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Effects of Zinc Oxide Nanoparticles on Growth and Secondary Metabolite Accumulation in In Vitro Culture of Ocimum basilicum L.

Çinko Oksit Nanopartiküllerinin Ocimum basilicum L.'nin İn Vitro Kültürlerinde Büyüme ve Sekonder Metabolit Birikimi Üzerine Etkileri

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Abstract

The study investigated the effects of zinc oxide nanoparticle (ZnO-NP) on the growth, secondary metabolite accumulation, and antioxidant activity of *Ocimum basilicum* L. (basil) shoots in vitro. Various concentrations of ZnO-NP (5, 10, 20, 40 and 50 mg/L) were applied to assess their impact on growth parameters such as shoot elongation, node and leaf numbers, shoot multiplication rate, root formation percentage, and root length. Results showed that lower ZnO-NP concentrations stimulated shoot elongation, while higher concentrations negatively affected root development. Total phenolic content (TPC) and antioxidant activity were assessed in methanolic extracts, with higher TPC observed in roots compared to stems. The antioxidant capacity, evaluated using DPPH and CUPRAC assays, showed variable responses to ZnO-NP concentrations, with higher antioxidant activity (SC_{50}: 0.77 mg/mL and 0.512 mmol TE/g plant, respectively) detected at 40 mg/L ZnO-NP concentration in roots. Additionally, the study analyzed rosmarinic acid content, the main phenolic compound in *O. basilicum*, and found that its accumulation increased with ZnO-NP application, especially in roots at the 40 mg/L ZnO-NP concentration with the value of 35.98 µg phenolic/mg plant. Overall, the findings suggest that ZnO-NP application influences growth, secondary metabolite accumulation, and antioxidant activity in *O. basilicum*, with effects varying based on concentration and plant part.

Keywords: *Ocimum basilicum*, ZnO nanoparticle, Antioxidant activity, Rosmarinic acid, Micropropagation

Özet

Bu çalışmada, çinko oksit nanopartikülünün (ZnO-NP) *Ocimum basilicum* L. (fesleğen) sürgünlerinin in vitro büyümesi, ikincil metabolit birikimi ve antioksidan aktivitesi üzerindeki etkileri araştırılmıştır. Çeşitli ZnO-NP konsantrasyonları (5, 10, 20, 40 ve 50 mg/L) sürgün uzaması, nod ve yaprak sayıları, kardeşlenme sayısı, kök oluşum yüzdesi ve kök uzunluğu gibi büyüme parametreleri üzerindeki etkilerini değerlendirmek için uygulanmıştır. Sonuçlar, düşük ZnO-NP konsantrasyonlarının sürgün uzamasını uyardığını, yüksek konsantrasyonların ise kök gelişimini olumsuz etkilediğini göstermiştir. Metanolik ekstraktlarda toplam fenolik içerik (TPC) ve antioksidan aktivite değerlendirilmiş, köklerde saplara kıyasla daha yüksek TPC gözlenmiştir. DPPH ve CUPRAC analizleri kullanılarak değerlendirilen antioksidan kapasite, ZnO-NP konsantrasyonlarına değişken tepkiler göstermiş, köklerde 40 mg/L ZnO-NP konsantrasyonunda daha yüksek antioksidan aktivite (sırasıyla SC_{50}: 0,77 mg/mL ve 0,512 mmol TE/g bitki) tespit edilmiştir. Ayrıca, çalışmada *O. basilicum*'daki ana fenolik bileşik olan rosmarinik asit içeriği analiz edilmiş ve ZnO-NP uygulamasıyla, özellikle de köklerde 40 mg/L ZnO-NP konsantrasyonunda 35,98 µg fenolik/mg bitki değeriyle birikiminin arttığı bulunmuştur. Genel olarak, bulgular ZnO-NP uygulamasının *O. basilicum*'da büyümeyi, ikincil metabolit birikimini ve antioksidan aktiviteyi etkilediğini, etkilerin konsantrasyona ve bitki kısmına göre değiştiğini göstermektedir.

Anahtar Kelimeler: *Ocimum basilicum*, ZnO nanopartikül, Antioksidan aktivite, Rosmarinik asit, Mikroçoğaltım

Abbreviations: TPC, Total phenolic content; GAE, gallic acid equivalent; ZnO-NP, Zinc oxide nanoparticle; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; CUPRAC, Cupric Reducing Antioxidant Capacity

1. INTRODUCTION

Ocimum basilicum L. (basil), a member of the Labiatae family, is a medicinal and aromatic plant species cultivated in many countries and naturally grows in the Mediterranean, Africa, America and Asia (Grayer et al., 1996). Although it does not grow naturally in the flora of Türkiye, it is cultivated in some regions (Karaca et al., 2017). Basil leaves are used in folk medicine as a cure for numerous ailments, including cancer, kidney problems, convulsions, influenza, diarrhea, malaria, epilepsy, fevers, gout, nausea, asthma, sore throat, toothache and bronchitis (Khalid et al., 2006; Duke et al., 2008; Shahrajabian et al. 2020). These leaves are a source of secondary metabolites particularly essential oil and phenolic compounds that exhibit antioxidant, antimutagenic, anti-inflammatory, antiviral, antifungal, antibacterial, antitoxic, hypolipidemic, antihyperglycemic, immunomodulatory, hepatoprotective, insect repellent and depigmenting properties (Burt 2004; Khalid et al., 2006; Gülçin et al., 2007; Duke et al., 2008; Hanif et al., 2011; Khair-ul-Bariyah et al. 2012; Karataş, 2022). Phytochemicals belonging to flavonoid, alkaloid, phenolic and terpenoid groups have been reported as bioactive constituents in *O. basilicum*. Previous studies on the determination of phytochemicals contained in this

species have reported that the most important and predominant phenolic is rosmarinic acid, an ester of caffeic acid (Açıkgöz 2020; Zeljković et al. 2020; Teoflović et al. 2021).

