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Effects of different doses of zeatin, kinetin, and gibberellic acid biostimulants on growth and biochemical parameters during the seedling development stage of Istanbul Oregano (*Origanum vulgare* L. ssp. *hirtum*)

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Abstract

This research was conducted to determine the effects of different doses of zeatin, kinetin, and gibberellic acid biostimulants, which are plant growth and development regulators, on the growth parameters and some biochemical parameters of Istanbul oregano (*Origanum vulgare* L. ssp. *hirtum*). The experiment was carried out in a greenhouse setting according to the "Completely Randomized Experimental Design" with three replications. In the study, seedling and root lengths, seedling and root fresh weights, seedling and root dry weights, chlorophyll a, chlorophyll b, total carotenoid content, total phenolic content, and antioxidant activity (CUPRAC and FRAP) parameters were examined. The results of the study showed that all biostimulants increased the growth and biochemical parameters compared to the control, except for seedling dry weight. The highest plant height was obtained from the 200 ppm dose of gibberellic acid, while the highest values in growth parameters, except for root dry weight, were obtained from the 50 and 100 ppm doses of kinetin. The highest values for chlorophyll a, total chlorophyll, total carotenoid, and FRAP antioxidant activity were found at the 40 ppm dose of zeatin, while the highest values for total phenolics and CUPRAC antioxidant activity were observed at the 100 ppm dose of kinetin.

Keywords: Antioxidant, Biostimulants, *Origanum*, Seedling Development, Total Phenolic Contents

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INTRODUCTION

The genus *Origanum*, belonging to the Lamiaceae family, is represented by 39 species, predominantly spread across the Mediterranean region (Sozmen et al., 2012). In Turkey, there are 23 species of this genus, 14 of which are endemic (Duman, 2000). In Turkey, there are four subspecies of *Origanum vulgare*. These are: O. vulgare subsp. gracile, O. vulgare subsp. hirtum, O. vulgare subsp. viride, and O. vulgare subsp. vulgare (Sarikurkcu et al., 2015).

The subspecies *O. vulgare* ssp. hirtum, known as Istanbul oregano in Türkiye, is known to be among the best oreganos in terms of essential oil quality and concentration, and its popularity is increasing (Skoufogianni et al., 2019).

Turkey holds a leading position in the global oregano market, with oregano being one of the country's significant export items. Turkey provides approximately 80% of the world's oregano trade, showcasing its effectiveness in this field. Around 90% of the country's oregano exports come from species belonging to the genus *Origanum*. Among these species, *Origanum onites* L. and *Origanum vulgare* subsp. *hirtum* are particularly the most cultivated and harvested (Bayram and Arabacı, 2021). It has been reported that the essential oil content of Istanbul oregano in the wild ranges from 1% to 6.1%, whereas in cultivated conditions, it ranges from 1.2% to 5.7% (Tınmaz et al., 2009).

Carvacrol and thymol are reported to be the main components of the essential oil in plants belonging to the genus *Origanum*, followed by components such as γ-terpinene, p-cymene, linalool, terpinen-4-ol, and sabinene hydrate (Azizi et al., 2009).

Istanbul oregano, used as a spice from its herb and leaves, and its oil is utilized in numerous industries. Oregano is known as a common spice in Mediterranean cuisine and stands out for its versatile uses. Particularly, its essential oil is used across various sectors due to its strong antioxidant, antibacterial, and antifungal properties. In the food and beverage industry, it is preferred for preserving products and keeping them fresh for a longer duration, while it also holds significant importance in the cleaning, cosmetics, and pharmaceutical fields. Chosen in complementary medicine, oregano is used as a nutritional supplement in aquaculture and as a nectar source in beekeeping, making it a highly functional plant. (Beltrán et al., 2018; Dutra et al., 2019; Guan et al., 2019).

Plant biostimulants are generally any stimulant substance (synthetic or natural) or microorganisms applied to plants in different forms and times with the aim of enhancing nutrient content, improving abiotic stress tolerance, and/or improving crop quality characteristics (Patrick 2015).

