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Research Article

In vitro ZnO Nanoparticles Enhanced Pea (*Pisum sativum* L.) Seedlings Growth

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Abstract

Zinc is a minor micronutrient that is also involved in carbohydrate, protein synthesis metabolisms. The present study was carried out to analyze in response to DNSA, proline, protein and MDA (Malondialdehit) responses in the form of zinc oxide nanoparticles (ZnO NPs) in *Pisum sativum*, for a period of 21st and 35th days. Two *P. sativum* (Maro Tarım and Kars) were used as the material in the presence of 0.8 ppm and 1.8 ppm ZnO nanoparticulate. The length and biomass of plants increased significantly upon ZnO NPs application. The activation of shoot and root length in two tested ecotypes was remarkably increased by ZnO. Accumulation of Zn increasedin presence of 0.8 ppm Zn^+ nanoparticle in *P. sativum*, which lower concentration more affected than higher concentration in terms of growth parameters. The amount of protein showed an increase, while those of DNSA and proline response to ZnO NPs in the higher concentration. However, there were significant differences between control and ZnO treatments in response to DNSA and proline. Malondialdehyde content displayed a gradual increase in leaf samples of *P. sativum* plants. The results suggest that lower application of ZnO NPs (0.8 ppm) could be promoted to the development process of plants and can be stimulated as a Zn regulator factor for crop physiological mechanisms.

Key words : *Pisum sativum*, *in vitro* culture, growth parameters, ZnO NPs

*In vitro*da ZnO Nanopartikülleriyle Geliştirilmiş Bezelye (*Pisum sativum* L.) Fidelerinin Büyümesi

Özet

Çinko, karbonhidrat, protein sentezi metabolizmalarında da yer alan mikro elementtir. Bu çalışma, *Pisum sativum*da 21 ve 35 gün boyunca çinko oksit nanoparçacıkların uygulanması sonucu (ZnO NP'ler) DNSA, prolin, protein ve MDA tepkilerini analiz etmek amacıyla yapılmıştır. Materyal olarak iki *P. sativum* ekotipine (Maro Tarım ve Kars) 0.8 ppm ve 1.8 ppm ZnO nanopartikül muamele edilmiştir. ZnO NP'lerin uygulanmasıyla bitkilerin uzunluğu ve biyokütlesi önemli ölçüde arttı. Test edilen iki ekotipte sürgün ve kök uzunluğunun aktivasyonu, ZnO ile önemli ölçüde arttırılmıştır. Büyüme parametreleri açısından daha düşük konsantrasyon yüksek konsantrasyondan daha fazla etkilenen *P. sativum*'da 0.8 ppm Zn^+ nanoparçacık varlığında Zn birikimi arttı. Total protein , prolin ve DNSA miktarı ZnO nanopartiküllerindeki konsantrasyonun artışıyla doğru orantı göstermiştir. Bununla birlikte, DNSA ve proline yanıt olarak kontrol ve ZnO muamelesi arasında önemli farklılıklar vardı. MDA içeriği, *P. sativum* bitkilerinin yaprak örneklerinde kademeli bir artış göstermiştir. Sonuçlar, daha düşük ZnO NP'lerin (0.8 ppm) uygulanmasının bitkilerin gelişim sürecine desteklenebileceğini ve baklagilfizyolojik mekanizmaları için bir Zn düzenleyici faktör olarak uyarılabileceğini göstermektedir.

Anahtar kelimeler : *Pisum sativum*, *in vitro* kültür, büyüme parametreleri, ZnO nanopartiküller