Many medicinal and aromatic plants are mellifluous in phenolic compounds with diverse and powerful biological activities. These compounds play a role in defense against UV, drought, heat, pathogens, predators, interspecies competition and pollination in plants. In medical terms, these compounds play an important role in the removal of free radicals that cause cell damage and thus show the ability to prevent the formation of chronic degenerative diseases such as cancer, cardiovascular and neurodegenerative diseases (Ncube et al., 2012; Briskin, 2000). In particular, environmental factors are known to significantly affect the plant growth and synthesis of secondary metabolites. Based on this information, studies have been conducted to investigate the effects of stressors such as temperature, light, UV, drought and many other factors on plant growth and accumulation of secondary metabolites (Nascimento and Fett-Neto, 2010).

In recent years, in vitro plant tissue culture systems have been evaluated as an important and effective tool for the potent production of medicinal and aromatic plants and their bioactive phytochemicals (Espinosa-Leal, 2018). In this way, the destruction of natural habitats can be prevented, plants that are superior in terms of content and yield can be created, and continuity can be ensured in the production of valuable plant secondary products regardless of time and seasonal conditions. For this purpose, various studies have been carried out on the production of rosmarinic acid, the main phenolic of *O. basilicum*, under *in vitro* conditions in many plant species and the changes in secondary metabolite content have been revealed (Tepe and Sokmen, 2007; Bauer et al.2009; Krzyzanowska et al. 2012; Kračun-Kolarević et al.2015). There are many reports evaluating the amount of rosmarinic acid in callus cultures, cell suspension cultures, shoot, root and adventitious root cultures of *O. basilicum* (Srivastava et al. 2016; Verma et al. 2016; Duran et al. 2019; Nazir et al. 2019; Açıkgöz 2020; Biswas 2020). However, there are no studies in which zinc nanoparticles were applied under *in vitro* conditions and their effects on physiological and biochemical parameters were determined.

Exposing the organs, tissues or cells of the plant to biotic and abiotic stressors is the leading method used to increase the production of secondary metabolites in plants. Studies have shown that secondary metabolites are produced as defensive responses of plants to such stressors (Zhoua and Wu, 2006). Newly, studies on the use of nanoparticles as elicitors that affect various morphological, physiological and biochemical mechanisms in plant metabolism have become widespread, especially *in vitro*, and it has been reported that they play an

important role in the biosynthesis of valuable plant secondary metabolites (Nair, 2016; Kim, et al., 2017; Gonçalves et al., 2021; Nourozi et al., 2021). Zinc is an essential micronutrient required for many events in plants such as cell membrane stability, synthesis of lipids, synthesis of cytochrome and chlorophyll, carbohydrate, protein and nucleic acid metabolism, photosynthesis, regulation of gene expression, cell transparency, the structure of enzymes and activity of phytohormones (Sousa et al., 2009; Torre-Roche et al., 2020; Mumivand et al., 2021). Zinc, which plays a critical role in various physiological processes for plants and is required in small amounts for plant growth and development, can cause negative effects on plant growth and development when its concentration exceeds the tolerable limit. This limit may vary depending on plant tissue and plant species. When the amount of zinc reaches or exceeds this limit, results such as increased ROS production in the plant and negativities in growth and development may occur. Besides these, zinc oxide nanoparticles (ZnO-NPs) have been frequently used to promote the production of bioactive secondary metabolites in medicinal plants (Gonçalves et al., 2021; Nekoukhou et al., 2022; Inam et al., 2023). These nanoparticles can be used in medicinal plants and can contribute positively or negatively to the production of the target compound.

In this study, it was aimed to determine the effects of ZnO-NP applied at different concentrations (5, 10, 20 and 40 mg/L) on growth and secondary metabolite accumulation in i*n vitro* shoots of *O. basilicum* as well as their antioxidant activities. Although there are some previous studies on *in vitro* micropropagation of *O. basilicum* and determination of essential oil and phenolic composition of plant materials collected from nature, to the best of our knowledge, there is no report investigating the changes in plant growth and development and secondary metabolite content as a result of ZnO-NP treatment under in vitro conditions. Therefore, the present study is the first report in which some physiological and biochemical effects of ZnO-NP exposure on this plant species were determined.

