Assessment of the Effects of Newly Fabricated CaO, CuO, ZnO Nanoparticles on Callus Formation Maintenance of Alfalfa (*Medicago Sativa* L.) Under *In Vitro* Salt Stress

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Abstract

Nanoparticles play an important role in plant adaptation to abiotic stress, especially in response to salt stress. In this study, two *alfalfa* lines (*Erzurum*, and *Muş*) were used as the material for the response NaCl to CuO, ZnO and CaO nanoparticles (NPs). CaO is evident to be higher effective than CuO, ZnO in callus induction from leaf explants. The antioxidant enzyme activities were also determined in the callus cultures. The maximum activity in MDA analysis was observed from callus treated of 50 mM NaCl with 0.8 ppm CuO NPs. The callus induction stage without salt treatments indicated a best result in 0.8 ppm CaO NPs for H₂O₂ value compared to the other NPs. The callus induction stage without salt treatments indicated a best result in 0.8 ppm CaO NPs for POD value compared to the other NPs for POD activity. The best response in protein rate was obtained from callus induction stage and callus formation stage after 50 mM treatment NaCl with 0.8 ppm CuO. LSCM analysis evident that the NPs could mitigate the negative effects of NaCl stress by the elimination of stress severity in callus cells. SEM analysis was supported the results obtained by LSCM analysis. Our findings suggest that CuO, CaO and ZnO NPs can offer a simple and effective method to protect *alfalfa* callus from NaCl stress severity.

Key Message

Engineered CaO, CuO and ZnO confer a protective response to salt stress in *alfalfa* callus maintenance. This response verifies with laser scanning confocal analysis and SEM analysis.

Introduction

Agricultural productivity is significantly constrained by biotic and abiotic environmental factors (Ji et al. 2013). Salt stress is a growing issue which is becoming a serious environmental stress around the world (Jalili et al. 2009; Zhu et al. 2016; Jalili et al. 2019). Salt-induced damage in product yield presumably exceeds damages from all other reasons, since both the severity and periods of the stress are crucial. Excess concentrations of various types of salts such as carbonates, calcium, chlorides, sulfates, magnesium, potassium and sodium, define various salinity growth mediums. Various control strategies have been supposed to challenge salt stress (Yazıcılar et al. 2021; Gao et al. 2016). Salt stress decreases root growth, stem expansion, leaf length, disrupts water-use capacity, and lowers plant water activity. Plants exhibit a difference of metabolic and physiological reactions at cellular and whole-organism levels against salt stress, thus making it a confusing event (Bezirganoglu 2017). The salt resistance trait is regulated by many physiological defensive strategies through complex enzymatic controlling pathways (Parida et al. 2005; Parihar et al. 2015). Metabolic process affects growth by challenging plants with photosynthesis, protein synthesis and lipid metabolisms. Physiological strategies like osmotic, ionic, oxidative stress and hormonal imbalances are affected due to salt stress. Salinity influences growth cope with of plants by the excess of Na⁺ and Cl⁻ ions in the medium that reduces the osmotic potential and inhibits the nutrients and water uptake (Gao et al. 2016). It is important to understand how salt stress challenges signals on the various levels of cells to activate the adaptive process in the plant (Manchanda and Garg 2008). Nanotechnology plays an important role in research

tools that solutions to the multiple agriculture-related matters. Nanotechnology has a larger application than biotechnology containing gene transformation, genomics, proteomics and bioinformatics and other technologies (Vijayakumar et al. 2010; Kim et al. 2017). Nanotechnologies can provide to enhance product capacity in less yield crops which is contributing in sustainable agriculture.

Moreover, nanotechnology has helped new potential for enhancing the structure of foods, flavour, higher protein content, and improved nutritional values. Nanoparticles have assisted to develop crop productivity by introducing such qualities as biotic resistance and increased abiotic stress resistance to the crops. CuO and ZnO oxide nanoparticles protect the plants against stress factors, through improving the activity of cytosolic enzymes (Alabdallah and Alzahrani 2020). Many studies have reported that various nanoparticles have both favourable and unfavourable effects on exogenous application of nanoparticle for the plant development processes, which are concentration-dependent and related to application strategy and targeted plant species (Kim et al. 2017; Ruttkay-Nedecky et al. 2017; Wang et al. 2016).

