig. 1). The working field location was carried out in 76.8 ha afforestation land at the Kastamonu Regional Directorate of Forestry, Tosya Property directorate, chief Akseki Business, Karasapaca by village F32 / D4 section number (Turkey forest number 230/231). The location of the study is 41 2′ 55.05″ N, 34 4′ 16.45″ E. The study area is located in the south, at an altitude of 910 m, average rainfall of 4.775 mm, average annual temperature of 41.5 °C, a slope range 31% to 60%, and a soil structure of sandy clay. A total of 34,500 black pine seedlings were planted (with 1.5 m intervals) in the work field within the scope of the Soil Conservation Implementation Project.

Fig. 1. Geographical location of Tosya district where the samples were taken in Karasapaca afforestation area (Taş 2006)

Experimental Designs, Preparation of Plant Samples and Melatonin Treatments

In this study, melatonin was purchased from Sigma-Alrich (St. Louis, MO, USA) and was dissolved in 200 μL ethanol followed by dilution with water (ethanol: water v/v = 1/10.000). Five different EMS doses (0 μM/control, 250 μM, 500 μM, 1000 μM, and 1500 μM) and 2 different application forms (root-dipping and needle-spraying) were applied to a total of 60 seedlings with 3 replications. In total, 10 seedlings were removed from each repeat (3x10). The root, stem, and needle tissues of 10 seedlings with 3 replicates removed were combined separately and used in element analysis. Materials collected at root, stem, and needle tissues were examined in the analysis. In the first application method of the melatonin substance, at the beginning of the vegetation period, the roots of the seedlings were soaked in melatonin for 30 min (root-dipping) before planting. For the second application method, melatonin substance was applied by spraying only to the needles of the seedlings. The samples were collected towards the end of the vegetation season at the end of November.

Laboratory Pretreatment and Mineral Element Analysis

Plant samples were made homogeneous using a mechanical grinder (Fritsch P- 15, Germany). Two grams of the dried samples were placed in 10 mL of concentrated HNO_3 at room temperature for 24 h and then burned (at 180 °C) using a CEM Mars 6 Microwave Reaction System (CEM Corporation, Matthews, NC, USA). Distilled water was added to the solution obtained from the filtrate. Element analysis was performed to determine the concentration of Na, K, Ca, Fe, Al, Mg (ppm) / Cr, Co, Cu, Mn, Ni, P, Se, Si, Ag, S, Zn, and Mo (ppb) by inductively coupled plasma optical emission spectroscopy

(ICP-OES; model Spectroblue FMX36, Spectro, Germany), using the Spectro smart analyzer software.

Statistical Analyses

The ICP-OES data obtained were evaluated with SPSS software (IBM, Armonk, NY, USA). For normality, the Shapiro-Wilk test was performed within each group and between two groups. Nonparametric tests (Kruskal-Wallis test) were applied as statistical tools to treat the data. When Kruskal-Wallis was tested, $p<0.05$ was found for a statistically significant element. The difference between two independent universes was determined by the Mann-Whitney U test with the Bonferroni's correction.

RESULTS AND DISCUSSION

To investigate the effects of the amount of EMS, various elements were measured. The elements of potassium, copper, nickel, selenium, silicium, and silver were significantly different ($p<0.05$) between the two different application methods (root-dipping and needle-spraying) of melatonin. However, there was no significant difference ($p<0.05$) in sodium, calcium, iron, aluminum, magnesium, cadmium, chromium, cobalt, manganese, phosphorus, sulfur, zinc, and molybdenum.

Compared with the control group, root application (dipping) of melatonin caused a significant difference in potassium, copper, selenium, silicium, and silver. Spray application caused a significant difference in calcium, copper, nickel, selenium, silicium, and silver, according to Bonferroni’s correction. Compared with the control group, statistically, the K element in the direct root-dipping of melatonin material and the Ni element in the direct needle-spraying made a difference between the application groups. Melatonin inhibits the operation of large conductance calcium-activated potassium channels; it blocks endothelial potassium channels to minimize flow-induced nitric oxide release (Geary *et al*. 1998).

The 18 elements investigated made a statistically significant difference ($p<0.05$) in all sapling tissues (root, needle, stem). These results are similar to those in the literature (Balzer and Hardeland 1996; Van-Tassel and O'Neill 2001a; Hernández-Ruiz *et al*. 2004; Arnao and Hernandez-Ruiz 2006; Ye *et al*. 2017).