2. MATERIALS AND METHODS

2.1. Plant material and Seed Germination

Mature seeds of *O. basilicum* were germinated in MS medium and seedlings were used as explants for further experiments. Seeds were obtained from a commercial company. Surface sterilization of the seeds was achieved in accordance with the study submitted in the literature (Bektaş et al., 2016). Briefly, mature seeds were kept in a 5% sucrose solution (with 1-2 drops of commercial bleach) for 12 hours and then the seeds taken from this solution were treated

with H_2O_2 for 30 minutes. Seeds were washed 3 times (5 min each) with sterile distilled water and transferred to Murahige&Skoog including vitamins (MS) medium supplemented with 2% sucrose and 0.7% phyto agar. Incubation conditions for all cultures were designed at 25 °C, 16 hours of light under cold white fluorescent light (50 µmol m^{-2} s^{-1}) and 8 hours of darkness.

2.2. Shoot Proliferation and ZnO-NP Treatment

After germination, the seedlings were incubated in the same environment for a total of 60 days. Nodal explants (3-4 explants from each seedling) were obtained from these seedlings, approximately 1 cm in length and containing a single node. These explants were subcultured every 30 days by transferring them to the above-mentioned MS medium in order to provide a sufficient number of nodes to be used as explants for further experiments. Cultures were incubated at 25 °C and 16/8 h light/dark photoperiod regime under cold white fluorescent light (50 µmol m^{-2} s^{-1}).

To determine the effect of nanoparticles, ZnO-NP was added to the MS medium at concentrations (5.0, 10.0, 20.0, 40.0 and 50.0 mg/L) frequently used in the literature (Lin and Xing, 2007; Mahajan et al., 2011; Salama et al., 2019). Higher and lower concentrations than these were also tested, but since no significant inhibition of seedling growth was detected at high concentrations (browning in most of the explants) and no differences were detected at lower concentrations from the data obtained from the control group and the 5 mg/L concentration, they were not evaluated in this study. ZnO-NP (Purity:>99.5%, Size: 30-50 nm) were purchased from Nanografi Company (Cat. No: NG04SO3802, Ankara, Turkey). The stock solution (10 mg/mL) of ZnO-NP prepared in distilled water was sterilized by autoclaving and added to the autoclaved nutrient media in appropriate amounts before solidification. ZnO-NP free medium was used as the control group. All media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) phytoagar and sterilized in an autoclave (15 minutes at 121°C). Cultures incubated at 25°C in a 16/8 hour (light/dark) photoperiod regime (cold white fluorescent lamps, 50 µmol m^{-2} s^{-1}) for 30 days (4 weeks). At the end of the incubation period, shoot length, number of leaves and nodes, number of multiple shoots, percentage of root formation, number and length of roots of the seedlings were measured. For each application, 25 magenta cups, each containing 4 explants, were created. Each experiments were performed triple.

2.3. Extraction of Plant Materials

After the harvesting of seedlings, plant materials (roots and aerial parts) were dried in the oven (40 °C) and powdered, respectively. The extraction of powdered plant material was performed

by using methanol (99%) with mobile maceration method by shaking on an orbital shaker for 24 hours at 25 °C. The filtrate obtained after the extraction was subjected to pre-filtering with the filter paper, and then to the final filtering process with a 0.45µm diameter filter. The obtained extracts were strained and supernatants were collected. Some of the obtained methanolic extracts were evaporated and the extract yields were calculated and the remained extracts were stocked at 4 °C in closed containers for further studies.

2.4. Analysis of Total Phenolic (TPC) Contents

The total phenolic content of the methanolic extracts was determined by the Folin-Ciocalteu method using the gallic acid standard curve (Singleton and Rossi 1965). Results are given as milligram gallic acid equivalents per gram of extract (mg GAE/g extract).

2.5. Antioxidant Activity

2.5.1. DPPH Activity

The DPPH radical scavenging activity of the extracts was performed according to the DPPH (2,2-diphenyl-1-picrylhydrazil) method (Molyneux, 2004). Briefly, 750 µL of methanolic extract was mixed with an equal amount of 100 mM DPPH solution, and the absorbance was measured at 517 nm (Mapada, UV-6100PC) after incubating for 50 minutes at ambient temperature. The SC_{50} value was calculated by using the absorbance-concentration graph prepared by measuring the changes in the absorbance of DPPH• treated with different extract concentrations. A low SC_{50} value indicates high radical scavenging (antioxidant) activity. All experiments were performed in triplicate

2.5.2. CUPRAC Assay

The determination of Copper (II) Ion Reducing Antioxidant Capacity (CUPRAC) involves the utilization of a procedure developed by Apak et al. (2004). 1 mL of $CuCl_2$ solution (10 mM), 1 mL of neocuproine ethanolic solution (7.5 mM), and 1 mL of NH_4CH_3COO buffer solution (1 M, pH=7) were combined in a test tube. Subsequently, 0.2 mL of the sample followed by 0.9 mL of water were added and vortexed. After approximately 30 minutes of incubation, measurements were taken at 450 nm. The results were expressed as µmol of Trolox equivalents (TE) per gram of the plant material.

