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CaO and Graphene Oxide Enhances Drought Stress from Callus Tissues of *Medicago Sativa* L. Cultivars

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ABSTRACT: Drought stress can be described as multidimensional stress factors affecting plants' growth, development, and productivity. In order to reduce the adverse impact of drought stress, a plethora of attempts have been employed. Among those attempts, nano-engineered particles have gained a remarkable attention. Of the relevant particles, calcium oxide (CaO) and graphene oxide (GO) have been well-documented to positively regulate and mediate the plant growth system through shifting physiological biochemical and molecular aspects of the plant. The solo impacts of the nanoparticles are well-known but their interactions were not assayed for *Medicago sativa* L. cultivars. For that reason, the present study investigates the impact of CaO NPs and GO on the response and regulation of the defensive mechanism in alfalfa (*Medicago sativa* L.) callus in drought stress-suffered cultivars.

The activation of CaO-GO can be induced with mannitol in the callus of alfalfa cultivars. Dry and fresh weight values were determined in callus samples. There were significant differences between cultivars and concentration. In terms of MDA, H_2O_2, proline content, it was observed that the Ca^{2+} NPs application was important, and it showed a strong link with the resistance degree of cultivars. Erzurum cultivar was observed for better proline content with 1.5 ppm GO. MDA activities demonstrated an increasing trend concerning concentrations of mannitol and nanoparticles. The MDA highest activity was observed with 1/2 ppm CaO+0.5/1.5 ppm GO (0.1849 mg/g FW) in the Erzurum. However, the Erzurum cultivar responded with better H_2O_2 content with 100 mM mannitol +0.5 ppm (0.1017 mg/g FW). This result has presented, under *in vitro* conditions, that the supplementation of CaO and GO can importantly reduce the negative impacts of drought stress on alfalfa callus; additionally, it has been seen that the dosages of nanoparticle and mannitol are also important.

Keywords: Alfalfa, nanoparticules, mannitol, *in vitro* culture

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INTRODUCTION

Drought is one of the most significant abiotic stress, emerging due to many reasons, *viz.* excessive salt, alters temperatures, and lower rainfall and the other environmental factors. Drought is a multi-dimensional stress and defined as a water deficit or period without significant rainfall, which negatively impacts crop growth and developments (Xiong et al. 2020; Ayyazet al., 2021; Bano et al., 2021). A few plant species have developed strategies drought stress, but these mechanisms are complex physiological conditions and rely on the crop species. General, annual plants have a mechanism called escape and tolerance in response to drought stress. It shows a broad range of mechanisms such as proline, carbohydrates, maintaining cell homeostasis, decreased photosynthetic accumulation and altered gene expression as well as enzyme activities (Shi et al., 2021a; Castroluna et al., 2014). Nanotechnology has a significant role in industry, food, agriculture, health and pharmaceutical sectors (Kokura et al., 2010; Singh et al., 2008; Tiede et al., 2008). Recently reports have reported that small particles are participated in different morphological and biochemical pathways that control crop growth and development, as well as abiotic stress responses (Arora et al. 2012; Regier et al. 2015). The application of nanoparticles in agriculture is important to prevent the use of pesticides, increase crop yield, and minimize environmental conditions factors (Le 2019; Usman et al. 2020). Ca^{2+} is a macro element that acts as a secondary regulator in plant growth and development (Bothwell and Ng 2005). Ca^{2+} is an important in cell signal transmitted for gene function and cell division and is participated a several of plant life cycle including hormone regulations, cell growth, light signaling and stress response. It provides intercellular communication in the plasma membrane, as well as conferring resistance in response to environmental stress factors (Sanders et al., 20002; Kapilan et al., 2018). Ca^{+2} has a high importance during drought defence mechanisms and inhibits the expanding of drought via several series of physiological and molecular changes (Kong et al., 2013; Shao et al., 2008). Graphene, a new type of nanomaterial, is commonly due to its many traits. Graphene oxide (GO) is one of the most important graphene derivatives as it can be modified and contains many oxygen-containing hydrophilic functional groups (Nazari et al., 2020). GO has greater surface activity and better biocompatibility than graphene (Chong et al., 2014). In recent years, it has a significant function in the agricultural field, especially in improving plant growth and development. *M. sativa* is a perennial forage legume plant with high nutritional value. *M. sativa* is the most preferred plant species among leguminous forage crops due to its longevity and being more resistant to environmental conditions (Jiang et al 2006). In addition, *M. sativa* is used as a model plant in tissue culture and genetic engineering studies, since it has a higher *in vitro* regeneration ability among legumes (Salih et al., 2019). The aim of this study was to increase the resistance elicited by the synergistic effect of Ca^{2+} NPs and GO from drought stress in *M. sativa* callus. The previous have clearly reported the positive effects of either solo CaO or Go treatments. However, their interactions or combinational treatments have not been investigated. We designed and hypothesized that combination treatments of CaO and GO would enhance the tolerance of alfalfa agaisnt drought stress.

