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Influence of ABA and CaO Nanoparticles on Antioxidative Potential of Calli Derived from Two Einkorn (*Triticum monococcum*) Ecotypes

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Abstract

CaO nanoparticles (NPs) are important macromolecules that act as signal transducers in plants' growth and survival processe s. This study analyzed the responses of two different (Incedere and Kurekdere) *Triticum monococcum* landraces to various an tioxidant and physiological processes under tissue culture conditions. Two *T. monococcum* landraces were cultivated in 1-and 10-mM mannitol, consisting of 0.5 ppm and 1.5 ppm Cao NPs. CaO NPs significantly enhanced the activation of growth factor s in the two tested landraces. TOS, DPPH, CUPRAC, PPO, and GR were significant with Ca2+ NP application and demonstrat ed a high level associated with the tolerance degrees of the varieties. When the results of the total antioxidant test were analyz ed, it was detected that oxidant levels decreased significantly when CaO NPs were treated at increasing concentrations. While the antioxidant capacity of CaO NPs was limited at low concentrations (10-30 µg/mL) in the first 7 days, a high promotion wa s detected at higher concentrations (50 µg/mL). The activity increased in the second week, and the antioxidant effect continue d, especially in the 30 and 50 µg/mL groups. While a significant increase was observed in the results of wheat samples treated with CaO NPs in the 1st week, it was seen that the rise in copper ion-reducing activity became more balanced in the 2nd week. This trend shows that CaO NPs activate phenolic metabolism in short-term applications, but cellular regulation mechanisms c ome into play in the long term and balance the enzyme activity. The 2nd week's data show that GR activity reached a plateau l evel in certain dose groups. It is demonstrated that GR activity in the 50 µg/mL group did not change compared to the 1st wee k , but a slight decrease was observed in the 10 and 30 µg/mL applications. ABA and CaO NPs were observed to positively affe ct wheat development.

Keywords: *Triticum monococcum*, Callus, CaO NP, ABA, Callus

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Introduction

Einkorn (*Triticum monococcum* L. ssp. *monococcum*) is a diploid (2n = 2x = 14) primitive wheat and a close relative of durum (*Triticum turgidum* ssp. *durum*) and bread (*Triticum aestivum* ssp. *aestivum*) wheat. It originated in the Karacadag Mountains in Türkiye and was distributed to Europe during the Green Revolution (1). Due to its yields on poor soils, *T. monococcum* is still growing in cultivated lands as the essential cereal crop of many agricultural regions such as South Europe, Minor Asia, the Caucasus, and North Africa. Today, *T. monococcum* is one of the most critical grown food crops broadly cultivated in Türkiye, Balkan countries, southern Italy, southern France, Spain, and Morocco (2,3). *Triticum monococcum* is cultivated for its whole grain and enriched grain nutrition, folic acids, fiber, minerals, and vitamins. Various polymorphism structures, small genome sizes, and easy cultivation procedures are detected in *T. monococcum* (4). Extensive studies on modern wheat breeding programs have been started, followed by investigations on resistance to pests and diseases, tolerance to abiotic stresses, and functional foods. *Triticum monococcum* has been used extensively for the development of cultivars resistant to stress conditions (5). It exhibits high yield potential, grain quality, carotenoids, tocopherols, and phenolic acids (6). It is currently used in bread wheat production because einkorn possesses interesting nutritional traits, including low gluten content and minimal toxicity. Einkorn is known as a main source of micronutrients. The macro- and micronutrients and fibre contained in cereals and cereal-based products are important for the growth and development of plants; a deficiency of these elements can lead to reduced growth and development. Nanobiotechnology, as a biotechnology and agricultural field, has many novel applications, including nano-pesticide fertilizers, herbicides, or genes, improving seed sprouting, expansion, and plant conservation against environmental stresses. Nanotechnology has more comprehensive applications than biotechnology, such as gene transformation, genomics, proteomics, bioinformatics, and other technologies (7,8). Nanotechnologies can improve yield volume in less yielding crops, which contributes to sustainable agriculture. Furthermore, nanotechnology has assisted novel potential for improving the quality of foods, flavor, advanced protein content, and enhanced nutritional values. Nanoparticles have supported the development of yield productivity by introducing such qualities as biotic tolerance and increased environmental stress tolerance to the crops (9). Nanoparticles (NPs), including a class of metal-oxide (CaO, TiO2, and CuO—ZnO) with physicochemical properties, called nanomaterials (NM), have suggested new opportunities in developing plant growth to improve crop productivity. NPs have matchless physicochemical features and great potential as scaffolds for biomolecule interaction. With their high pertinence to in vitro applications, the use of NPs for maintaining and controlling callus development is a promising and worthy topic due to their increased effectiveness, resistance, progression, and, incredibly, their high specific surface area, which can induce interactions with living cells (10). Calcium (Ca^{2+}) is the primary plant nutrient, which is the central task of Ca^{2+} in plant expansion to ensure structural support to cell walls. Calcium is also well-known as a subsidiary precursor when plants are mechanically or biochemically injured. CaO is among the most favorable heterogeneous base catalysts due to its relatively high base sites and nontoxicity. CaO NPs can improve the plant's agronomic traits by eliminating oxidative stress (11). ABA is a significant phytohormone, and its functions and biosynthesis have been extensively studied at almost all enzymatic steps