2.6. HPLC Analysis of Rosmarinic Acid

Rosmarinic acid contents of shoots and roots were determined by using RP-HPLC-DAD. These analyses were achieved on the Thermo Scientific Dionex Ultimate™ 3000 system (Thermo Scientific, Bremen, Germany). Chromatographic separation was carried out on a Thermo Scientific™ Hypersil™ ODS C18 HPLC (250 mm×4.6 mmx5 µm) column (Thermo Scientific, USA) at temperature of 30°C using a mobile phase, consisting of 2% (v/v) acetic acid in water (A), 70% (v/v) acetonitrile in water (B) at a flow rate 1.2 mL/min, under gradient elution conditions. The gradient was used as follows: zero-time condition was 5% B and it was increased to 60% B in 26 minutes. Rosmarinic acid was monitored 315 nm (Bektaş, 2020; Bektaş vd., 2022). Chromatogram areas were calculated using the integration measurement method and the mAU detection unit.

2.7. Statistical analysis

All experiments were conducted using a completely randomized design, and the significance of the results was evaluated through one-way analysis of variance. The means of the treatments were then compared utilizing Tukey's test, where statistical significance was set at $P \leq 0.05$. Additionally, to ensure the reliability and reproducibility of the findings, the experiments were repeated triple and consistent results were obtained.

3. RESULTS AND DISCUSSION

3.1. Seed Germination and Effects of ZnO-NP application on Growth Parameters

It was observed that mature seeds commenced germination from the 10th day after being introduced into the culture. Variations were noted, with some seeds germinating early (at day 10) and others initiating germination later. Consequently, the germination rates of the seeds were calculated as of the 30th day post-transfer into the culture medium, resulting in an observed germination rate of 88%. Among the germinated seeds, 75% successfully progressed into healthy seedlings, completing their development.

Nanoparticles are substances that interact with plant cells and can affect plants positively or negatively depending on their type, size and concentration, causing various physiological and biochemical changes in cells (Navarro et al., 2008; Thuesombat et al., 2014). Generally, low concentrations stimulate plant growth and development (Farag et al., 2017), while high concentrations can have toxic effects. In our study, different concentrations of ZnO-NPs were applied to *O. basicilicum* shoots to investigate their effects on growth parameters.

Figure 1 shows a representative image of *O. basilicum* seedlings grown under *in vitro* conditions after a 1-month incubation period. Table 1 presents empirical data illustrating the impact of various concentrations of ZnO-NP on multiple parameters associated with plant growth. The parameters assessed include shoot elongation, node and leaf numbers, shoot multiplication rate, root formation percentage, and root length. To ensure the reliability of the data, the mean values and standard variations were calculated from three independent replicates. Observationally, as the ZnO-NP concentration escalates from 5.0 to 50.0 mg/L, there are discernible alterations across several measured variables. Specifically, there's a progressive rise in shoot elongation, indicating increased shoot growth from 61.20 to 76.01 mm with ascending concentrations. However, other parameters such as node and leaf numbers, as well as shoot multiplication, exhibit relatively subtle fluctuations despite the concentration changes. At lower concentrations of ZnO-NP (5.0, 10.0, 20.0 mg/L), there seems to be a trend of slightly increased shoot elongation compared to the control group. However, at higher concentrations (40.0, 50.0 mg/L), shoot elongation appears to decrease, indicating potential negative effects on shoot growth. The shoot multiplication rate remains constant across all concentrations and the control group, indicating that ZnO-NP concentrations do not significantly influence shoot multiplication. There are slight fluctuations in node and leaf numbers across different ZnO-NP concentrations, but no clear pattern emerges. This suggests that ZnO-NP may not have a significant impact on node and leaf development.

Figure 1. Representative image of *O. basilicum* seedlings grown *in vitro* after a 1-month incubation period. The picture belongs to seedlings grown in medium supplemented with 5.0 mg/L ZnO-NP.

Of particular interest is the inverse relationship observed between ZnO-NP concentration and root development. Notably, the percentage of successful root formation declines from 100% at lower concentrations (5.0 to 20.0 mg/L) to 75% at 40.0 mg/L and to 0% at 50.0 mg/L. This decline is mirrored in root length, diminishing from 133.58 to 100.13 mm with the escalation in ZnO-NP concentration. Root formation seems to be adversely affected at higher concentrations of ZnO-NP (40.0, 50.0 mg/L), with a decrease in root formation percentage compared to the control group. Additionally, root length decreases notably at higher

Table 1. Impact of ZnO-NP concentrations on in vitro growth parameters of *O. basilicum*.

	Concentration (mg/L)	Shoot Elongation (mm)*	Node Number*	Leaf number*	Shoot Multiplication (shoots/per explant)	Root Formation (%)	Root length (mm)*
ZnO-NP	Control	61.20±1.33d	3.16±0.37b	6.33±0.75b	2.00	100	153.75±3.11a
	5.0	70.47±1.12b	3.58±0.50a	7.16±1.00a	2.00	100	133.58±2.23b
	10.0	66.03±1.66c	3.41±0.50ab	6.83±1.00ab	2.00	100	118.75±2.47c
	20.0	65.93±1.53c	3.38±0.49ab	6.77±0.98ab	2.00	100	108.27±3.26d
	40.0	76.01±1.34a	3.69±0.46a	7.38±0.93a	2.00	75	104.22±7.69e
	50.0	56.07±1.30e	3.22±0.42b	6.44±0.84b	2.00	0	-

* Same letters in each row were not significantly different at $P < 0.05$ (Tukey's range test). The means of the three replicates are given with their standard deviations.