When the application of melatonin substance in only different dose groups compared to the control group was evaluated, there was no significant difference ($p<0.05$) between sodium, iron, aluminum, magnesium, chromium, cobalt, manganese, nickel, and sulfur. However, a significant difference ($p<0.05$) was found in the potassium, calcium, cadmium, copper, phosphorus, selenium, silicium, silver, zinc, and molybdenum (Fig. 2).

As the impact of melatonin on the elements was examined, it was seen that 500 µM and 1000 µM doses of melatonin resulted in an increase in the amount of the elements compared to the control group (0 µM). When the statistical results were evaluated, it was more accurate to determine use of the low and middle dose.

Zhang J. *et al*. (2017) noted that high concentrations of melatonin (100 and 200 mM) had an opposite effect and even decreased root growth in seedlings. Similar results were obtained in other studies (Chen *et al*. 2009; Sarropoulou *et al*. 2012; Wang *et al*. 2016; Marta *et al*. 2016; Sharif *et al*. 2018; Chen *et al*. 2020). As a result, several studies have shown that utilizing EMS in plants has an impact on the amount of elements present.

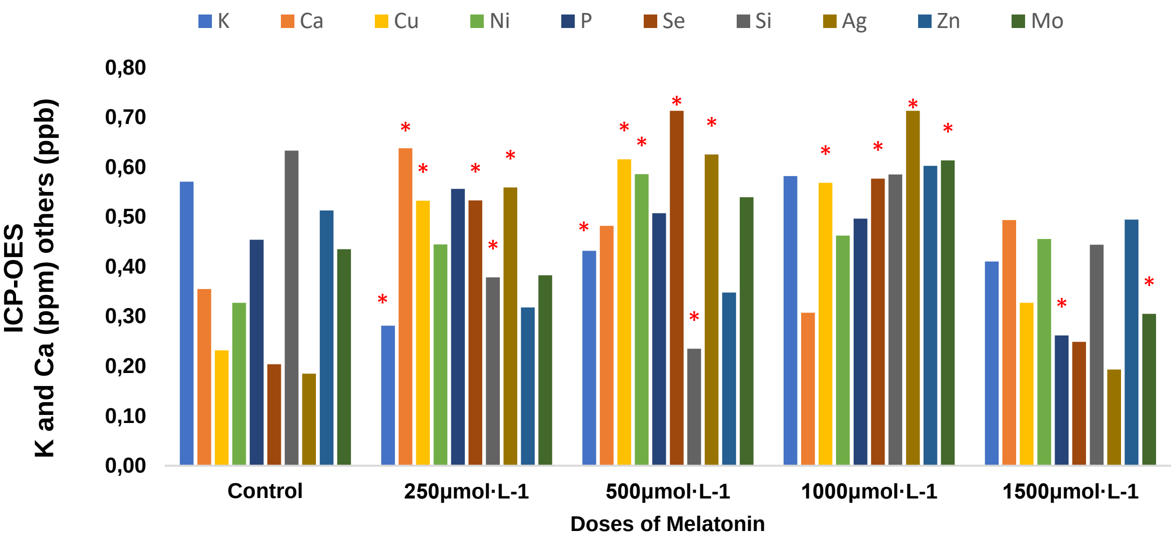

Fig. 2. Comparison of the elements that are meaningful only with respect to the dose compared with the control group. * Statistically different from the control group according to Bonferroni's correction

The interaction of the melatonin substance with 4 different doses and 2 different administration forms (root-dipping / needle-spraying) was compared with the control group. While there was a significant difference ($p<0.05$) in the elements of potassium, calcium, copper, phosphorus, selenium, silicium, silver, zinc, and molybdenum, no significant difference was found in the elements sodium, iron, aluminum, magnesium, chromium, cobalt, manganese, nickel, and sulfur (Table 1).

Table 1. Change in Macro/Micro Elements according to Different Melatonin Treatments (4 doses+control (Cont) and Method of Administration [root-dipping (Dip) + Needle-Spraying (Spray)]