through molecular-genetic, biochemical, and physiological approaches (12). Endogenous ABA levels significantly promote response to abiotic stresses, and they adjust some aspects of biochemical responses to a variety of biotic and abiotic stresses (13). Several researchers report that both the adaptation and survival of plant cells and tissues to various abiotic stress conditions may be improved by exogenous ABA (14). It is usually in vitro culture media to improve somatic embryogenesis and promote somatic embryo quality by improving dehydration tolerance and inhibiting precocious germination. ABA is also applied to induce somatic embryos to transform a stable phase in in vitro culture mediums and during synthetic seed studies (15). Recently, many studies have been published on the act of in vitro culture on enviromental e stress and the improvement of stress-resistance plants through in vitro selection (16,17). However, the effects of CaO NPs and ABA on the in vitro culture of einkorn wheat are currently not well known. This study aimed to determine the effects of ABA+CaO NPs treatments on oxidative stress in callus tissues of two landraces of einkorn.

Materials and Methods

Plant Material and Callus Induction: In our study, two ecotypes (İncedere and Kürekdere) of einkorn (Triticum monococcum) were used as the material for the response to CaO NPs and ABA applications. The mature seeds were disinfected with 1% sodium hypochlorite for 5 min, washed three times with ddH2O, and incubated with autoclaved water overnight at 4 °C. Mature embryos, aseptically obtained from swelling seeds and placed with the scutellum side up, were cultured in vitro on hormone-free MS medium (18). The explants were in total darkness for a total of one month at 25 ± 1 °C. After one month, callus induction appeared and was used for enzyme activity studies.

CaO NPs and ABA Treatments: The calli were transferred onto the callus development medium, including MS basal medium (pH 5.7), and 0.8 % agar was added with 2 mg/L of 2,4-D for 1 week in a growth cabinet at 28 °C, under a 16/8-h photoperiod condition. The calli were transferred to two distinct media containing CaO NPs and ABA, with treatments applied during the first and second weeks, in response to the CaO NPs+ABA treatments. In the first week, compact callus was transferred on hormone MS medium (18) 4 mg L–1 2,4-D (2,4-dichlorophenoxyacetic acid) in the presence of 1 and 10 mm ABA, consisting of 0.5 ppm and 1.5 ppm CaO NPs. For the second week, the callus was transferred to hormone-free MS medium (18) 4 mg L–1 2,4-D in the presence of 1 and 10 mm ABA, consisting of 0.5 ppm and 1.5 ppm CaO NPs for two weeks.

Determination of DPPH● Free Radical Removal Activity: DPPH● free radical removal activities of callus samples were performed according to a modification of the Blois method (19). 1 mM DPPH● solution was used as a free radical. Callus was transferred to the test tubes successively, and their total volume was complemented to 3 mL with distilled water. Then, 1 mL of DPPH● solution was added to each sample. After incubating for 30 minutes in the dark at room temperature, the absorbance changes at 517 nm were measured against the blank sample formed from ethanol. The control sample was prepared using 3 mL of ethanol and 1 mL of DPPH● solution. Reduced absorbance gives the remaining amount of DPPH● solution, i.e., free radical removal activity. BHA (Butylated Hydroxyanisole) and α-tocopherol were used as standard antioxidant compounds. DPPH● free radical calculations were made according to the following equation.