Similar to the results obtained from our study, Zaeem et al. (2020) reported that there was a decrease in root and shoot length with increasing ZnO-NP concentration in *Linum usitatissimum* plant and that the roots were more affected by this situation due to direct contact with the nanoparticle. They reported that the rooting rate and root length were at optimum level up to 5-35 mg/L concentration of ZnO-NP concentrations, and the values of these parameters decreased when the concentration was higher than this concentration. They attributed this unfavourable situation in root formation to the role of Zn in auxin synthesis. It has also been reported that Zn can act as an activator or cofactor for some enzymes that have important roles in rooting (Mildvan, 1970). In addition, a study suggested that the negative effects of nanoparticles on root elongation may be due to the non-porous nature of the agar medium, water accumulation and less available dissolved oxygen (Lee et al., 2010).

3.2. Total Phenolic Contents of Extracts

The amounts of total phenolic substances in methanolic extracts obtained from roots and aerial parts of *O. basilicum* seedlings grown at different ZnO-NP concentrations are presented in

Table 2. According to the results obtained, the amounts of total phenolic substances in the roots were 2 to 3 times higher than the amounts obtained from the stems. The amounts of total phenolic substances obtained from the stems of ZnO-NP-treated seedlings (except 20.0 mg/L ZnO-NP treatment) were higher than the amounts of total phenolic substances of seedlings without nanoparticle treatment (control). In the extracts obtained from the stems of the seedlings, the highest total phenolic content was found in 40.0 and 50.0 mg/L ZnO-NP treatments. In the roots, it was determined that the total phenolic matter amounts calculated in the other ZnO-NP treatments were higher than the control except for the total phenolic matter amounts of the seedlings grown in the medium containing 20.0 mg/L ZnO-NP. The highest total phenolic matter content in roots was calculated at low concentrations of ZnO-NP (5.0 and 10.0 mg/L) in contrast to that in stems. The lowest accumulation of total phenolic substances in both stems and roots as a result of ZnO-NP exposure was found in seedlings grown at 20.0 mg/L concentration.

It is known that plant secondary metabolites can be synthesised more when plants are exposed to abiotic or biotic stressors. In response to the addition of elicitors to growth media, plants activate their defence systems (Nourozi et al., 2019a, b). It has been reported that the application of nanoparticles as elicitors generally increases the production of secondary metabolites in parallel with the increase in the amount of ROS. ROS is the fastest response of plants to stress factors and plays a dual role in both triggering the defence system of plants and increasing cell damage or disrupting signal transduction (Dat et al. 2000). As mentioned in the literature, it is obvious that nanoparticles induce ROS in plants (Marslin et al. 2017; Ranjan et al. 2021) and their effects on plants have been found to be concentration-dependent. It has been reported that high concentrations are toxic, while low concentrations cause beneficial effects (Jalil and Ansari 2019).

The mechanisms of action of nanoparticles in plants are not yet clearly known. However, it is suggested that plants become susceptible to toxicity caused by NPs due to oxidative stress caused by ROS (Wang et al., 2011). Free radicals are usually produced as a result of oxidative stress. Hydroxyl radicals, superoxide anions and hydrogen peroxide are a few examples of free radicals that damage cells, cellular membranes, nucleic acids and proteins (Ghorbanpour et al., 2015). Phytoalexins, such as phenolic compounds, can accumulate in higher amounts in damaged tissues and cells when free radicals show negative effects (Wen Wang and Yong Wu, 2010). The accumulation of phenolic compounds in relevant organs allows plants to respond to stress when ROS are produced (Janas et al., 2009). The resulting

free radicals are detoxified by phenolic compounds (Mittler, 2017). Although elicitors are reported to increase the amount of phenolic compounds, the concentration of the elicitor used is important for plant growth and development. Application of elicitors at high concentrations can lead to cell, tissue, or organ death (Kim et al., 2004). As mentioned in the previous sections, in our study, it was observed that ZnO-NP applied at high concentrations caused regression in the growth and development of *O. basilicum* seedlings and even root formation did not occur at the highest concentration of 50.0 mg/L. Therefore, the results of the roots of the seedlings grown at that concentration could not be presented.

Table 2. Biochemical analysis results of methanolic extracts obtained from *O. basilicum* seedlings grown at different ZnO-NP concentrations

	ZnO-NP Concentration (mg/L)	TPC (mg GAE/g plant)*	DPPH (mg/mL)*	CUPRAC (mmol Troloks/g plant)*	Rosmarinic Acid Content (µg phenolic/mg plant)*
Aerial parts	Control	10.63±0.37f	0.359±0.011c	0.210±0.001f	7.54±0.12f
	5.0	12.34±0.20e	0.328±0.027c	0.213±0.000f	5.78±0.17g
	10.0	13.31±0.22e	0.327±0.009c	0.209±0.000f	8.09±0.08f
	20.0	10.02±0.18f	0.424±0.014d	0.158±0.002g	5.29±0.17g
	40.0	14.92±0.38d	0.325±0.028c	0.209±0.004f	9.83±0.15e
	50.0	14.57±0.34d	0.334±0.014c	0.222±0.001e	7.38±0.15f
Roots	Control	26.84±0.91b	0.087±0.001ab	0.439±0.004c	22.04±0.59c
	5.0	31.54±0.26a	0.087±0.000ab	0.520±0.002a	31.22±0.86b
	10.0	32.21±0.06a	0.095±0.005ab	0.488±0.002b	30.88±0.27b
	20.0	24.98±0.10c	0.109±0.000ab	0.360±0.001d	20.27±0.53d
	40.0	27.49±0.15b	0.077±0.001a	0.512±0.005a	35.98±0.52a
	Trolox	-	0.121±0.001b	-	-

*Same letters in each row were not significantly different at $P < 0.05$ (Tukey's range test). The means of the three replicates are given with their standard deviations.