Introduction

Nanotechnology has affected each field of science and technology, of which plant biotechnology is an important part of these fields. Nanoparticles have many important agronomic functions in a large number plant species, where it appears to contribute to a developing plant's life cycle (Yazıcılar et al. 2021). It provides new chances and reduces the application of fertilizers and chemicals, which ensures environmentally friendly sustainable production. They can supply more agricultural production to be achieved in this way (Duhan et al. 2017, Sanzari et al. 2019). Several reports have been carried out on exogenous treatments of nanoparticles for the crop cultivation and maintenance processes, its impact on seedling regeneration and development in vitro is limited compared to exogenous treatments (Dimpka et al. 2019, Cunningham et al. 2018). ZnO is an essential trace element for plant systems which affects induction of seed germination, seedling development, defense mechanisms and pathogen inhibition as well as antioxidant and antimicrobial activity. Moreover, it also may function as a main component of various metabolic pathways and stimulate the metabolism of enzyme systems. It has further been reported that alterations in the endogenous levels of Zn^{+2} involve proteins and the synthesis of the nucleic acids (Rajput et al. 2021). Zinc oxide NPs have potential to improve the yield and development of food crops due to its superior properties, such as high specific surface area and small sizes, and fast response to block a large compass of pathogenic agents (Wang et al. 2016, Naseer, 2020, Faizan et al. 2020). But nowadays, the in vitro activity of ZnO NPs is still scarcely known. In vitro culture strategies are particularly useful in all fields of plant science because these strategies can support the agronomic enhancement of plants by reducing difficulty in exogenous treatments under field conditions (Bezirganoglu, 2017). The *P. sativum* is one of the most economically growth forage legumes commonly grown in the cool-season regions. It has been a significant grain forage plant for livestock, silage and in soil richness green manure, seeds indicating domesticated properties dating from almost 7000 years ago have been emerged in a historical sites nearby Turkey. Peas are cultivated for its high yield and richness in nutritions, organic acids, minerals, and vitamins. Various morphological structures, golden yellow, purple, and pod shapes are found in pea (Bezirganoglu et al. 2018). The goal of current study was to observe the seedlings growth and enzyme activities for pea by investigating different ZnO concentrations in vitro callus medium.

Materials and Method

Plant Material and ZnO Treatments

In this study, two *P. sativum* ecotypes (Kars, and Maro Tarım) were used as the material in response to ZnO NPs. The mature seeds were dis-infected with 1% NaOCl for 5 min, washed several times with sterile autoclaved water and rinsed with several changes of autoclaved water 12 hours at 4 °C. Then, mature seeds were incubated in plates including full MS medium Murashige and Skoog (1962) from four different ZnO NPs concentrations, such as in presence of 0.8 and 1.8 ppm and in absence of 0.8 and 1.8 ppm ZnO, medium in terms of leaves ZnO NPs 21st and 35th Days.

Proline Estimation

Proline amount was determined with the protocol of Bates et al. (1973). Seedling samples (100 mg) was powdered in 5 mL of 3% aqueous sulfosalicylic acid and centrifuged at 4 °C for 15 min at 4800 × g. Extract (2 mL) was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in test tubes. Samples were boiled for 1 h at 100 °C. The reaction was terminated in an ice bath and 4 mL of toluene was used for the reaction of the mixture extraction. The absorbance of color reaction product was measured at 520 nm using toluene as a blank. The proline concentration was determined from a calibration curve.

Soluble Sugar Determination

100 mg of leaf samples were powdered with 5mL of 2.5N HCl at cold. It was centrifuged at 9000 rpm for 10 minutes. The pellet part was discarded and 2 mL of supernatant was obtained and placed to the glass tube and 2 mL of DNSA (3,5 dinitrosalicylic acid) was added. It was incubated in a 90 ◦C water bath for 20 minutes. It was kept in the ice bath until it cools. For each sample, 100 µL per well was added in triplicate to 96 well plate. Measurements were made at 550 nm at the NanoDrop.

MDA (Malondialdehyde)

Malondialdehyde was measured following the protocol of Heath and Packer (1968) using liquid nitrogen. 0.4 grams of powder leaf sample was separated in 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) tricholoroacetic acid. The sample was kept at 98 °C for 30 min. and then immediately obtained into an ice bath. The pattern substance was centrifuged at 3000 ×g for 10 min. and the content of the supernatant was detected at 532 and 600 nm (Jaleel et al. 2007, Erdal, 2012).

Total Protein Analysis

P. sativum leaf samples (0.2 g) were homogenized to powder in a mortar using liquid nitrogen. Sample buffer [0.1 M $NaPO_4$ (pH 6.5), 1 M EDTA, 0.5 mM PMSF] was prepared to the eppendorf tube and mixed by vortex. The extracts were centrifuged at 13000 rpm for 10 min at 4 °C. The concentration of sample was performed by employing the method of Bradford (1976), and 30 g of the protein were separated in 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (Laemmli, 1970).