MATERYAL ve METOT

Plant Material and Callus İnduction

In our study, two different *M. sativa* cultivars (Erzurum and Konya) were used as the material for the response to CaO NP and GO nanoparticulate. The mature seeds were sterilized with 1% NaOCl for 5 min, washed several times with sterile distilled water. Then, The mature seeds were transferred in plates including full MS medium (Murashige and Skoog 1962) for 2 weeks at 25 ± 1 and in 16 h

light/8 h dark photoperiod at 1500 lx illumination intensity. Leaf explants were removed aseptically using forceps and placed on MS medium (Murashige and Skoog 1962) with 2 mg L^{-1} glycine, 1 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 1 mg L^{-1} kinetin 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. The leaf explants were incubated in total darkness at 25 ± 1 °C temperature for one month.

Drought stress treatment

Erzurum and Konya leaf explants were used for callus induction in MS (Murashige and Skoog 1962) medium consist of 1 mg L^{-1} 2,4-D and 1mg L^{-1} kinetin for 1 month. Then, callus samples were placed under two different drought stresses, (Murashige and Skoog 1962) 1 mg L^{-1} 2,4-D and 1mg L^{-1} kinetin in the presence of 50–100 mM mannitol consists of 0.5 ppm and 1.5 ppm Grafen oxide and Ca^{2+} nanoparticulate. For the GO nanoparticle, callus was transferred to 1 mg L^{-1} 2,4-D and 1mg L^{-1} kinetin in the presence of 50 and100 mM mannitol 0.5 ppm and 1.5 ppm GO nanoparticulate for one month. For the CaO nanoparticule, callus was transferred to 1 mg L^{-1} 2,4-D and 1mg L^{-1} kinetin in the presence of 50 and100 mM mannitol 0.5 ppm and 1.5 ppm Ca^{2+}nanoparticulate for one month. The experiment period was one month.

Determination of callus dry and fresh weight

The total number of leaf explants, callus colour, characteristics and induction percentage in a petri dish was evaluated after 1 months. The callus induction percentage and the fresh weight of the callus were measured. Number of days obtained for the explant to generate callus was calculated in each cultivar. The fresh mass of callus was obtained after 35 days of incubation and expressed as gram. Callus patterns of accepted fresh weight were dried to fixed weights in an oven adjusted at 50°C for 24 h (Uzun,1997).

Quantification of proline content

For quantification, 0.1 g callus sample was homogenized with 3% sulfosalicylic acid ($C_7H_6O_6S$). The homogenate obtained was added to ninhydrin and glacial acetic acid and kept in a 100ºC sub-bath for 1 hour. Toluene was added to the resulting mixture and 520 nm measurement was made in the spectrophotometer. Proline standard graphics were created and calculations were made (Rodriguez et al., 2005).

Determination of malondialdehyde (MDA) content

Regarding MDA content, 0.2 g *M. sativa* callus were homogenized with 0.1% TCA (Trichloroacetic acid). 20%TCA and 0.5% TBA (Thiobarbituric Acid) were added to the obtained homogenate and incubated in a 98ºC water bath. The resulting homogenate was measured at 532 and 600 nm in the spectrophotometer. (Erdal, 2012).

Determination of hydrogen peroxide (H_2O_2)

For assaying content of H_2O_2, 0.2 g *M. sativa* callus (Erzurum and Konya) were homogenized with TCA and KH_2PO_4 and KI were added to the resulting supernatant. The resulting homogenate was measured at 390 nm (Velikova et al., 2000).

Statistical analysis

This study was carried out in 3 repetitions. Two-way ANOVA test was performed using SPSS 25.0 program. The means were separated using Duncan test (p<0.005)

RESULTS AND DISCUSSION

Previous studies have shown that plant macro and micro elements play a basal function in alleviating drought stress in plant species (Cheong et al. 2007). However, despite the significant signalling role of CaO in plant defences to various environmental stresses, researches for the potential role of CaO and GO in drought stress resistance in *M.sativa* have not been elaborately studied. In our study, determining the responses of *M.sativa* to mannitol stress and nano treatment of CaO and GO displayed that the higher fresh weight in callus is associated with higher proline accumulation in callus which positively increases the MDA and H_2O_2. We clearly display that the treatment of GO and CaO to *M.sativa* plants could be a beneficial method to enhance drought tolerance. The fresh and dry values revealed a large range of variation among cultivars for mannitol stress treatments, ranging from 0.0120 to 0.114 g. For Erzurum cultivar, the best value was found in *control* (0.0932 g) and 1.5 ppm GO (0.0933 g) treatments with the same amounts, followed by '50 mM mannitol+2 ppm CaO+ 0.5 ppm GO (0.1140 g)' and 100 mM mannitol (0.0841 g). For Konya cultivar, the highest callus weight was obtained 100 mM mannitol+1.5 ppm GO (0.1280 g), followed by 50 mM mannitol +0.5 ppm GO (0.1260 g) concentration and 1 ppm CaO+ 0.5 ppm GO (0.1212 g) (Table. 1).