3.3. Antioxidant activity of Extracts

The results of DPPH and CUPRAC tests performed to evaluate the antioxidant activities of the extracts of seedlings grown in MS medium containing various ZnO-NP concentrations are given in Table 2. Measurements at two different sampling points, stem parts and roots, revealed the effects of ZnO-NP concentrations on antioxidant capacity. Accordingly, in both DPPH and CUPRAC analyses, the antioxidant capacity results of the extracts obtained from the roots were higher than those obtained from the stem parts.

When the DPPH radical scavenging effects were analysed, it was observed that the SC_{50} values obtained from the extracts were similar to the control. It was determined that the calculated SC_{50} value (0.077 mg/mL) of the methanolic extract obtained from the roots of the seedlings growing in the medium containing only 40.0 mg/L ZnO-NP nanoparticles was lower than the control (0.087 mg/mL), i.e. it had higher activity. The extract obtained from the stems of seedlings grown in the medium supplemented with 20.0 mg/L ZnO-NP showed lower activity than the control. It was observed that the activities of the extracts obtained from the stem parts did not change with increasing ZnO-NP concentration, while the activity increased at the highest concentration tested in the roots, and there was no difference in the activities of the samples at other concentrations.

In CUPRAC analysis, as in DPPH analysis, higher activity values were measured in roots compared to stems. When the copper (II) ion reduction antioxidant capacity values obtained from the stems of the experimental groups were examined, it was observed that the values were similar to or lower than the control except for the concentration of 50.0 mg/L. In the roots, the values obtained from the seedlings growing in the medium supplemented with 20.0 mg/L ZnO-NP were lower than the control, while the values obtained from the other treatments were higher than the control. The highest antioxidant activity was calculated as 0.520 and 0.512 mmol TE/g plant in the media treated with 5.0 and 40.0 mg/L ZnO-NP, respectively. The lowest activity was detected at 0.158 mmol TE/g plant from the stems of seedlings growing in medium containing 20.0 mg/L ZnO-NP. When the antioxidant capacity values obtained in both CUPRAC and DPPH analyses were compared, it was also observed that the highest and lowest activities were at similar treatment concentrations and plant parts.

Nanoparticles are an inductive signal for the induction of oxidative stress in plants in response to their possible toxicity and also for the production of antioxidant secondary compounds (Anjum et al., 2019). Non-enzymatic antioxidants such as phenolic compounds have the ability to control oxidative stress due to their high antioxidant capacity. In a study on the flax plant, it was reported that the increase in TPC and TFC was reported to counteract the oxidative stress caused by nanoparticle application (Zaeem et al., 2020). As in this study, nanoparticles have been shown to be abiotic elicitors that positively or negatively affect plant physiology and secondary metabolite production, since they increase the potential antioxidant potential of plants (Choi and Hu, 2008; Venkatachalam et al., 2017). In a study, it was observed that ZnO-NPs applied to callus cultures produced from Stevia rebaudiana increased secondary metabolite production (Javed et al., 2018). The reason behind the excessive metabolite

production as a result of nanoparticle treatments is still unclear, but the aforementioned studies and some existing reports have demonstrated that ZnO-NPs are potential abiotic elicitors *in vitro* cultures for the production of medically important metabolites (Sharafi et al., 2013; Javed et al., 2017). In addition to these studies, there are studies in the literature in which ZnO-NPs were found to negatively affect plant growth and development. Among these studies, in a study similar to the results obtained in our study, it was reported that the application of ZnO-NPs above 35 mg/L decreased the ability of plants to withstand ROS accumulation and caused a decrease in biomass (Stadtman and Oliver, 1991; Mittler et al., 2004). Because Zn shows toxic effect above a certain concentration. High Zn concentration causes denaturation of proteins in plants, delayed plant growth and development, and as a result, decreased production of secondary metabolites responsible for activities.

3.4. Rosmarinic Acid Contents

The amounts of rosmarinic acid determined by HPLC analyses in the methanolic extracts of *O. basilicum* seedlings exposed to different ZnO-NP concentrations and seedlings grown in the control group are presented in Table 2. In the studies, 18 different components were firstly determined by HPLC and different phenolic components were determined with different level ranges, but it was determined that rosmarinic acid was the main component in all samples and had a share of approximately 98-99% of all analysed components (Figure 1 and 2). For this reason, instead of interpreting the increase and decrease rates of all phenolic components in all groups separately, analyses were performed and completed on rosmarinic acid, which is the dominant phenolic component of the Lamiaceae family to which *O. basilicum* species belongs, with the idea that rosmarinic acid is the main cause of effective bioactivity. According to the results presented in Table 2, it was observed that the amount of rosmarinic acid determined in the roots was higher than in the stems, similar to the total phenolic content, DPPH and CUPRAC analyses. The highest amount of rosmarinic acid determined in the stem parts was analysed in seedlings grown at 40.0 mg/L ZnO-NP concentration with a value of 9.83 µg phenolic/mg plant. In roots, the highest rosmarinic acid content was found in seedlings exposed to 40.0 mg/L ZnO-NP. This value is also the highest amount of rosmarinic acid detected in both roots and stems. The lowest amount of rosmarinic acid was determined as 5.29 µg phenolic/mg plant in the stems of seedlings grown in the medium containing 20.0 mg/L ZnO-NP. The lowest amount of rosmarinic acid was determined in the roots in the same medium (20.27 µg phenolic/mg plant). The values obtained from these groups were lower than the values obtained from their control groups. It was observed that the amount of rosmarinic acid increased with increasing