Statistical Analysis

Experiment was conducted three replications. ANOVA test was performed by SPSS 25.0 and means were evaluated by Duncan test at the 0.05 significant degree.

Results and Discussion

The differences among the ZnO treatments were detected to be significant on root and shoot length for Maro Tarım. The highest root length was 0.8 ppm of ZnO^+ and 1.8 ppm of ZnO^- treatment, the lowest root and shoot growth were obtained from standart ZnO^- treatments (Fig.1 b,c,a). The impact of the applications on shoot and root growth in Kars was also detected to be significant. The highest root length was standart ZnO^- and 0.8 ppm of ZnO^+ treatment (Fig.2 a,c), the lowest root length was 0.8 ppm of ZnO^- shoot growth were obtained from 1.8 ppm of ZnO^+ treatments (Fig.2 b,d).

Figure 1. Effect of ZnO NPs on Roots and Leaf Growth of *P. sativum* Ecotypes a: MS Zn^-, b: 0.8 ZnO^+, c: 1.8 ZnO^-

Figure 2. Effect of ZnO NPs on Roots and Leaf Growth of *P. sativum* Ecotypes a: MS Zn^- , b:0.8 ZnO^-, c: 0.8 ZnO^+ , d: 1.8 ZnO^+

Table 1 greatly displays that proline contents were remarkably influenced in seedlings stage of two pea ecotypes for 0.8 ppm and 1.8 ppm of ZnO NPs treatments. Proline content revealed significantly variation between pea samples for ZnO applications, ranging from 0.020 to 0.150 nmol g^{-1} FW in 21st days of Kars ecotype. The maximum content was obtained from seedlings treated of standart ZnO^- NPs in 21st days in Kars ecotype. The Maro Tarım in 21st days revealed the best result in standart ZnO^+ NPs for proline content compared to the other concentrations. Though the highest proline content was detected in the treatments of standard ZnO^+ in seedlings, the lowest proline content was found in seedling for 1.8 ZnO^- and 1.8 ppm of ZnO^+ NPs (Table 1).

Table 1. Change in proline ratios at 6 diferent medium concentrations

Treatment (ppm)	**Proline (nmol g^{-1} FW)**			
	Maro (21st Day)/Kars (21st Day)		**Maro (35th Day)/Kars(35th Day)**	
MS Zn^+	0,071±0,010[a]	0,079±0,010[b]	0,018±0,010[b]	0,019±0,010[c]
Zn^+ 0,8	0,061±0,012[a]	0,020±0,010[d]	0,021±0,010[b]	0,018±0,012[b]
Zn^+ 1,8	0,030±0,011[b]	0,020±0,009[d]	0,019±0,010[b]	0,023±0,005[a]
MS Zn^-	0,060±0,009[a]	0,150±0,008[a]	0,014±0,004[b]	0,029±0,010[c]
Zn^- 0,8	0,061±0,011[a]	0,074±0,004[b]	0,058±0,012[a]	0,019±0,010[a]
Zn^- 1,8	0,030±0,011[a]	0,041±0,012[c]	0,019±0,011[b]	0,014±0,005[c]

a–cMeans in the same column with diferent superscript letters difer signifcantly (*P≤0.05*)

The MDA content of pea ecotypes was applications to increase gradually in presence of ZnO NPs. All dosages of ZnO were detected to induce gradual promotes in MDA value and the highest MDA value was found in Maro tarım ecotype in 21st days exposed to standart ZnO^- (Table 2). Although there were significant differences between ZnO^+ and ZnO^- in Maro Tarım ecotype. There were no differences between ZnO^+ and ZnO^- in Kars ecotype. It was detected that the value of MDA in seedlings was greatly linked to ZnO concentration and genotype in tissue culture conditions and the lowest MDA level was found with 1.8 ppm ZnO^- in 35 days.