Macro /Micro Element	Control	250 µM		500 µM		1000 µM		1500 µM		H / P Values
		Dip	Spray	Dip	Spray	Dip	Spray	Dip	Spray	
	Means									
Na (ppm)	45.50	36.83	55.61	42.89	46.28	58.50	42.89	33.50	47.50	6.709 $^{/ns}$
K (ppm)	57.06	27.00	29.22	48.33	38.00	53.11	63.22	24.44	57.56	25.047 **
Ca (ppm)	35.50	51.78	75.78	33.11	63.22	42.22	19.22	52.67	46.00	31.342 ***
Fe (ppm)	37.50	38.06	39.00	54.00	60.50	53.44	40.89	34.11	60.00	12.492 $^{/ns}$
Al (ppm)	41.50	38.00	41.00	53.00	56.00	52.89	44.00	28.00	59.11	10.859 $^{/ns}$
Mg (ppm)	39.28	41.00	40.44	50.67	53.67	52.22	38.11	39.78	60.56	7.594 $^{/ns}$
Cr (ppb)	43.50	43.00	41.00	46.00	58.00	52.22	42.00	32.00	53.78	6.583 $^{/ns}$
Co (ppb)	36.28	38.00	38.44	54.89	58.11	53.67	43.67	36.33	59.33	11.457 $^{/ns}$
Cu (ppb)	23.17	53.33	53.11	60.22	62.89	57.78	55.89	13.44	52.00	39.094 ***
Mn (ppb)	46.61	37.22	32.78	56.00	54.00	45.00	51.78	34.00	51.00	8.144 $^{/ns}$
Ni (ppb)	32.72	45.89	43.00	52.11	65.00	50.33	42.11	33.00	58.11	14.599 $^{/ns}$
P (ppb)	45.39	53.33	57.89	45.00	56.44	55.22	44.00	14.22	38.11	19.314 *
Se (ppb)	20.39	55.00	51.56	70.78	71.78	67.44	47.89	5.22	44.56	6.671 ***
Si (ppb)	63.28	28.67	47.00	21.67	25.33	72.89	44.11	32.78	56.00	38.464 ***
Ag (ppb)	18.50	56.78	55.00	59.89	65.11	76.33	66.22	5.00	33.67	71.647 ***
S (ppb)	47.28	46.11	54.11	32.89	59.67	48.33	51.33	23.00	45.00	13.045 $^{/ns}$
Zn (ppb)	51.28	33.56	30.00	35.11	34.44	61.44	59.00	27.89	71.00	27.387 **
Mo (ppb)	43.50	46.00	30.50	48.11	59.72	72.67	50.00	30.50	30.50	30.931 ***

Thus, different forms of application of exogenous melatonin were found to have different effects on the plant's melatonin absorption. Similar outcomes have been obtained previously. Melatonin-induced changes in plant growth and tissues system architecture may be due to a signaling mechanism similar to that of mineral nutrients (Dawood and El-Awadi, 2015; Li *et al*. 2016; Zhang R. *et al*. 2017; Sharif *et al*. 2018).

When the dual interactions between the examined seedling tissues (root, needle, stem) and different dose groups of EMS were determined, there were significant differences ($p<0.05$) in all elements (Fig. 3; Fig. 4).

Compared to the control group, the amounts of the metal elements calcium, nickel, copper, selenium, and silver analyzed in the root, needle, and stem tissues of the 250 µM dose application group were increased. Amounts of aluminum, iron, magnesium, cobalt, copper, selenium, silver, nickel, and calcium in the 500 µM dose application group, and copper, selenium, and silver amounts of elements in 1.000 µM dose application group were also increased compared to the control group (Fig. 3; Fig. 4).

Compared to the control group, the amount of sodium analyzed in the root and needle tissue was increased in the 250 and 500 µM doses application groups; the analyzed amount of chromium in the 500 µM dose application group and those of potassium, sodium, zinc, molybdenum, and chromium in the 1000 µM dose application group were also elevated (Fig. 3; Fig. 4). The amount of lead element analyzed on root and stem tissue was increased in 250, 500, and 1000 µM doses application groups compared with the control group (Fig. 4). The amount of manganese analyzed on the needle and body tissue was increased in the 500 µM dose application group compared with the control group (Fig 3). The amounts of silicon and sulfur, which were analyzed on the body tissue were increased in the 1000 µM dose application group compared to the control group (Fig. 4).

Figures 3 and 4 show that the value of the EMS is revealed by the increases in macro/micro elements responsible for the realization of plant metabolic activities. Likewise, it increased the amount of macro/micro elements that help maintain plant physiological processes. Melatonin has an important modulating influence on the mineral element composition of forest tree plants. Many studies have reported that melatonin functions in the binding, regulation, stimulation, amelioration, and enhancement of elements (Posmyk *et al.* 2008; Erdal and Dumlupinar 2011; Tan *et al.* 2012; Turk and Erdal 2015; Aguilera *et al.* 2015; Dawood and El-Awadi 2015; Li *et al.* 2016; Zhang J. *et al.* 2017; Zhang R. *et al.* 2017).