concentration (except for the amount of rosmarinic acid determined at a concentration of 20.0 mg/L) up to a concentration of 40.0 mg/L ZnO-NP in both roots and aerial parts.

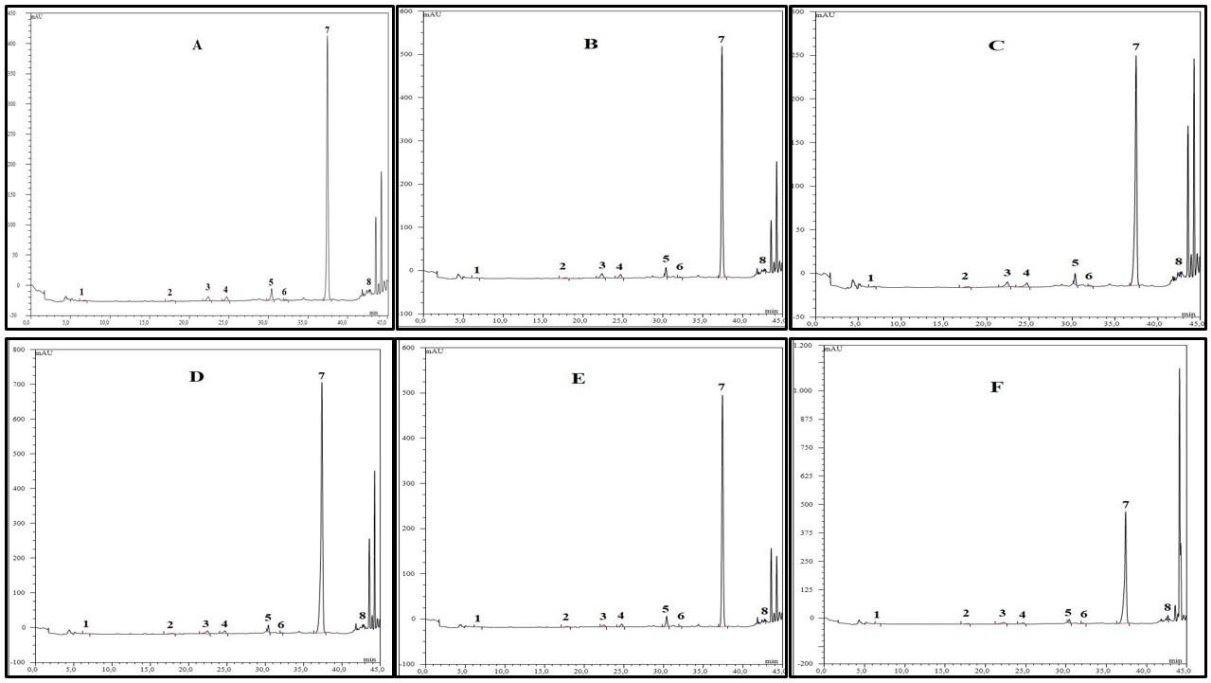

Figure 2. HPLC chromatograms of shoots of *O. basilicum* seedlings exposed to different ZnO-NP concentrations under *in vitro* conditions. A: 5.0 mg/L ZnO-NP, B: 10.0 mg/L ZnO-NP, C: 20.0 mg/L ZnO-NP, D: 40.0 mg/L ZnO-NP, E: 50 mg/L ZnO-NP, F: Control, 1: gallic acid, 2: protocatechuic aldehyde, 3: catechin, 4: caffeic acid, 5: *p*-coumaric acid, 6: ferulic acid, 7: rosmarinic acid, 8: quercetin.

The main role of rosmarinic acid, which is one of the important plant secondary metabolites and reported as the dominant phenolic component of members of the Lamiaceae family, is thought to contribute to a number of plant defence mechanisms under biotic and abiotic stress conditions. In this context, by applying various elicitor and signalling compounds to plants, plant defence mechanisms can be triggered and the yield of plant secondary metabolites involved in these defence mechanisms can be increased. For this purpose, there are several studies in which the changes in the amount of rosmarinic acid in shoot, callus and suspension cultures of different plant species in the presence of plant growth regulators, precursor compounds and elicitors were examined and positive results were observed (Santos-Gomes et al. 2003; Tepe and Sokmen 2007; Costa et al. 2012; Roy and Mukhopadhyay 2012; Costa et al. 2013; Kamalizadeh et al. 2019; Bektaş 2020; Taghizadeh et al. 2021; Karataş 2022). Among these studies, there are also studies in which nanoparticles such as Fe_3O_4 and TiO_2 applied as elicitors promote rosmarinic acid accumulation. However, no report investigating the changes in rosmarinic acid accumulation as a result of ZnO-NP applications was found in the literature. On the other hand, there are studies in which ZnO-NPs were applied to different

plant species and the changes in their different secondary compounds were investigated and as a result, metabolite production was reported to increase (Chamani et al. 2015; Javed et al. 2018; Wang et al. 2023). In our study, it was observed that ZnO-NP application increased the accumulation of rosmarinic acid in the aerial parts of *O. basilicum*, especially in the roots.