However, the physiological and molecular responses of callus cells to NPs are still unclear. *In vitro* cell cultures provide promising technology for the rapid production and stress-tolerant cultivars improved in short period of time (Bezirganoglu et al. 2017; Elmaghrabi and Ochatt 2006). The use of NP can influence the plant metabolism for enhancing the plant development and the capacity of ROS scavenging. The green derived nanoparticles from plants are economical and eco-friendly (Iqbal et al. 2020; Gohari et al. 2020). Callus culture is the suitable resources for rapid production of shoot quality and bio active phytochemicals. Callus can be stimulated further for the *in vitro* propagation of whole plants and used to deliver NPs into plants. The treatments of NPs in callus culture increased their content of bioactive molecules in response to biotic and abiotic stress factors (Yazıcılar et al. 2021; Bezirganoglu 2017). The improvement of novel strategy to characterize and clarify NPs in cells and tissues would support a better understanding of the potential effects during *in vitro* culture of plant tissues. To date, microscopy techniques have been used to study the assimilation and accumulation of NPs in plants under *in vitro* conditions. *Alfalfa* is used livestock feed and is superior to other forage crops in terms of nutritional quality. It has a rich and long history and is one of the earliest crops domesticated by human (Putnam et al. 2001). *Alfalfa* is cultivated for its rich source of proteins, carbohydrates, vitamins, minerals and dietary fibres. It also cultivated for its high yield and nutritional feeding quality, as well as its role on N fixation, soil conservation (Sakiroglu and Brummer 2017). However, *alfalfa* production is severely reduction by salt stress. Plants thrive by maintaining cell division and proliferation. Copper is an important micronutrient with many functions including redox reactions and participating the synthesis of chlorophyll and metabolism of carbohydrate and protein. Tolerance of diseases and crop yields can be influenced by deficiency of Cu (Dimpka et al. 2012) Zn is essential micronutrient participating directly in metabolic activities in plants such as formation of protein and carbohydrate and synthesis of chlorophyll as well as involving in the synthesis of auxin and indole acetic acid IAA from tryptophan. It was found that Zn key functions in regulation of redox systems and conservation of plant cells in response to oxidative stress. Zn deficiency plants have decreased crop productivity and reduced quality of crops (Lian et al. 2019). Understanding the response of *alfalfa* plants toward salinity stress at the nanobased level and developing salt-resistant cultivars are the vital mandates for its effective management. Till date, no

previous research article is available in literature to show the effects of, CuO and ZnO NPs on callus biomass formation and production of antioxidants in callus cultures of *alfalfa*. Therefore, the over all objective of the current study was to evaluate the possible effects of NPs on callus induction, biomass formation and extension of desirable levels of resistance to salt stress in *alfalfa*. Moreover, free radical scavenging activity and the antioxidant enzyme activities were also determined in the callus cultures.

Materials And Methods

Plant material and Callus induction

In our study, two *alfalfa* lines (*Erzurum*, and *Muş*) were used as the material for the response to CaO, CuO and ZnO NPs nanoparticulate. The mature seeds were sterilized with 1% NaOCl for 5 min, washed several times with sterile distilled water and rinsed with several changes of sterile distilled water overnight at 4°C. The mature seeds were cultivated in Petri dishes containing full MS medium (Murashige and Skoog 1962) for 30 days at 25±1 and in 16 hours light / 8 hours dark photoperiod at 1500 lux illumination intensity. Leaves were removed aseptically using forceps and placed on MS medium (Murashige and Skoog 1962) with 2 mg L⁻¹ glycine, 4 mg L⁻¹ 2,4-D, 100 mg L⁻¹ myo-inositol, 0.5 mg L⁻¹ nicotinic acid, 0,5 mg L⁻¹ pyridoxine HCl, 0.1 mg L⁻¹ of thiamine HCl vitamins, 1.95 g of MES, 50 mg L⁻¹ of ascorbic acid, 20 g of sucrose, solidified with 7 g of agar and the pH adjusted to 5.8 before autoclaving. In sterilization of the vitamins and hormones, 0,22 µm of porous cellulose nitrate filters were used and added 0.8 ppm CaO, CuO and ZnO NPs. The leaves were incubated in total darkness at 25±1°C temperature for one month.