To sum up, the present study showed that exogenous melatonin supplementations applied to improve planting quality in forest trees have an important effect on the macro/micro amount of elements in the plant. However, more research is needed to prove melatonin's ability to promote plant development and quality seedlings, including experimental proof of the formed complex. The exogenous application of melatonin in forestry may be a new frontier to be explored.

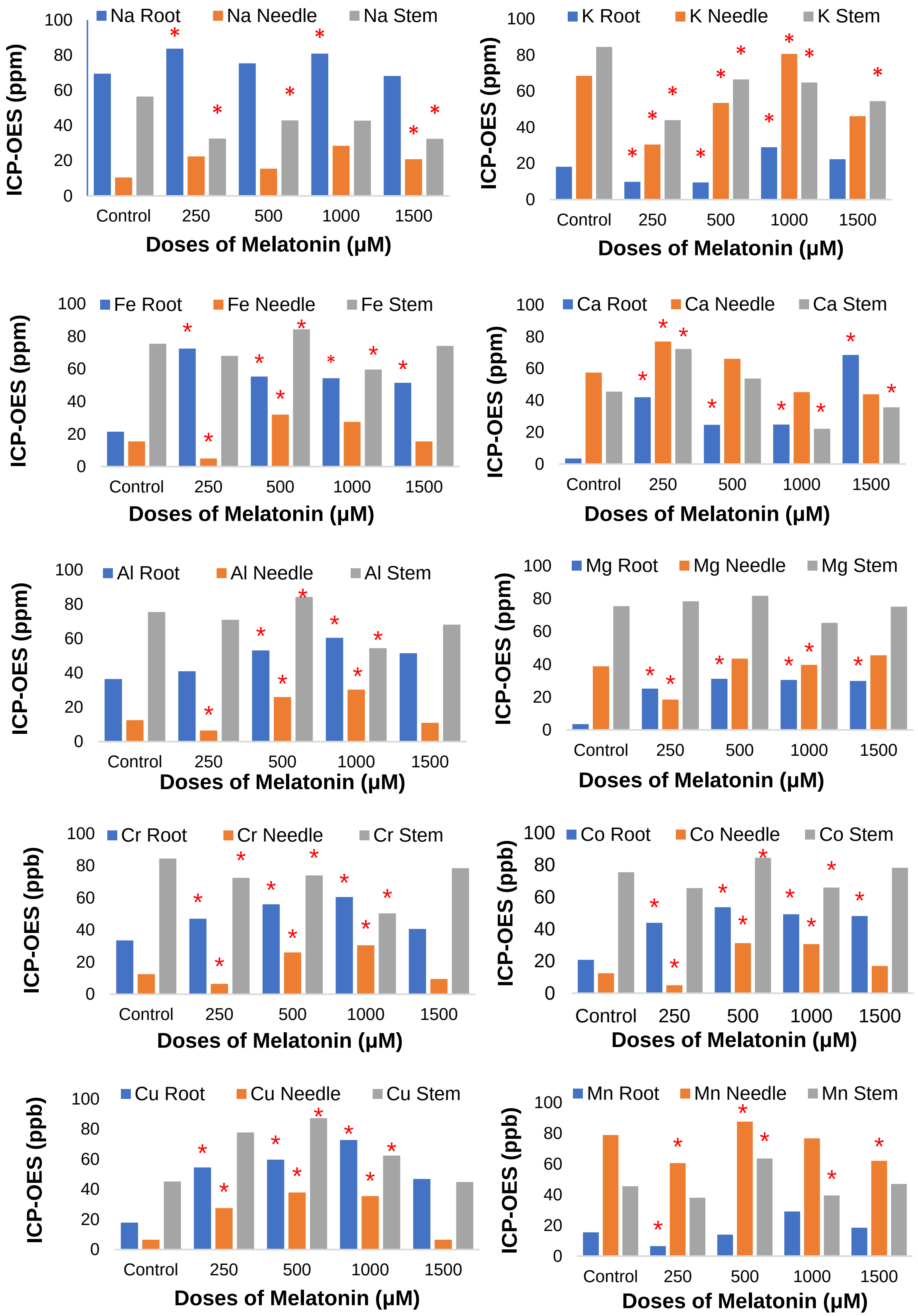

Fig. 3. Comparison of the different melatonin treatments and various seedling tissue from the different macro/micro elements
* Statistically different from the control group according to Bonferroni's correction.