DPPH Free Radical Removal Activity (%) = (1- (A numune)/Akontrol)x 100

A sample refers to the absorbance value found after adding the sample to the DPPH● radical solution, and a control refers to the absorbance value of the control sample containing only the DPPH● radical solution. BHA and α-tocopherol were used as positive controls.

Determination of Cupric Reducing Antioxidant Capacity: Cupric Reducing Antioxidant Capacity (CUPRAC) analyses of callus samples were carried out by a modified procedure of the CUPRAC method, developed by (20). Callus samples were transferred to the test tubes, then 0.25 mL 0.01 M copper (II) chloride ($CuCl_2$) solution and 0.25 mL 7.5 x 10–3 M ethanolic neocuprin solution were added successively. Then, 0.25 mL of 1 M ammonium acetate buffer solution was added. Subsequently, after incubation for 30 minutes at room temperature, the absorbance change was read using the Epoch ™ Microplate UV-Vis Spectrophotometer at 450 nm against the blend of distilled water. For the control, distilled water was used instead of the sample. The increased absorbance level shows more copper reduction capacity.

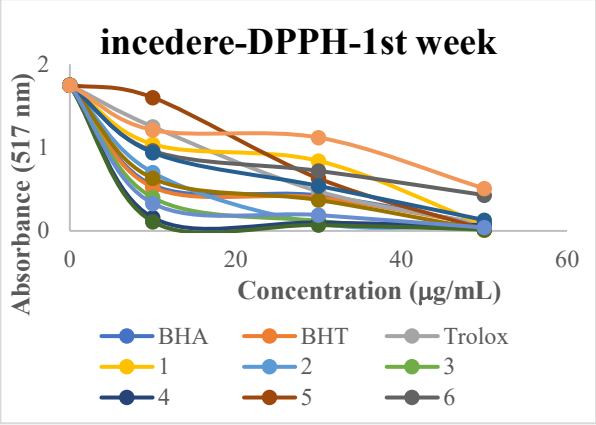
Determination of PPO Activity: To determine PPO activity, 0.5 g frozen leaves were ground to a powder under liquid nitrogen. The frozen plant powder was added to the extraction solution (0.05 M sodium phosphate containing 5 mM ascorbic acid and 1% (w/v) polyvinyl pyrrolidone at pH: 6.5). The suspension was centrifuged at 20000 x g for 15 min at 4 °C. The supernatants were filtered through Whatman No: 4 filter paper and assayed for the enzymatic activity. PPO activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (UV-1208 Shimadzu JAPAN). Then, 50 µl of crude extract was added to a 3 mL substrate mixture containing 0.20 M sodium phosphate buffer (pH: 6.5), 25 mM catechol. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme that can cause an increase in absorbance of 0.001/minute (21,22).

Determination of Glutathione Reductase Activity: Glutathione reductase (GR) (EC 1.8.1.7)) activity determination is based on monitoring the oxidation of NADPH at 340 nm. Activity measurement was performed by measuring the change at 340 nm for 3 min in 1 mL of a mixture containing 50 mM potassium phosphate (pH=7) buffer, 2 mM Na2 EDTA, 0.15 mM NADPH, 0.5 mM GSSG, and 100 µL of enzyme extract (234,2).

Statistical Analysis: Each experiment was repeated three times. Analysis of variance was conducted using a one-way ANOVA test using SPSS 21.00, and means were compared by the Duncan test at the 0.05 confidence level.

Result

DPPH• Free Radical Scavenging Activity Findings: The DPPH radical scavenging activity of CaO NPs was compared with the standard antioxidants BHA (Butylated Hydroxyanisole), BHT (Butylated Hydroxytoluene), and Trolox. While the antioxidant capacity of CaO NPs was limited at low concentrations (10-30 µg/mL) in the first round, a significant increase was observed at higher concentrations (50 µg/mL). In the second round, the activity increased, and the antioxidant effect continued, especially in the 30 and 50 µg/mL groups (Figures 1,2).