Green synthesis and structural characterization of CaO, CuO and ZnO NPs

Preparation of Plant Extract

The walnut shells to be used in the green synthesizing of CaO, CuO and ZnO NPs NPs were collected from the walnut gardens in Erzurum in August-September 2020 and kept in the refrigerator at +4 °C until they were studied.

25 grams of walnut shells were first washed with distilled water, then the walnut shells were broken. The solid particles were then separated from the solution by filtration using filter paper (Watman 1). CaO, CuO and ZnO NPs were synthesized using obtained walnut shell extract 0,1 M Ca(NO₃)₂, Zn(NO₃)₂ and Cu(NO₃)₂ solutions.

Green Synthesis of CaO, CuO and ZnO NPs Nanoparticles

The synthesis of CuO, CaO and ZnO NPs was synthesized by using the walnut shell extract as reducing agent, using the green synthesis method previously used by Nadaroglu et al 2017.

Characterization of CaO, CuO and ZnO NPs

The CaO, CuO and ZnO NPs characterization obtained was carried out within the Eastern Anatolia High Technology Application and Research Center (DAYTAM) affiliated to Atatürk University. Scanning Electron Microscopy (SEM) and XRD analysis were used for the characterization of CaO, CuO and ZnO NPs. Information about the size and morphological properties of nanoparticles synthesized in this way was obtained.

Salt stress treatment

Erzurum and *Muş* leaf explants were used for callus formation in MS (Murashige and Skoog 1962) medium containing 4 mg L⁻¹ 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.125 mg kinetin including 0.8 ppm CaO, CuO and ZnO NPs nanoparticulate. The total culture duration was one month. Then, callus was obtained from 50 mM salt stress, such as one week and two week medium in terms of salt stress treating callus exposure times. Callus was transferred to hormone MS medium (Murashige and Skoog 1962) 1.0 mg/l 2,4-D 2,4-dichlorophenoxyacetic acid) and 1 mg/l kinetin in the presence of 50 mM NaCl including 0.8 ppm CaO, CuO and ZnO NPs.

MDA (Malondialdehyde)

Malondialdehyde was measured using the method of (Heath and Packer 1968) using liquid nitrogen. 0.4 grams of ground callus material was dispersed in 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The sample was boiled at 98 °C for 30 min. and then quickly taken into an ice bath. The sample content was centrifuged at 3000 ×g for 10 min. and the value of the supernatant was monitored at 532 and 600 nm (Heath and Packer 1968; Jaleel et al. 2007; Erdal 2012).

H₂O₂ (Hydrogen peroxide)

H₂O₂ (Hydrogen peroxide) content was measured using the method of Sergiev et al. (1997). 0.4 g of callus material was homogenized in 4 ml of trichloroacetic acid and centrifuged at 4 °C for 15 min. at 13000 rpm. 2 ml of extract was mixed with 0.8 ml of KH₂PO₄ and 1.6 ml of KI in test tubes. The absorbance of the callus sample product was measured at 390 nm using a standard curve with H₂O₂ solutions (Velikova et al. 2000).

POD (Peroxidase)

The activity of POD (Peroxidase) was measured following the procedure established by Chance and Maehly (1955) by adding 100 µL of the callus extract to 3 mL of assay solution, which contained 13 mM guaiacol, 5mM H₂O₂ and 50mM Na-P buffer (pH 6.5). The POD (Peroxidase) activity was determined in absorbance at 470 nm of protein. The total soluble protein contents were determined by BCA (Bicinchoninic Acid) protocol (Yee et al. 2003; Erdal 2012).

Sectioning with microtome

Having been kept in 10% formaldehyde for 3 days, callus structures were taken to the cassettes and left for an overnight wash. Then, it was kept in 70, 80, 96% ethyl alcohol, one hour apart, respectively. Absol-I, Absol-II, Xylol-I, and Xylol-II were kept for 1 hour, respectively. The calluses were embedded in parafilm, and a section of 6µm was taken (Rolls et al. 2012).

Laser scanning confocal microscope (CLSM)

Callus, sectioned with microton, are kept for about 30 minutes with 1% rhodamine. Then it is passed through distilled water 3 times. Fluorescence images were obtained with a Nikon Eclipse TE2000 Confocal Laser Scanning Microscope C1si. Samples were excited with the 488 nm line of an argon laser and dye emission was collected at 520 and 610 nm. The DCF fluorescence was visualized in a single optical section of the callus. All images were obtained at the same depth (Minta et al. 1989).