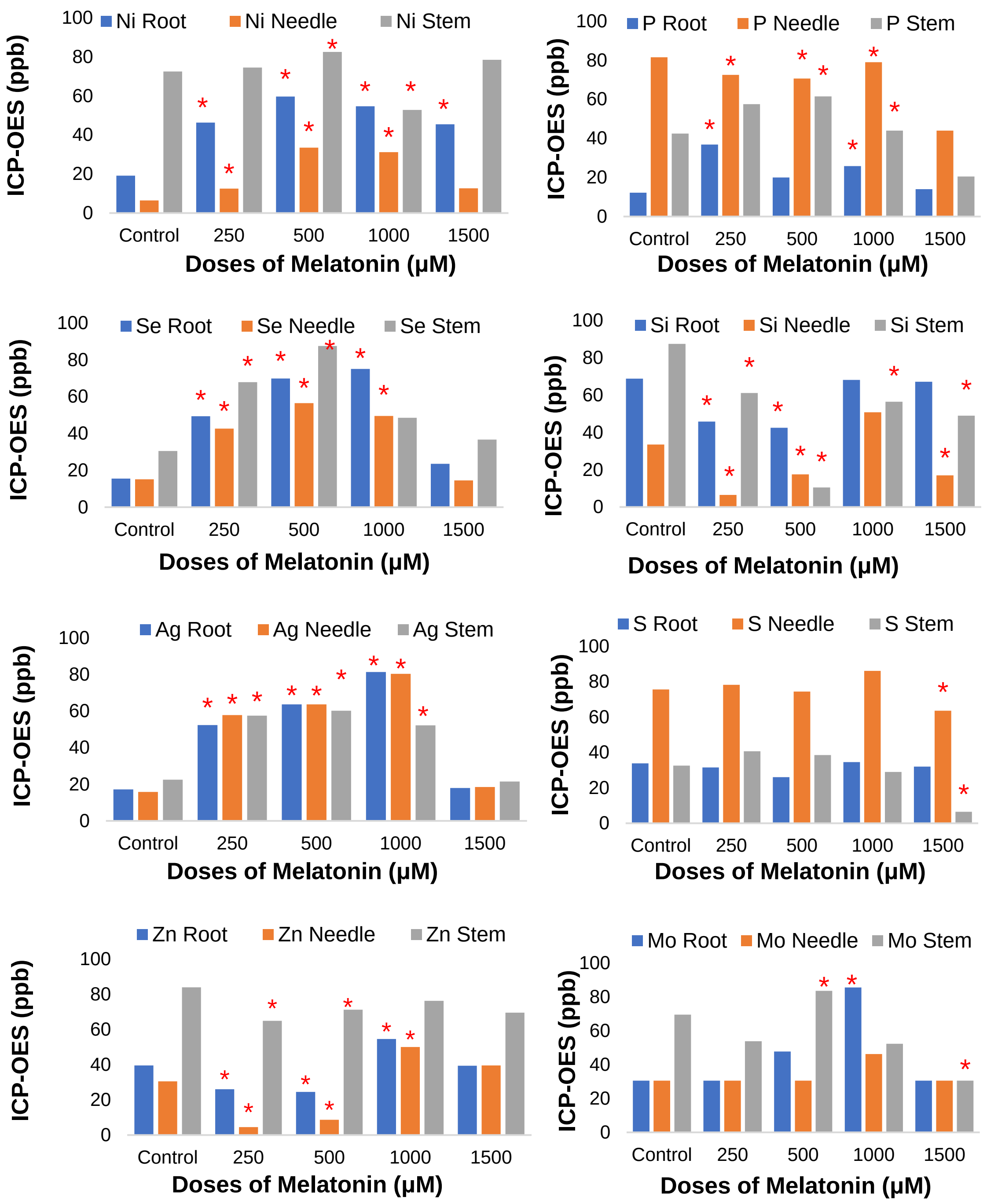

Fig. 4. Comparison of the different melatonin treatments and various seedling tissue from the different macro/micro elements
* Statistically different from the control group according to Bonferroni's correction.

CONCLUSIONS

1. High dose applications of exogenous melatonin supplementations (EMS) inhibited the elemental amounts of some macro / micro components in the wood tissue.
2. Different doses of EMS caused variable mobility in different tissues of the plant in

terms of macro / micro amount of elements.

3. EMS needle-spraying applications of seedling had a greater impact on macro / micro amount of elements the control group.

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