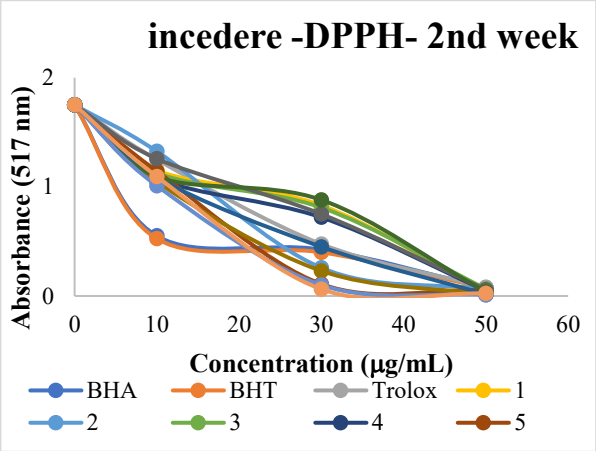
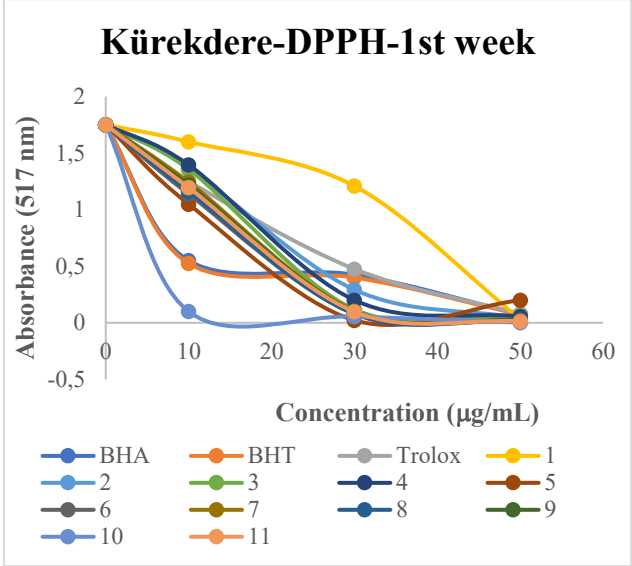


Figure 1. Comparison of DPPH radical scavenging activity of wheat samples treated with CaO NPs and ABA at various concentrations (10-50 µg/mL) with standard antioxidants BHA, BHT, and trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs



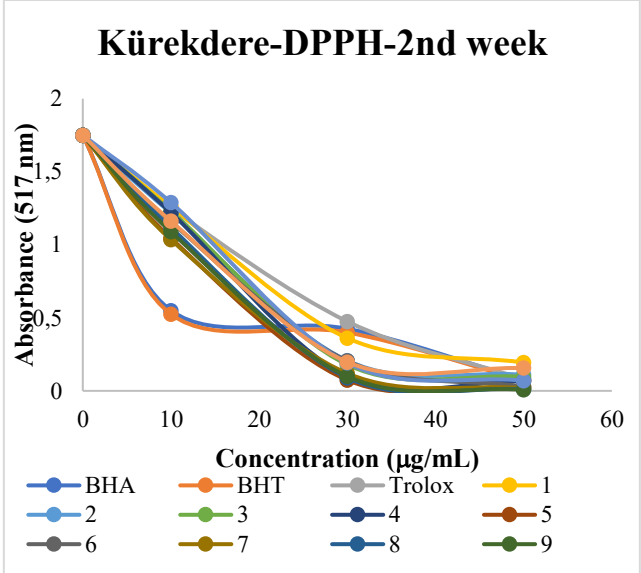
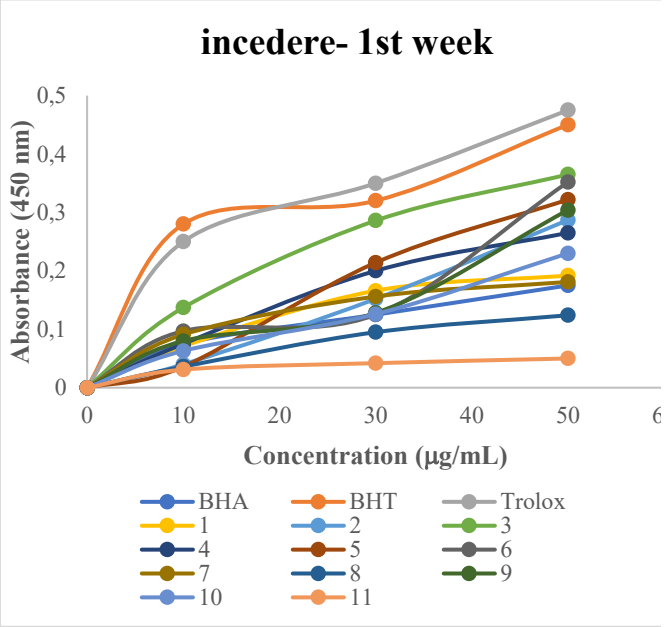