Scanning Electron Microscopy

Alfa callus tissues were prefixed in 5% buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 2 h at room temperature. After dehydration through a graded ethanol series, samples were dried with a CPD (CO₂ critical-point drying) system, sputter-coated with gold (Jeol JFC-1100 E ion-sputtering system) and observed with a scanning electron microscope (HITACHI S-4700).

Statistical Analysis

Each experiment was repeated three times. Analysis of variance was conducted using a one-way ANOVA test using SPSS 13.0 and means were compared by Duncan test at the 0.05 confidence level.

Results

Green synthesis of Ca NPs

Characterization of CaO, CuO and ZnO NPs

Surface morphology examination of synthesized CaO, CuO and ZnO NPs was carried out using Zeiss brand Sigma 300 model scanning electron microscope (SEM). From the SEM images given in Figure 1A, it can be seen that CaO NPs have a particle size ranging from 35 to 160 nm. SEM images of CaO show that synthesized CaO NPs are porous, the structure is regular and has a very pleasant layered structure. When the SEM image of the obtained CuO NPs was examined (Figure 1B), it was determined that the CuO NPs were spherical and their dimensions were distributed between 20-45 nm (Gultekin et al, 2017; Gultekin et al. 2020). When the SEM images of ZnO NPs were examined (Figure 1C), it was determined that ZnO NPs were agglomerated and compatible with XRD results. It was observed that the structures of ZnO NPs were spherical and varying in size between 17-65 nm. In addition, when the surfaces of ZnO NPs are rough, it is clearly seen from Figure 1C. The findings obtained are also compatible with the literature (Rajendran and Sengodan 2017; Nadaroglu and Alayli 2020).

XRD analysis

The spectra of the XRD analysis (X-ray diffraction) of the CaO NPs structure are shown in Figure 2. 28.75 ° indicates 34.16° (111), 47.11°, 50.86 ° (311), 54.60 ° (222), 62.60 °, Ca²⁺ and carbohydrate units and CaO NPs (Sahu et al. 2017; Yazıcılar et al. 2021). The findings obtained confirmed that the structure of CaO NPs was successfully formed. XRD and crystallographic analysis of zinc nanoparticles by green synthesis method using walnut shell extract are given in Figure 3A. Characteristic peaks of the XRD spectrum that can be indexed at 2θ = 11.39°, 22.24°, 36.09°, 49.22°, facets (111), (200), (220) are consistent with the literature. Zn NPs structures were determined to be cubic (fcc) zinc nanocrystals (Nadaroglu and Alayli 2020). XRD and crystallographic analysis of CuO nanoparticles synthesized by green synthesis method are given in Figure 2C. 2θ = 32.2°, 39.62°, 58.9°, 70.3° of the XRD spectrum showed characteristic peaks that can be indexed at facets (110), (111) and (202) (Figure 2C). It has been determined that CuO NPs structures have a spherical structure (Gultekin et al. 2020).

MDA (Malondialdehyde)

The callus induction stages of the *Erzurum* and *Muş* genotypes produced as a result of ZnO, CuO and CaO NPs application against NaCl were evaluated. Also, salt-free ZnO, CuO and CaO NPs applied callus formation stages were evaluated. Table 1. clearly display that MDA activities were greatly affected in callus formation stage of two *alfalfa* lines in presence of 0.8 ppm NPs after salt treatments. MDA values indicated a large range of variation among tested samples for salt stress treatments, ranging from 0,0168 to 0,0466 nmol g⁻¹ FW. The maximum activity was observed from callus treated of 50 mM NaCl with 0.8 ppm CuO NPs. The callus induction stage without salt treatments indicated a best result in 0.8 ppm CaO NPs for MDA value compared to the other NPs. Although the highest membrane damage was found in the treatments with 0.8 ppm CuO 50 mM NaCl in callus induction stage, the lowest membrane damage was found in 'callus formation stage' for 0.8 ppm CaO NPs 50 mM NaCl (Table 1).