ZnO treatments caused different effects on the DNSA content. There was a detectable difference among cultivars and concentrations (Table 3). DNSA content revealed significantly variation between pea samples for ZnO supplies, ranging from 0,932 to 1,867 nmol g^{-1} FW in 21st days. The DNSA content in Maro tarım was higher than that of the other concentrations under ZnO^- 0.8 ppm treatments, which peaked at 1.867 nmol g^{-1} FW in 21st days. The highest value was obtained (1,608 nmol g^{-1} FW) from seedlings treated of 0.8 ppm ZnO^+ NPs in 21st days in Kars ecotype. There was also a detectable difference of DNSA in control and ZnO treatments between 1,941 and 2,632 g^{-1} FW for the 35th day. The DNSA content in Maro tarım was higher than that of the other concentrations under standard ZnO^+ treatments, which peaked at 2,402 nmol g^{-1} FW in 35th days. The highest value was obtained (2,632 nmol g^{-1} FW) from seedlings treated of 1.8 ppm ZnO^+ NPs in 35 days in Kars ecotype.

SDS PAGE analysis displayed that expected protein bands are easily detectable in the Kars and Maro Tarım ecotypes. However, no accumulation of protein bands was detected in the Maro Tarım ecotype "0.8 ppm Zn^+" and Kars 0.8 ppm Zn^- in 35th days (Fig. 3b and 3d). Comparison of protein accumulation in ZnO treatments, in Kars ecotypes, displayed that protein bands accumulation was higher after treatments with ZnO^+; Similarly, Maro Tarım displayed higher protein bands accumulation after ZnO^+ treatments. According to the result of protein bands intensity, 0.8 ppm and 1.8 ppm ZnO^+ in Kars 35th days exhibited the most abundant protein bands, followed by standart Zn^+ Maro Tarım 21st and 35th days

Table 2. Change in MDA ratios at 6 diferent medium concentrations

Treatment (ppm)	**MDA (nmol g^{-1} FW)**			
	Maro Tarım (21st Day)/Kars (21st Day)		**Maro Tarım(35th Day)/Kars(35th Day)**	
MS Zn^+	0,058±0,010[a]	0,028±0,012[b]	0,043±0,002[a]	0,032±0,001[b]
Zn^+ 0,8	0,049±0,004[a]	0,029±0,006[b]	0,041±0,005[a]	0,027±0,003[bc]
Zn^+ 1,8	0,022±0,003[b]	0,033±0,004[b]	0,043±0,003[a]	0,030±0,002[bc]
MS Zn^-	0,066±0,003[a]	0,027±0,001[b]	0,033±0,001[b]	0,033±0,005[ab]
Zn^- 0,8	0,049±0,01032[a]	0,031±0,006[b]	0,015±0,001[c]	0,039±0,009[a]
Zn^- 1,8	0,065±0,006[a]	0,044±0,001[a]	0,044±0,003[a]	0,025±0,005[c]

a–cMeans in the same column with diferent superscript letters difer signifcantly (*P≤0.05*)

Table 3. Change in DNSA ratios at 6 diferent medium concentrations

Treatment (ppm)	**DNSA (nmol g^{-1} FW)**			
	Maro Tarım (21st Day)/Kars (21st Day)		**Maro Tarım(35th Day)/Kars(35th Day)**	
Sdt Zn^+	1,744±0,187[ab]	1,316±0,138[ab]	2,402±0,156[a]	2,403±0,024[b]
Zn^+ 0,8	1,732±0,074[ab]	1,608±0,223[a]	2,309±0,056[a]	2,236±0,006[c]
Zn^+ 1,8	1,528±0,144[ab]	0,932±0,353[bc]	2,059±0,075[b]	2,632±0,022[a]
Std Zn^-	1,437±0,344[b]	1,566±0,420[a]	2,031±0,259[b]	2,300±0,059[c]
Zn^- 0,8	1,867±0,162[a]	1,463±0,076[ab]	2,052±0,028[b]	1,941±0,089[d]
Zn^- 1,8	1,534±0,087[ab]	0,571±0,420[c]	2,230±0,029[ab]	2,399±0,044[b]

a–cMeans in the same column with diferent superscript letters difer signifcantly (*P≤0.05*)