Figure 2. DPPH radical scavenging activity of wheat callus samples treated with CaO NPs at various concentrations (10-50 µg/mL). Trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

CUPRAC findings: The copper ion (Cu^{2+}) reduction capacities of wheat callus samples treated with CaO NPs, determined by the CUPRAC method, were analyzed in comparison with standard antioxidants (BHA, BHT, Trolox). While a significant increase was observed in the results of wheat callus samples treated with CaO NPs during the first week, the enhancement in copper reduction activity appeared to stabilize and

become more balanced by the second week.This situation shows NPs can lead to different biochemical interactions in plant tissues over time (Figure 3, 4).



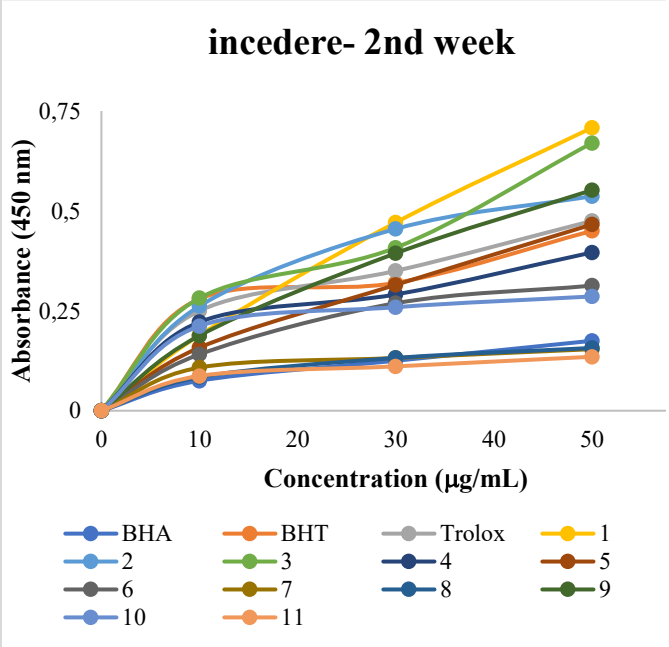
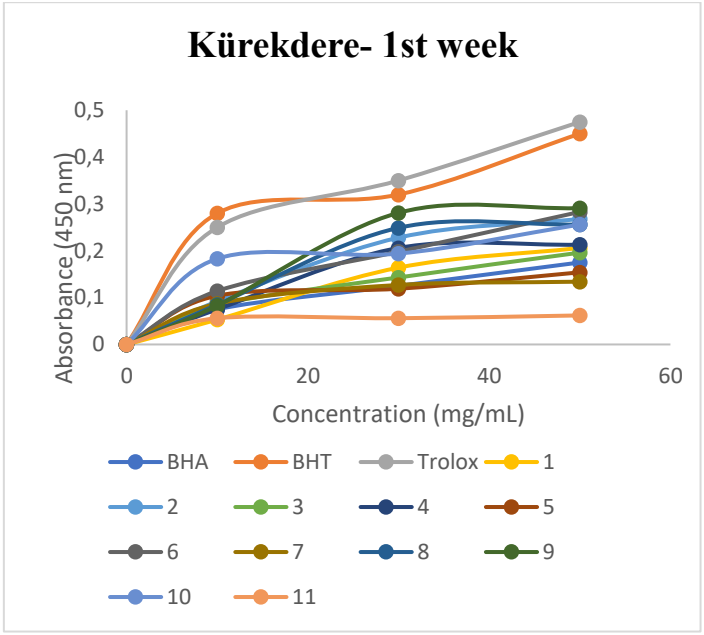


Figure 3 cupric (Cu^{2+}) reducing activities of wheat samples treated with CaO NPs at various concentrations (10-50 µg/mL). Trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs



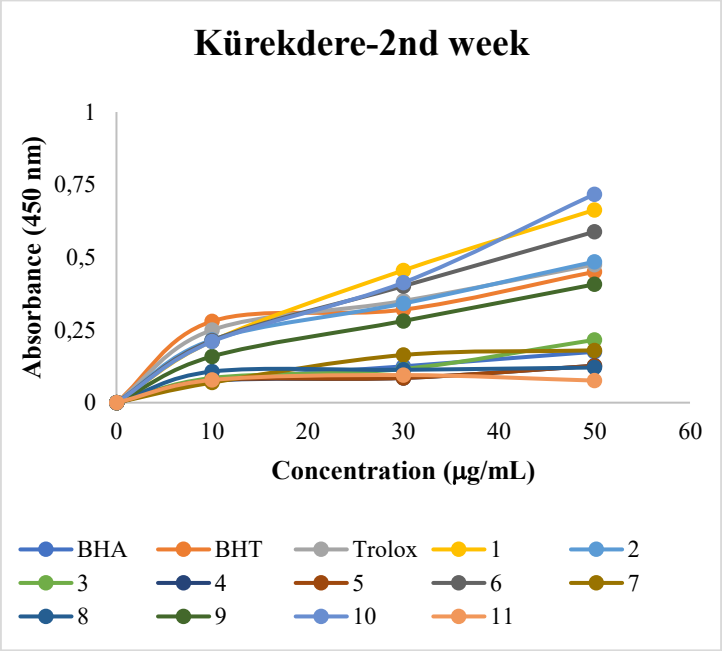


Figure 4. Cupric ion (Cu^{2+}) reducing activities of wheat samples treated with CaO NPs at various concentrations (10-50 µg/mL). Trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

PPO Activity Findings: Polyphenol oxidase (PPO) is an enzyme that plays a role in plant defense mechanisms through the oxidation of phenolic compounds. Changes in PPO activity were analyzed to evaluate the effects of CaO NP applications on phenolic metabolism. The first-week results showed that CaO NPs treatment caused a dose-dependent increase in PPO activity ($p < 0.01$). It was determined that PPO activity increased by 42.8% in the 50 µg/mL CaO NPs applied group compared to the control group. The second week's data revealed that PPO activity remained stable in the high-concentration groups (30 and 50 µg/mL) compared to the first week ($p > 0.05$). Still, a slight decrease was experienced in the 10 µg/mL application ($p < 0.05$). This trend shows that CaO NPs activate phenolic metabolism in short-term applications, but cellular regulation mechanisms come into play in the long term and balance the enzyme activity (Figure 5).