H₂O₂ (Hydrogen peroxide)

There were significantly differences among 1. week *Muş* callus and the other groups (Fig. 2). Table 1 clearly display that H₂O₂ activities were significantly affected in the tested callus of two *alfalfa* lines in presence of 0.8 ppm NPs. H₂O₂ values indicated a large range of variation among tested samples for salt stress treatments, ranging from 0,0134 to 0,0979 nmol g⁻¹ FW. The callus induction stage without salt treatments indicated a best result in 0.8 ppm CaO NPs for H₂O₂ value compared to the other NPs. Although the highest membrane damage was found in the treatments of 0.8 ppm CuO NPs in the callus induction stage, the lowest membrane damage was found in callus formation stage for 0.8 ppm CuO NPs (Table 1).

POD (Peroxidase)

Table 1 clearly display that POD activities were significantly affected in callus formation stage of two *alfalfa* lines in presence of 0.8 ppm NPs. POD values indicated a large range of variation among tested

samples for salt stress treatments, ranging from 0,1035 to 1,666 nmol g⁻¹ FW. The maximum activity was observed from callus treated of 50 mM NaCl. The callus induction stage without salt treatments indicated a best result in 0.8 ppm CaO NPs for POD value compared to the other NPs. Although the highest membrane damage was found in the treatments of 50 mM NaCl, the lowest membrane damage was found in 'callus induction stage' for 0.8 ppm CuO NPs (Table 1).

Protein Analysis

The analysis displayed that protein levels have stronger effects in the *Muş* and *Erzurum* callus (Fig. 6). However, few accumulations of protein were detected in the control callus except 0.8 ppm CuO. CuO displayed lower protein accumulation prior to NaCl. The best response was obtained from callus induction stage and callus formation stage after 50 mM treatment NaCl with 0.8 ppm CuO. The best response was obtained from callus induction stage and callus formation callus without NaCl with 0.8 ppm ZnO (Table 1).

Laser Scanning Confocal Microscope (LSCM)

LSCM was used as a visual marker to verify the distribution of nanoparticles in stable callus culture. NaCl free callus and callus which applied the 50 mM NaCl alone were used as a control. The analysis displayed that the CaO, CuO and ZnO NPs are obviously traceable in the *Erzurum* and *Muş* callus tissues. However, The NaCl stress inhibited in terms of nanoparticles was at different degrees. CuO exhibited a better response than CaO and ZnO NPs in response to NaCl stress. In the first week, accumulation of NPs inside the cell was lower activity by NaCl than in the second week in terms of *Erzurum* and *Muş* genotype. According to the result of the confocal analysis, CuO exhibited the most abundant callus induction stage, followed by ZnO exhibited at callus induction stage, 50 mM NaCl ZnO callus induction stage and finally, 50 mM NaCl CuO callus induction stage (Fig. 7).

SEM analysis of callus structures

SEM detection indicated that each genotype callus type had various callus structures. There was a continuous amorphous sphere, termed extracellular matrix, on the callus surface. It was also detected that cultivars belonging to the same genotypes share similar cell structure and shapes. The soft and compact character of the callus in *Erzurum* and *Muş* convert to granular- mucilage resembles, mostly likes a membranous surface and wrinkled cell mass structure under SEM detections (Fig. 8).

Discussion

Alfalfa is an important high nutritional feeding quality and N fixation, which have a great potential for wildlife habitat and soil conservation. However, abiotic stress such as salinity which causes adverse effects on germination, plant vigour and crop yield, thereby affecting annual development and resulting in serious economic losses (Sakiroglu and Brummer 2017). Various cultural and chemical control strategies have been attempted to address these concerns, but the strategies were only partially powerful. In