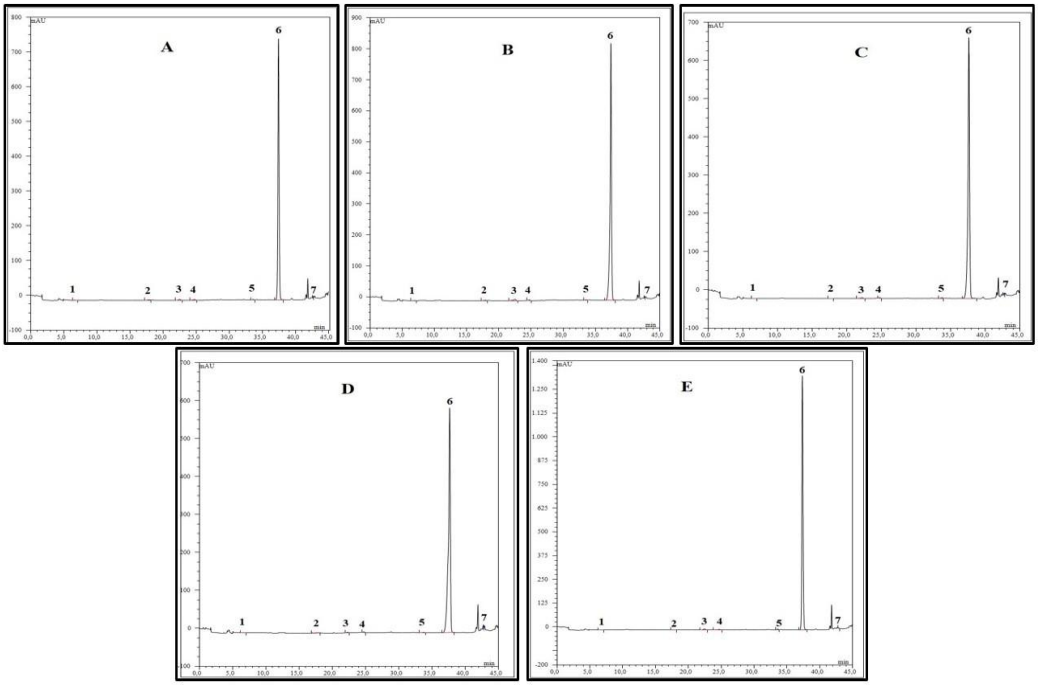

Figure 3. HPLC chromatograms of roots of *O. basilicum* seedlings exposed to different ZnO-NP concentrations under *in vitro* conditions. A: 5.0 mg/L ZnO-NP, B: 10.0 mg/L ZnO-NP, C: 20.0 mg/L ZnO-NP, D: 40.0 mg/L ZnO-NP, E: Control, 1: gallic acid, 2: protocatechuic aldehyde, 3: catechin, 4: caffeic acid, 5: ferulic acid, 6: rosmarinic acid, 7: quercetin.

4. CONCLUSIONS

In conclusion, the effects of zinc oxide nanoparticles (ZnO-NPs) on growth, secondary metabolite accumulation and antioxidant activities of *Ocimum basilicum* L. were investigated under controlled in vitro conditions. Results revealed that ZnO-NP treatment showed dose-dependent effects on various physiological and biochemical parameters of *O. basilicum*. Low concentrations of ZnO-NP showed stimulatory effects on shoot elongation and secondary metabolite accumulation, while higher concentrations led to negative effects, especially on root development. This highlights the importance of optimising nanoparticle concentrations to achieve a balance between growth promotion and potential toxicity. The study also showed that ZnO-NPs were able to enhance the production of phenolic compounds, especially rosmarinic acid, and antioxidant activities in *O. basilicum*. These findings indicate the potential of ZnO-

NPs as elicitors to enhance the medicinal properties of basil. However, careful evaluation of nanoparticle concentration is required to avoid adverse effects on plant growth and development. Future research should focus on elucidating the mechanisms underlying nanoparticle-induced changes in plant physiology and investigating their applicability under field conditions to promote sustainable agricultural practices. Overall, this study adds valuable insights into the interactions between nanoparticles and medicinal plants, paving the way for further advances in plant biotechnology and pharmacology

DECLARATIONS

On behalf of all authors, the corresponding author states that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

Funda YILMAZ BAYDU and Ersan BEKTAŞ contributed jointly to design of the study and the execution of the experimental work. Funda YILMAZ BAYDU performed the initial data analysis and drafted the manuscript. Ersan BEKTAŞ contributed to data analysis interpretationof the results, and critically reviewed the manuscript. Both authors read and approved the final version of the manuscript.

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