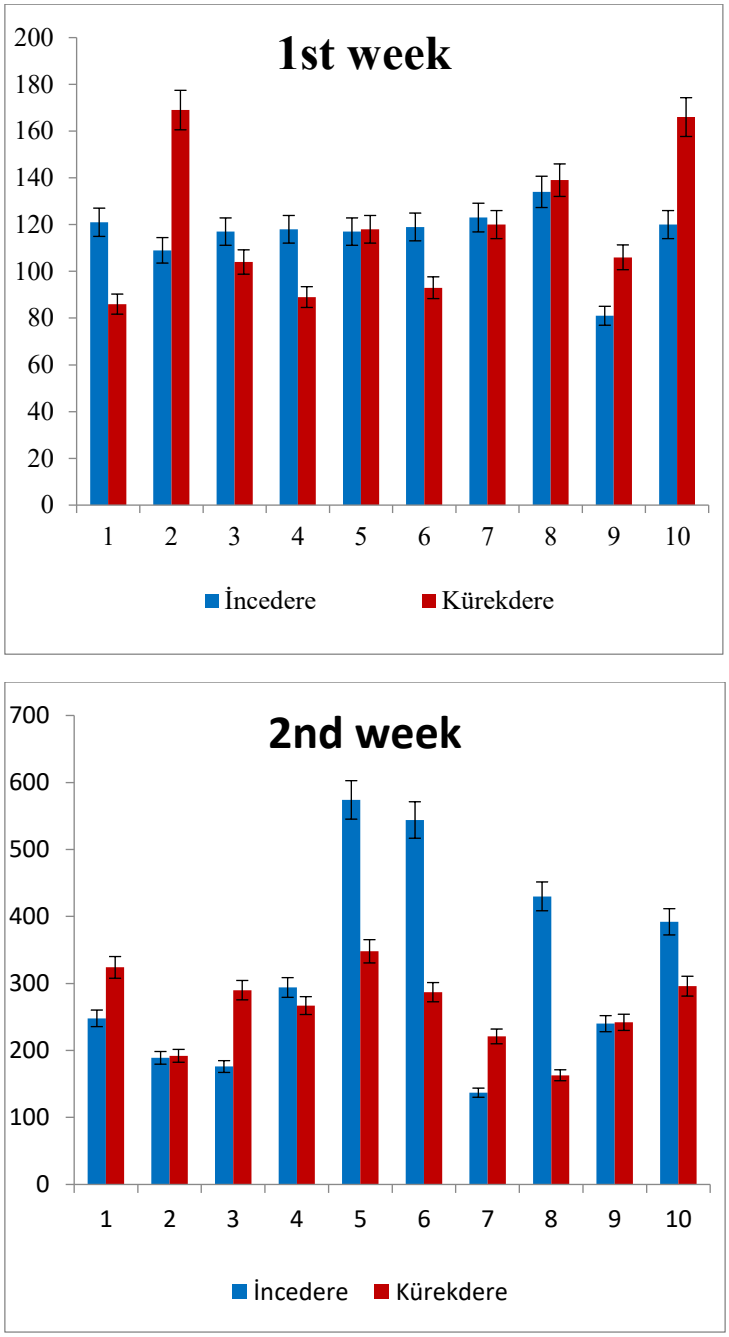


Figure 5. PPO enzyme activity in the 1st and 2nd weeks of Incedere and Kürekdere. 1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

Glutathione Reductase Activity Findings: Glutathione reductase (GR) is an antioxidant enzyme that is critical in maintaining cellular redox homeostasis. Our study determined that CaO NPs application in the 1st week caused a significant increase

in GR activity ($p < 0.05$). In particular, it was determined that GR activity increased by 24.6% and 38.2% in 30 and 50 µg/mL CaO NPs applications, respectively, compared to the control group. The 2nd-week data show that GR activity reached a plateau in certain dose groups; GR activity in the 50 µg/mL group did not change compared to the first week ($p > 0.05$), but a slight decrease was observed in 10 and 30 µg/mL applications ($p < 0.05$) (Figure 6).

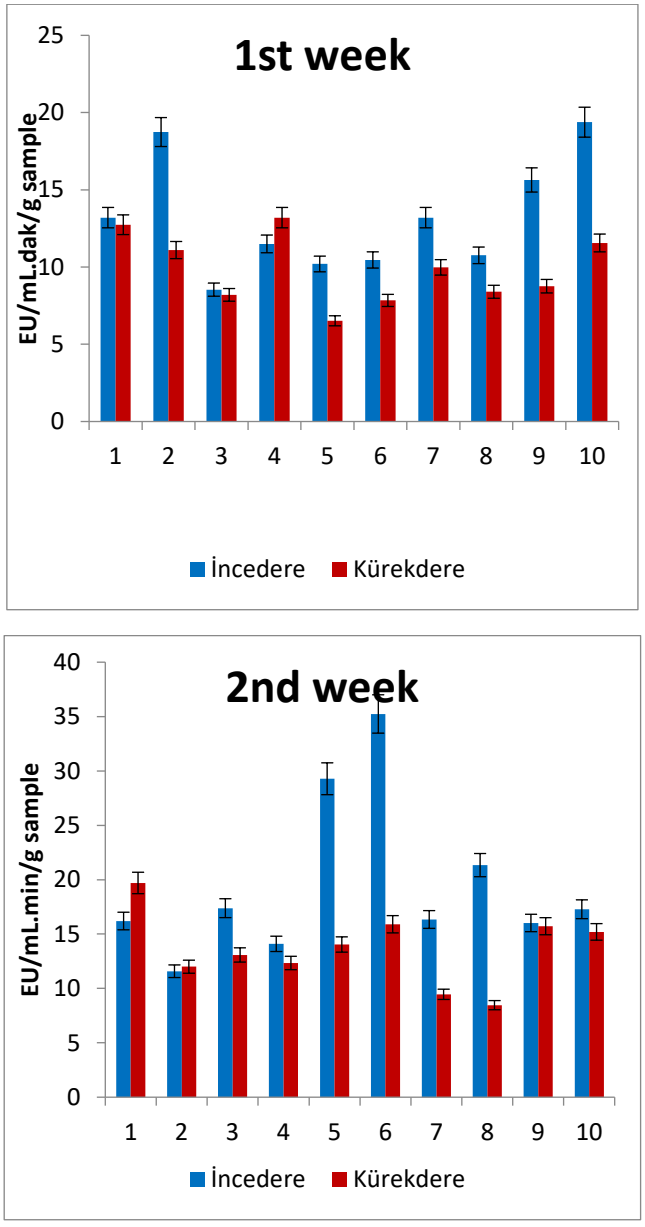


Figure 6. Effect of CaO NPs and ABA applications Glutathione reductase enzyme activity. 1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