comparison, nanotechnology supplied new options to produce resistance plants against abiotic stress factors (Iqbal et al. 2020; Gohari et al. 2020). In fact, many studies have been reported on the plant species use of nanoparticles in response to stress factors. In this study, in vitro callus induction responses of *alfalfa* under NaCl and CaO, CuO, ZnO were examined and present study displayed significant differences in their responses to the NPs. The results indicated that NPs had a promoting effects callus induction, while control callus postponed the day of callus induction. It was detected that with treatment NPs the degree of callus induction also highly increased. The obtained results that compact callus with globular structure and yellowish colour callus were formed from leaf explants within 1 months, while there was callus induction in the NPs within a shorter time 1 months. Callus were induction in NPs treatments with various frequencies. The highest frequency of callus induction from leaf explant of *Muş* genotype was detected on the medium containing 0.8 ppm CaO. Among tested treatments, CaO is evident to be higher effective than CuO, ZnO in callus induction from leaf explants. The fresh weight in *Muş* callus in the presence of 0.8 ppm CaO was heavier than those of CuO and ZnO in equal concentration (data not shown). It was proved that considerable uptake and accumulation of the nutrient elements occurred. Noticeably the size all tested NPs examined in this study did identical, with a value of 20 -160 nm but varying uptake and accumulation functions of those three types of NPs were detected, and the genotype dependent uptake for NPs occurred in the callus. Previous studies have shown a positive correlation between NaCl and nanoparticles accumulation in various plant species (Wang et al. 2019; Javed et al. 2017). Ca^{2+} ions are rapidly carried by membrane channels that are available on the plasma surface (Berridge et al. 2000; Ditta and Arshad 2016; Liu et al. 2016). This obviously confirms that CaO in the culture medium was quickly responsive and effective accumulation. In terms of confocal analysis, assessment of NPs functions in the presence of NaCl was based on the expanded coloration, tissue damage, and amount of the cell survival. In our cases, the results of laser scanning confocal analysis and SEM demonstrate the delivery of NPs *alfalfa* callus is supported related to the uptake of nutrient elements and translocate from culture medium (Fig. 7). CuO nanoparticle application in the *Muş* genotype at the 1st week provided an expected improvement on NaCl stress. CuO application showed a dense distribution in the cell by preventing the adverse effect of 50 mM salt faster than ZnO and CaO application. The best reponse of ZnO nanoparticle application was obtained in the 2nd week of the *Muş* genotype compared with the 1st week and the 2nd week. ZnO accumulated intensely in certain parts of the cell in 1st week application, and as the period extended, it simultaneously distributed into the cell and prevented the negative effect of salt. These indicate that term alterations of defense responses to NaCl could be a main process. One or two weeks post NaCl application, control callus displayed salt severity on the callus tissues, resulting in a tissue damage within less than 7 days (Fig 7). By contrast, *Muş* and *Erzurum* genotypes are 1st application CuO NPs survived longer than 2 weeks, indicating considerably improved resistance to NaCl. Our detection of NPs in callus tissues following a fairly aggressive 2nd applications suggests that CaO, CuO and ZnO was present, not merely on the extracellular matrix, but that it penetrated inside the callus cells. SEM analysis supported with confocal analysis results in indicating that CuO was disrupted the extracellular matrix of *alfalfa* callus 7 days post exposure (Fig 7c). 14 days post exposure, CuO was clearly located inside callus cell tissues. It was

detected that the formation of callus structure of *alfalfa* callus subject to media including various NPs such as CaO, CuO and ZnO can be added to the culture media. It was noticed that no harmful for cell wall in callus of the control while harmfuls were detected in cell wall of callus exposure to 50 mM NaCl. This is evident that even lower doses NaCl dramatically increased on the harmful cell wall of the callus tissues. CaO, CuO and ZnO influence for induction NaCl stress various concentrations of NaCl. Callus produces an amorphous mass of extreme cell wall in response to exposure to different time-periods (Fig. 8). Accumulation nanoparticles and its adverse effects on applied callus tissues highly depend upon the genotype and exposure time. For example; 7 days post exposure, ZnO NPs exhibited membranous structures to be extracellular matrix and related to neighboring cells, and they were intensely ZnO that accumulated and present in the 50 mM NaCl. In contrast, CuO NPs showed partial rough and mucilage-like on the fibroblast in *Erzurum* genotypes. Although the CaO NPs exhibited wrinkle and rough structures at 1st week of *Erzurum* genotype. In 1st week of the application of 50 mM NaCl of *Muş* genotype mostly granular structures and nodular callus segments were showed. Interestingly the CaO NPs exhibited mucilage-like structures and differentiation of callus primordia at 2nd week of *Muş* genotype and 2nd week of the application of 50 mM NaCl of *Erzurum* genotype wrinkle structures were shown (Fig. 8). Obviously, CaO, ZnO and CuO are greatly reactive and are able to pass through the cell membrane in both cases and recover the callus from NaCl stress. It is evident that callus exposed to NaCl in the 2nd week will provide higher physiological properties regardless of nanoparticle types which are linked to the regeneration capacity in callus cells. This verifies us that chemical composition and structural regulating on the callus tissues might play important functions in morphological formation. Medium conditions in tissue culture induced activation of various cellular defense strategies, adjusting cell adaptation under new environmental conditions. These findings are consistent with one of the first reports on formation of the ECM mediated on the outer surface layer (Zari et al. 2015). They suggested that ECM formation might be a stress response of explants, revealed by specific tissue culture conditions. NaCl severity resulted in an obvious increase in MDA values in callus. A reduction of MDA was also observed in callus formation stage subject to 0.8 ppm CaO 50 mM NaCl period. Based on genotype, the NPs applied callus had considerably higher MDA values compared with 1st and 2nd weeks. This indicated that NaCl-activated stress severity was alleviated by ZnO, CuO and CaO (Fig. 3). These results indicated that the effect of NPs was linked to the callus structure and genotypes. These findings are in agreement with one of the reports in absence of NPs mediated tolerance to NaCl stress potato callus (Rizwan et al. 2015). Levels of MDA in callus induction stages higher activity than callus formation stage in callus cells of both genotypes. The obtained results suggested that MDA activity from callus induction stage was higher promoting NPs uptake and binding capacity in the cytosol even without NaCl. This verifying that the NaCl induced stress was mitigated by nano-Cao, CuO and ZnO. Under stress conditions, H₂O₂ is generated and mediates crosstalk among metabolic process. Therefore, the H₂O₂ is a likely signaling molecule that participates to the events (Soleymanzadeh et al. 2020). In our cases, the results verified that the production of H₂O₂ is dependent on genotype and nanoparticles types and this was confirmed by the statistical test. However, application of *Muş* genotype with 1st week was significantly different from 2nd week as well as *Erzurum* genotype. and there was higher H₂O₂ production at the callus formation stage in the presence of CuO