Zeatin, a member of the cytokinin biostimulant group, is a biostimulant that plays critical roles in plant growth and development, regulating various processes such as the separation of buds from the apical tip, expansion of leaves, formation of chloroplasts, slowing down the aging process, promoting the germination of seeds, and regulating the cell cycle (Havlicek et al., 1997; Mok and Mok 2001).

Kinetin, another member of the cytokinin biostimulant group, is reported to be a biostimulant that delays aging by affecting ethylene synthesis, contributes to growth and development by increasing cell division, and is effective in increasing chlorophyll synthesis (Toprak 2019). Gibberellic acid (GA), produced by plants, serves as a signaling molecule in processes such as germination, water uptake, initiation of flowering, fruit development, shoot elongation, and regulation of various metabolic events, working in conjunction with other phytobiostimulants responsible for these processes (Zhu et al., 2019; Khan et al., 2020).

This study has been conducted to determine the effects of foliar applications of zeatin, kinetin, and gibberellic acid biostimulants at different doses on the growth and biochemical parameters of the Istanbul oregano plant during the seedling development phase.

MATERIALS AND METHODS

Material

The study was conducted in the greenhouse of the Agricultural Sciences and Technologies Training, Application, and Research Center belonging to the Faculty of Agriculture, Sakarya University of Applied Sciences. The oregano seedlings used in the research were obtained from a commercial company.

Method

The experiment was conducted following a Completely Randomized Experimental Design with three replications. In the study, oregano seedlings known as "Istanbul oregano" were treated with biostimulants known to affect plant growth and development: zeatin (20, 40 mg/L), kinetin (50, 100 mg/L), and gibberellic acid (100, 200 mg/L). A total of 21 pots, each with a capacity of 2 liters, were used. These pots were filled with a homogeneous mixture prepared from finely sieved garden soil (3 parts) and Klassman TS1 brand peat (1 part). The ground in the greenhouse, where the study was to be conducted, was leveled with a rake and then rolled before placing the pots at intervals of 20 cm within rows and 30 cm between row space.

After placing the pots in the greenhouse, five randomly selected pots were each watered with 500 ml of water. Plates were then placed under the bottoms of the pots to collect the water that drained through. After the drainage process was complete, an average of 215 ml of water accumulated in the plates from each pot. The water retention capacity of the pots was calculated by subtracting the drained water from the 500 ml of water added to each pot.their water-holding capacities were measured to be 285 ml. Subsequently, the seedlings were planted in the pots at an approximate depth of 3 cm on November 1, 2023. During planting, the lower two leaves of the plants were removed by hand if they touched the soil to prevent fungal disease. Throughout the experiment, each pot was watered with approximately 100 ml of water once a week.

Kinetin and gibberellic acid hormones were dissolved in 96% ethanol, while zeatin hormone was dissolved in NaOH and completed to 1 liter with distilled water. The prepared biostimulant solutions were filled into 1-liter spray bottles, wrapped in aluminum foil to protect from light, and stored in a refrigerator. The initial foliar applications of the biostimulants were made on January 2, 2024, approximately two months after planting. Due to the experiment being conducted during the winter months, growth and development were slower compared to the summer months; therefore, the application of biostimulants was delayed. Foliar-applied biostimulants have been shown to produce effects such as improved leaf color, increased growth rate, and enhanced stress tolerance within a few days, as demonstrated in various studies on different plant species. For example, in a study by Khallouf et al. (2017), biostimulants were applied every two days and concluded on day 15. In another study by Shahzad et al. (2015), applications were made on days 5, 6, and 9, with observed positive effects. The trial lasted for an average of 2.5 months. During the period from the setup to the conclusion of the experiment, it was determined that the average daytime temperature was 15°C, while the nighttime temperature was 4°C (Anonymous 2024).

The roots of the plants were softened with water and separated from the soil. Subsequently, root lengths were measured and recorded with the help of a ruler. The fresh weights of the seedlings and roots were measured on a precision scale. The aerial parts and roots of the seedlings were placed in drying paper and then put in an oven at 35°C for 108 hours to dry. Afterwards, the dry weights of the seedlings and roots were measured.