Discussion

Nanoparticles (NPs), either alone or together with hormones, have been applied to several plant varieties and were detected in cells containing predominantly salicylic acid and ABA for improving growth and development in vitro cell culture. Upon the type and concentrations, the synergistic influence of NPs with plant tissues affects many physiological and biochemical changes during the cellular growth process (25). In this study, treatments of CaO NPs+ABA greatly influenced the callus improvement and the process of precious antioxidant compounds. CaO NPs+ABA at two doses was implemented in vitro on callus developed and maintained in the MS media in composition with 4 mg L–1 2,4-D (2,4-dichlorophenoxyacetic acid), including 0.5, 1.5 ppm CaO NPs nanoparticulate and 1 and 10 mM ABA. Based on the CUPRAC rates, samples of first-week cultured calli in the presence of CaO NPs enhanced hormone response and greatly promoted CaO NPs markers as compared to control callus, and there was an upwards in Cuprac amount that was collected in the presence of CaO NPs. Conversely, samples of second-week cultured callus cells in the presence of Ca2+ NPs have a more stable hormone response, without changing CUPRAC rate, as compared to the untreated callus (Figure 3, 4). This event was considered to be a positive feedback treatment through signal transduction. The feedback organizing results between CUPRAC and Ca2+ NPs have been reported on adaptation and developmental conditions in various plant species (12). These findings agree with those published by (26) without nanoparticle treatments in the study on Saponaria prostrata plant extract by two different in vitro bioanalytical methods, including CUPRAC and FRAP. Our study demonstrated that under CaO NPs conditions, the DPPH content declined by a larger level than in the untreated callus in both the İncedere and Kürekdere ecotypes of *T. monococcum* (Figure 1, 2).

The positive impacts of CaO NPs in ABA presence were deficient at 0.5 ppm than at 1.5 ppm concentration. Bursal et al. (2022) (27) found a parallel result for DPPH decreased in water extract compared to the control set of S. prostrata. However, their results indicated considerable free radicals scavenging activity when compared to the standards used. (28) found similar results in garlic extract with different solvents. PPO activity is closely associated with the synthesis and degradation of metabolites in plants. Callus types are essential factors affecting PPO activity (29). Both ecotypes were quickly observed for better PPO activities with Ca2+ NPs concentrations of 1 ppm and 10 ppm. Conversely, the same ecotypes responded with more stable PPO activity at 1.5 ppm CaO NPs 1- 10 ppm in a long time. Our results indicated that PPO activity varied remarkably depending on the CaO NPs+ABA degrees and period of treatments but independent of genotype (Figure 5). The first-week treatments showed a significant increase in GR activity, primarily due to the enhanced effect of CaO NPs, whereas in the second week, the activity slightly decreased (Figure 6). In the current study, the antioxidant enzyme GR activity is the lowest on the premier concentration of ABA 10 mM, presumably due to deactivation. These results suggest that the duration of treatment and GR activity may be regulated differently. These outcomes agree with those published by (30) in the study on Mung bean germinating seedlings fluoride stress at different concentrations for 5 days.

Conclusion

In this study, the synergistic effects of CaO NPs and ABA on callus development and antioxidant responses in *Triticum monococcum* ecotypes were evaluated under *in vitro* conditions. The combined application of CaO NPs and ABA significantly enhanced callus improvement and modulated key antioxidant parameters such as TOS, CUPRAC, DPPH, and PPO activities. Notably, CaO NPs treatments effectively reduced oxidative stress induced by ABA, especially at higher concentrations, suggesting their potential role in stress mitigation and cellular adaptation. The most pronounced changes were observed during the first week of treatment, where CaO NPs contributed to a heightened antioxidant enzyme response, particularly GR activity. However, this effect diminished over time, highlighting the importance of treatment duration. The observed decrease in DPPH content and modulation of PPO activity suggest that nanoparticle concentration and treatment period play critical roles in determining physiological responses, independent of genotype. Overall, these findings emphasize the promising role of CaO NPs-particularly in combination with ABA-as bioactive agents for enhancing stress resilience and promoting callus development in cereal crops.

Declaration of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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