NPs. Additionally, Application of long period callus cells with CuO considerably recovered stress severity at callus formation stage (Fig. 4). These results are contributed with the outcomes obtained on callus cells tissues in different *Triticale* genotypes (Yazıcılar et al. 2021). The POD activity with NPs in callus tissues was considerably decreased exposed control and in presence of 50 mM NaCl. Moreover; The results of this study displayed that calli decreased callus formation stages that the POD decreased; and these decreases were seen to have changed depending on the genotypes, type and period of the nanoparticles applied. This decrease was detected changeable depending on the applied NPs period. Although the maximum POD activity was recorded at in presence of NaCl, the minimum POD activity was recorded at 0.8 ppm CuO callus formation stage (Fig. 5). Based on our findings, decreases in POD activity in NPs-treated callus could be linked to promotion of callus development parameters and protective role of NP as direct or indirect. Additionally; Induction in POD activity may be explained that Cu^{+2} ions led to higher ROS generation, and initially promoted their antioxidant system to challenge the ROS but then lost capacity to regulate antioxidant enzyme activity. This is consistent with the earlier report indicating that POD activity is greatly linked to NPs ions. (Liang et al. 2018; Castiglione et al. 2014). It is evident that organs and tissues of plants exposed to environmental stress in long-term will gradually lose their biochemical and enzymatical structures. 1st week *Muş* applications exhibited an enough activity of POD that mainly due to rapid and efficient inducer of CuO NPs, whereas ZnO was slowly effective, which could probably be an outcome of stronger stability of ZnO. Stability is a main strategy determining the transformation, transport, fate, and toxicity of ZnO NPs in various growth media. This can be explained that the behavior of NPs depends on the intrinsic physiochemical properties and the chemistry of the surrounding different environment media. The nanoparticles uptake mechanisms can be influenced by several factors including salinity, total organic carbon, pH, redox potential, water properties like ionic strength, natural organic material (NOM), redox potential, and other chemical components influence the short- and long-term behavior of CuO NPs (Sousa and Texeira 2013; Conway et al. 2015). Applied long-term cultured callus cells with 0.8 ppm NPs exhibited the best response and considerably recovered ZnO NPs as compared to control callus in terms of protein content. After NaCl exposure, ZnO was found to be hardly recovered at callus induction stage and callus formation stage in response to NaCl stress as compared to 50 mM NaCl and control callus (Fig. 6). This explains that ZnO uptake, translocation and accumulation of ZnO-NPs by plants depend upon the distinct features of the NPs as well as on the physiology of the host plant. This can be attributed to that the effects of ZnO on callus cells may be dependent concentrations and strong systemic activity of the Zn^{+2} ions. These results were similar with an earlier report on expression in response to ZnO uptake (Lorez-Moreno et al. 2010; Zhao et al. 2012). The uptake and biotransformation of ZnO NPs in plants are not only related concentrations but also particle adhesion onto cell surface, therefore ZnO uptake may also arise due to particle dissolution in the culture medium. This suggested that the callus cells were slightly effective at NaCl severity in 0.8 ppm ZnO applied. As explained above, CuO was more effective than ZnO on callus cells in response to NaCl severity. It is well-known that callus cells within explant resources demonstrate intensive cell division and hence there is necessary for active synthesis of nucleic acids. Need for DNA production improves the synthesis of NTP. That is an early substrate for nucleic acid synthesis. Increased NTP synthesis increases pH level within cells. This statement confirms that protein synthesis is significant for plant growth and