Total Phenolic Content Analysis

The total phenolic content was assessed using the Folin–Ciocalteu method according to Waterhouse (2002). Initially, 250 µL of Folin–Ciocalteu reagent and 50 µL of the extract solution were added to a tube, with the total volume adjusted to 3 mL using distilled water. After a 5-minute incubation period, 750 µL of a 20% (w/v) Na_2CO_3 solution was added, and the tubes were mixed. The resulting solution was kept in the dark at room temperature for 90 minutes. The absorbance was then measured at 765 nm using a UV-Vis spectrophotometer (Agilent Cary-60, Santa Clara, CA, USA). A gallic acid standard curve was generated by repeating the procedure with concentrations of 50, 100, 150, 200, and 300 µg/mL. The total phenolic content was expressed as gallic acid equivalents using the standard curve (mg GAE/100 g of dry weight thyme).

Determination of FRAP Reducing Capacity

Initially, 0.3 M sodium acetate buffer (pH 3.6), 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution, 20 mM FeCl3, and 2 mM FeSO4 solutions were prepared. The working solution was obtained by mixing the buffer solution, TPTZ, and FeCl3 solutions in a 10:1:1 ratio. Absorbance measurements were taken at 593 nm using a 2 mM FeSO4 solution to create the standard curve, followed by measuring the samples at a minimum of three different concentrations. The results were reported as mg extract/µmol Fe2+ equivalents (Sachett et al. 2021).

Determination of CUPRAC Reducing Capacity

This method was based on a partially modified version of a previously reported procedure. Plant extracts were taken in different concentrations (10, 20, 40 µg) into tubes. Then, 0.25 mL of CuCl2 solution (0.01 M), 0.25 mL of ethanolic neocuproine solution, and 0.25 mL of CH3COONH4 buffer solution (1 M) were added. After incubating the mixtures in the dark for 30 minutes, absorbance values were measured at 450 nm against a blank (Ak and Gülçin 2008). The measurement results were evaluated by comparing them to trolox equivalents.

Photosynthetic Pigments (chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll a/chlorophyll b ratio, and total carotenoid content)

The analysis of photosynthetic pigments was conducted according to Lichtenthaler (1987). A 0.2 g (200 mg) fresh plant sample was extracted with 10 mL of 80% acetone and centrifuged at 4600 rpm for 15 minutes. The absorbance values of the aliquots taken after centrifugation were measured at wavelengths of 663, 645, and 470 nm using a spectrophotometer (PG T60 UV-VIS) and recorded. The calculations were made using the following formulas:

Chlorophyll a (µg g^{-1} FW) = 11.75 × A662 - 2.350 × A645
Chlorophyll b (µg g^{-1} FW) = 18.61 × A645 - 3.960 × A662
Total chlorophyll (µg g^{-1} FW) = chlorophyll a + chlorophyll b
Total carotenoid (µg g^{-1} FW) = (1000 × A470 - 2.270 × chlorophyll a) - (81.4 × chlorophyll b / 227)
where A is the absorbance value, and FW is the fresh weight.

Statistical Analysis

Statistical analyses of the data obtained were performed using the COSTAT (version 6.03) package program, and multiple comparison tests were performed according to the Least Significant Difference (LSD = 0.05) test.

RESULTS AND DISCUSSION

The effect of synthetic biostimulants on all growth parameters except root length in Istanbul oregano was found to be statistically significant at the 5% level. The highest value for seedling length was observed in the GA200 treatment with 12.16 cm, while the kinetin100 treatment yielded the best results for seedling fresh weight with 2.96 g, as seen in Table 1. In terms of root fresh and dry weights, the kinetin50 treatments produced higher results compared to other treatments, with 4.89 g and 0.70 g, respectively. For seedling dry weight, the control treatments performed relatively better with 0.72 g (Table 1).

Foliar applications of kinetin on *Ervatamia coronaria* plants increased the seedling fresh and dry weights, root fresh and dry weights, and root length compared to the control (Ashour et al. 2023). The kinetin biostimulant is reported to enhance chlorophyll content in plants, promoting the production of photosynthetic proteins, accelerating cell division, and breaking apical dominance in plants. This results in increased lateral branch and root formation, leading to increases in both fresh and dry weights of above-ground and below-ground parts (Lazar et al. 2003; Bielach et al. 2017).