ZnO nanoparticles are thought to play the most important function in providing growth and development in crops (Del Buono et al. 2021). Upon the type and concentration, interaction of nanoparticles with plant cells impacts several biochemical and molecular changes during plant life cycle. In this study, treatments of ZnO greatly influenced the seedlings growth, developments and physiological parameters. ZnO at two doses were tested *in vitro* on seedlings tissues in the MS media in the combination with 4 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.125 mg kinetin consisting 0.8, 1.8 ppm ZnO NPs. Individually the two concentrations of ZnOs, when tested higher concentrations remarkably increased the shoot length, root length, and leaf numbers (Fig 1,2). The presence of ZnO NPs may have both promote or adverse effects on seed germination, plant development, biochemical and molecular mechanisms depending on dosage or treatment time (Regni et al. 2022, Sturikova et al. 2018). The results displayed that ZnO NPs supplemented in the development medium at the dosages of 0.8 and 1.8 ppm promoted the length of shoots, the root length, and the number of hairy roots. Zn participates in nucleic acid synthesis, carbohydrates, lipids metabolism and protein activities. The detected promotion in plant development properties in tissue culture due to the supplementation of ZnO NPs is in agreement with earlier reports (El-Mahdy and Elazab, 2020, Hussain et al. 2018).

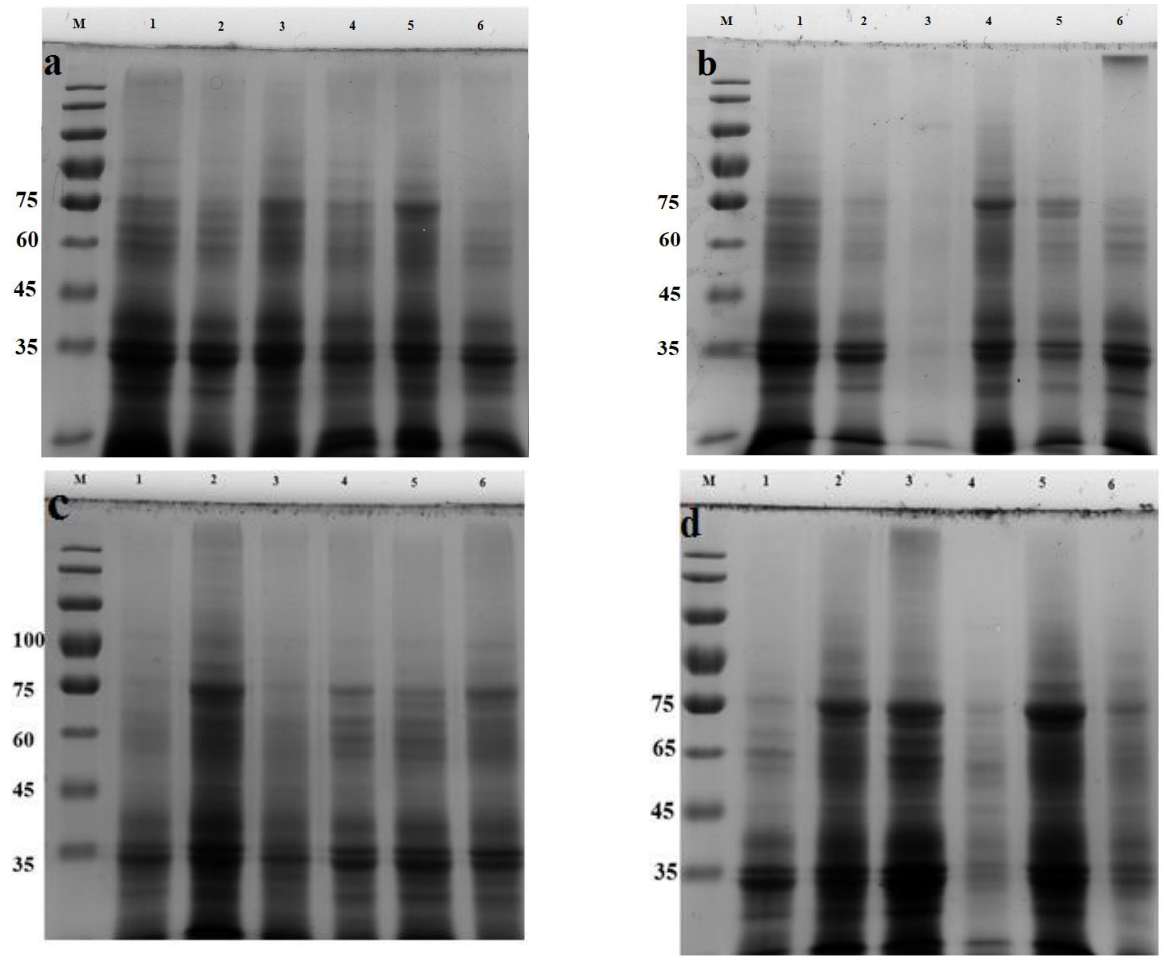

Figure 3 Change in total protein at 6 diferent medium concentrations a: Maro Tarım 21st Day, b: 35th Day, c: Kars 21st Day, d:35th Day (1: MS Zn^{+} 2: Std Zn^{-} 3: 0.8 Zn^{+} 4:0.8 Zn^{-} 5:1.8 Zn^{+} 6:1.8 Zn^{-})

In the present study, intensity of protein bands in tested pea ecotypes were effectively obtained with dosage of 0.8 and 1.8 ppm ZnO, resulting in increased plant growth. ZnO NPs in plants can strongly induce some cellular mechanisms involving growth and biomass production including protein synthesis. Our results proved a promotion in protein accumulation in the tissue culture dependent on ZnO NPs dosage applied and exposed time (Figure 3). The promoting effects of ZnO NPs were considerable at 0.8 ppm 35th days. A lower dose of ZnO NPs can significantly induce the synthesis of the protein. Therefore, promotion impacts of ZnO NPs are dependent on the dose and exposure time. Khan et al. (2021) have detected a gradually increased all growth parameters and the