development, which are highly sensitive to the NaCl stress. The result may have verified by the microscopy studies with a SEM in which display a reduction in the formation of continuous surface in presence of ZnO NPs. In fact, by extending the period ZnO, the formation of membranous structures as well as some wrinkle and amorphous compounds were detected. These effects are likely due to synthesis protein and inducing structural changes in protein and thus the formation of various conformational changes in the callus surfaces.

Conclusions

It was observed that NaCl resulted in altered pattern of CuO and ZnO in the callus tissues of the two *alfalfa* genotypes. NaCl tolerance in *alfalfa* can be improved by increase in NPs uptake efficiency. Moreover, callus induction stage in presence of CaO were affected but did not appear to have expected positive relationship with antioxidant enzyme activities under NaCl. It is inferred from the outcomes of the present study that decreased the adverse effects of NaCl on *alfalfa* callus tissues through improving MDA, H₂O₂ and protein rates and lowering the activity of POD in presence of NPs. In the near future, these CaO, CuO and ZnO can be application to the callus tissues of other plant species that improve to other abiotic stress factors.

Declarations

Author contributions Concept—IB; Design—IB and BY; Supervision—IB; Resource—IB; Materials—BY, FB, MŞ, HN and AA; Data Collection and/or Processing—MŞ and BY; Analysis and/or Interpretation— BY, FB and MŞ; Literature Search—BY and MŞ; Writing—IB; Critical Reviews—HN and AA.

Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interests exists.

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Tables

Due to technical limitations, table 1,2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

SEM images of CaO NPs (A), ZnO NPs (B) and CuO NPs (C)

Figure 2

XRD patterns of CaO NPs (A), ZnO NPs (B) and CuO NPs (C)

Figure 3

Changes in MDA acitivity alfalfa genotypes: 1: First week Muş, 2: First week Erzurum, 3: Second week Muş, 4: Second week Erzurum

Figure 4

Changes in H₂O₂ activity alfalfa genotypes: 1: First week Muş, 2: First week Erzurum, 3: Second week Muş, 4: Second week Erzurum 1: First week Muş, 2: First Erzurum, 3: Second week Muş, 4: Second week Erzurum ($p \leq 0.005$)

Figure 5

Changes in POD activity alfalfa genotypes: 1: First week Muş, 2: First week Erzurum, 3: Second week Muş, 4: Second week Erzurum 1: First week Muş, 2: First Erzurum, 3: Second week Muş, 4: Second week Erzurum ($p \leq 0.005$)

Figure 6

Changes in protein activity alfalfa genotypes: 1: First week Muş, 2: First week Erzurum, 3: Second week Muş, 4: Second week Erzurum 1: First week Muş, 2: First Erzurum, 3: Second week Muş, 4: Second week Erzurum ($p \leq 0.005$)

Figure 7

a Erzurum control, b Erzurum 50 mM NaCl, c Erzurum 0.8 ppm CuO (+), d Erzurum 0.8 ppm CaO 50 mM NaCl (-), e Muş control, f Muş 50 mM NaCl, g Muş 0.8 ppm ZnO (+), h 0.8 ppm CaO 50 mM NaCl

Figure 8

a Erzurum control, b Erzurum 50 mM NaCl, c Erzurum 0.8 ppm CuO (+), d Erzurum 0.8 ppm CaO 50 mM NaCl (-), e Muş control, f Muş 50 mM NaCl, g Muş 0.8 ppm ZnO (+), h Muş 0.8 ppm CaO 50 mM NaCl

Supplementary Files

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