Table 1. Effects of certain synthetic biostimulants on the growth parameters of İstanbul oregano plant

Biostimulants	Seedling length (cm)	Seedling fresh weight (g)	Root length (cm)	Root fresh weight (g)	Seedling dry weight (g)	Root dry weight (g)
Control	9.53 b	2.90 a	25.60	4.75 a	0.72 a	0.66 a
Zeatin20	8.47 b	2.30 ab	26.75	2.85 c	0.55 ab	0.43 bc
Zeatin40	8.33 b	2.78 a	28.75	3.73 b	0.61 a	0.50 b
Kinetin50	10.23 ab	2.71 a	29.00	4.89 a	0.62 a	0.70 a
Kinetin100	9.40 b	2.96 a	29.00	4.50 ab	0.66 a	0.65 a
GA100	10.00 ab	1.98 b	28.50	2.30 c	0.39 b	0.39 c
GA200	12.16 a	2.67 ab	29.00	4.47 ab	0.58 ab	0.67 ab
LSD(0.05)	2.48	0.73	ns	0.8	0.19	0.14
CV(%)	14.58	16.08	7.29	11.74	19.13	14.4

ns=not significant

Foliar applications of GA3 have been reported to increase the height and quality of *Araucaria heterophylla* plants (Gul et al. 2006), and spraying *Dahlia pinnata* plants with 100 or 200 ppm GA3 has been shown to enhance plant growth parameters (Yousef and Gomma 2008). Similar studies have demonstrated that GA3 foliar applications increase seedling length in *Hibiscus sabdariffa* L. (Alharby 2021), and wheat (Mirheidari et al. 2022), with comparable findings by Santos et al. (1998) and Srivastava and Srivastava (2007). Gibberellic acid applications in thyme have been shown to increase growth parameters, chlorophyll pigments, and essential oil content (Dadkhah et al. 2016), and gibberellic acid applications are reported to have positive physiological, morphological, and biochemical effects on plants (Taiz and Zeiger 2010). The increases in growth parameters due to gibberellic acid applications are attributed to the enhanced activity of enzymes such as carbonic anhydrase, nitrate reductase, and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) (Yuan and Xu 2001; Afroz et al. 2005; Aftab et al. 2010). Additionally, GA3 is reported to promote growth and development by stimulating cell growth and cell division (Taiz and Zeiger 2010). The findings of previous studies support our results.

Table 2. Effects of some synthetic biostimulants on chlorophyll pigments in İstanbul oregano plant

Biostimulants	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophylls (mg/g)
Control	98.69 b	116.13 bc	214.82 bc
Zeatin20	87.46 c	115.26 bc	202.71 cd
Zeatin40	112.55 a	130.51 a	243.06 a
Kinetin50	103.07 b	126.92 ab	229.99 ab
Kinetin100	84.46 c	101.93 d	186.40 d
Gibberellic acid100	84.42 c	103.03 cd	187.44 d
Gibberellic acid200	76.34 d	131.71 a	208.05 c
LSD (0.05)	7.47	13.11	19.77
CV (%)	4.61	6.35	5.36

When evaluating Table 2, it is evident that the applied hormones had a statistically significant effect on chlorophyll pigments at the 5% level. The highest values for chlorophyll a and total chlorophyll, were 112.55, 243.06, and mg/g, respectively, measured in the 40 ppm foliar applications of Zeatin hormone. For chlorophyll b pigment, the highest value was 131.71 mg/g, found in the 200 ppm applications of gibberellic acid.