soluble protein contents of ZnO NPs treatments on Rapeseed plants. In line with these results, our results stated that ZnO stimulated from the inducible impact of zinc on nutrient takeup and the promotion of protein synthesis. MDA content is well known to be a marker of oxidative metabolism. In pea seedlings, lipid peroxidation was gradually improved in ZnO treatments and the impacts of ZnO NPs on MDA content are strongly linked to the plant development mechanisms. 21st days ZnO supplied seedlings revealed a important promotion of MDA value (Table 2). However, after 1.8 ppm ZnO NPs, two ecotypes revealed higher dosages promptly declined the content of MDA. This result is in accordance with those published by Liang et al. (2021) in the study of high concentration ZnO-Quantum dot for lettuce growth. Their results demonstrate that high dosages (500 mg L^{-1}) of ZnO QDs can strongly promote the value of MDA, which revealed that high dosage of ZnO QDs stimulated stress in lettuce, outcoming in MDA. With regard to proline DNSA, ZnO NPs at the higher concentration improved the value of these molecules (Table 1 and 3). Both proline and DNSA value maintain a specific point of metabolic equilibrium in the plant cells, and when the crop is exposed to external factors, this equilibrium will be unstable. DNSA content at low concentrations of ZnO, increased significantly in an exposure time- dependent manner. On the other hand; High-concentration

ZnO treatment can importantly decrease the content of DNSA, which may be due to the injury of metabolic equilibrium, thus eliminating enzyme activity. Similar results were detected in proline contents in present study. This result is in disagreement with those published by Hashemi et al. (2019) in the study on ZnO nanoparticles for soybean. Their results demonstrate that as the concentration of nanoparticles, proline content decreased.

Conclusion

We detected that ZnO NPs applied on *P. sativum* seedlings exhibited significant impact to both 21st days and 35th days treatments than control seedlings and that the analysis of MDA, protein displayed that ZnO accumulation in seedlings is probably an active process induced by exposure time and dose.

Conflict of Interest Statement

The authors of the article declare that there is no conflict of interest between them.

Contribution Rate Statement Summary of Researchers

The authors declare that they have contributed equally to the article.

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