Exogenous cytokinins have been reported to enhance growth, water status, chlorophyll accumulation, antioxidant status, stomatal opening, and the functioning of the photosynthetic apparatus in Ricinus communis plants exposed to copper (Cu) heavy metal (Sameena et al. 2021). Similarly, cytokinins such as Zeatin, kinetin, benzyladenine, and thidiazuron have been reported to increase biomass production, chlorophyll pigments, and carotenoid content (Yu et al. 2024). Zeatin hormone applications in wheat have been shown to increase chlorophyll pigments, carotenoids, and non-enzymatic antioxidant substances (Ali et al. 2022). Many studies indicate that applications of Zeatin and other cytokinin group hormones increase chlorophyll pigments and carotenoid content in various plants compared to the control (Emami et al. 2011; Faraji et al. 2011; Ali et al. 2023). Cytokinin group hormones, including Zeatin, are reported to directly or indirectly influence the production of chlorophyll and other plastid pigments by re-regulating the gene regions encoding these pigments (Cortleven and Schmülling 2015). Gibberellic acid applications have been shown to increase chlorophyll pigments in stevia plants (Modi et al. 2011), and foliar applications of GA3 hormone have been reported to increase chlorophyll pigments in *Ficus benjamina* L. and *Spathiphyllum wallisii Regel* plants compared to the control (Salehi et al. 2014; Rahbarian et al. 2014).

Table 3. Effects of some synthetic biostimulants on biochemical parameters of İstanbul oregano plant

Biostimulants	Total Phenolics (mg/g GAE)	Total Carotenoids (mg/g)	CUPRAC (mM/g TE)	FRAP (mM/g AAE)
Control	0.35 bc	0.81 ab	4.73 a	0.80 b
Zeatin20	0.35 bc	0.70 ab	5.05 a	0.78 b
Zeatin40	0.43 ab	0.93 a	5.48 a	1.24 a
Kinetin50	0.38 ab	0.83 ab	5.85 a	1.15 a
Kinetin100	0.49 a	0.65 b	6.36 a	1.15 a
Gibberellic acid100	0.40 ab	0.69 ab	5.71 a	1.16 a
Gibberellic acid200	0.25 c	0.65 b	3.01 b	1.20 a
LSD (0.05)	0.12	0.25	1.68	0.29
CV (%)	18.49	19.52	18.56	15.9

Table 3 shows that the hormones applied during the seedling development stage of Istanbul oregano have a statistically significant effect at the 5% level on antioxidant activities determined by the CUPRAC and FRAP methods, as well as on the total phenolic content and total carotenoid levels. According to the CUPRAC method, which measures free radical scavenging activity, the total phenolic content was higher in the Kinetin100 treatments with 6.36 mM/g TE and 0.49 mg/g GAE compared to other treatments. Similarly, the FRAP method, which determines antioxidant activity, showed relatively better results in total carotenoid levels with 1.24 mM/g AAE and 0.93 mg/g in the Zeatin40 treatments (Table 3).

Kinetin has been reported to increase the content of phenolic compounds such as trigonelline, caffeine, and the main chlorogenic acid, 5-caffeoylquinic acid, in coffee plants (Campa et al. 2012). The current study is consistent with several reports indicating that kinetin enhances the production of phenolic and alkaloid compounds in explants of various species under different abiotic stress conditions (Steinhart et al. 1964; Angelova et al. 2001; Siahpoush et al. 2011). While the mechanism through which exogenous kinetin affects the metabolic pathways of these compounds is not yet fully understood, it has been suggested that kinetin positively regulates relevant transcription factors (Barciszewski et al. 1999) and directly enhances the activities of phenolic and alkaloid biosynthesis enzymes (Steinhart et al. 1964; Angelova et al. 2001).

CONCLUSION

In this study, the effects of synthetic biostimulants on Istanbul oregano were investigated, revealing significant effects on all growth parameters except root length. GA200 application was found to be the most effective in increasing seedling length, kinetin100 in seedling fresh weight, and kinetin50 in root weights. These findings indicate that biostimulants positively affect plant growth. Hormone applications were found to have significant effects on the increase of chlorophyll pigments, suggesting that considering the use of Zeatin and gibberellic acid to optimize plant growth could be beneficial. For Istanbul oregano seedlings during the development stage, it is recommended to use kinetin100 applications to increase antioxidant activity and total phenolic content, while Zeatin40 applications may be preferred to maximize total carotenoid levels.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Declaration of Interests

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

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