Table 1. Ratio of callus dry weight to fresh weight of *M. sativa* cultivars (Konya and Erzurum)

	Dry/Fresh Weight (g)	
Treatment	**Erzurum**	**Konya**
Control	0.0932 ± 0.00015^{c}	0.1100 ± 0.001^{p}
1 ppm CaO	0.0581 ± 0.00010^{ef}	0.0774 ± 0.00007^{n}
2 ppm CaO	0.0519 ± 0.01897^{f}	0.0702 ± 0.00078^{p}
0.5 ppm GO	0.0321 ± 0.00010^{ij}	0.0797 ± 0.00657^{l}
1.5 ppm GO	0.0933 ± 0.00010^{c}	$0,0966\pm0.0006^{o}$
1 ppm CaO+0.5 ppm GO	0.0430 ± 0.00010^{gh}	0.1212 ± 0.00073^{c}
1 ppm CaO+1.5 ppm GO	0.0091 ± 0.00010^{l}	0.0772 ± 0.00069^{h}
2 ppm CaO+0.5 ppm GO	0.0620 ± 0.00100^{e}	0.0780 ± 0.00095^{mn}
2 ppm CaO+1.5 ppm GO	0.0450 ± 0.00100^{g}	0.0539 ± 0.00008^{r}
1/2 ppm CaO+0.5/1.5 ppm GO	0.0260 ± 0.00100^{jk}	0.0740 ± 0.00095^{o}
50 mM mannitol	0.0081 ± 0.0001^{l}	0.0984 ± 0.00007^{h}
50 mM mannitol+1 ppm CaO	0.0910 ± 0.001^{c}	0.0493 ± 0.00009^{e}
50 mM mannitol+2 ppm CaO	0.0061 ± 0.0001^{l}	0.1013 ± 0.0009^{g}
50 mM mannitol+0.5 ppm GO	0.0230 ± 0.001^{k}	0.1260 ± 0.001^{b}
50 mM mannitol+1.5 ppm GO	0.0120 ± 0.001^{l}	0.0893 ± 0.00008^{j}
50 mM mannitol+1 ppm CaO+0.5 ppm GO	0.0370 ± 0.001^{hı}	0.1136 ± 0.00009^{d}
50 mM mannitol+2 ppm CaO+0.5 ppm GO	0.1140 ± 0.001^{a}	0.1213 ± 0.00059^{c}
50 mM mannitol+2 ppm CaO+1.5 ppm GO	0.0570 ± 0.001^{ef}	0.0053 ± 0.00001^{y}
100 mM mannitol	0.0841 ± 0.0001^{d}	0.0538 ± 0.00199^{r}
100 mM mannitol+1 ppm CaO	0.0323 ± 0.0001^{jk}	0.1212 ± 0.00072^{c}
100 mM mannitol+2 ppm CaO	0.0441 ± 0.0001^{g}	0.0181 ± 0.00008^{u}
100 mM mannitol+0.5 ppm GO	0.0552 ± 0.0001^{f}	0.0293 ± 0.00009^{t}
100 mM mannitol+1.5 ppm GO	0.1071 ± 0.0001^{b}	0.1280 ± 0.00004^{a}
100 mM mannitol+1 ppm CaO+0.5 ppm GO	0.1040 ± 0.0006^{b}	0.0135 ± 0.00006^{v}
100 mM mannitol+2 ppm CaO+0.5 ppm GO	0.0261 ± 0.0001^{kl}	0.1032 ± 0.00006^{f}
100 mM mannitol+2 ppm CaO+1.5 ppm GO	0.1061 ± 0.0001^{b}	0.0787 ± 0.00006^{lm}

1: Control, 2: 1 ppm CaO, 3: 2 ppm CaO, 4: 0.5 ppm GO, 5: 1.5 ppm GO, 6: 1 ppm CaO+0.5 ppm GO, 7: 1 ppm CaO+1.5 ppm GO, 8: 2 ppm CaO+0.5 ppm GO, 9: 2 ppm CaO+1.5 ppm GO, 10: 1/2 ppm CaO+0.5/1.5 ppm GO, 11: 50 mM mannitol, 12: 50 mM mannitol/1 ppm CaO, 13: 50 mM mannitol/2 ppm CaO, 14: 50 mM mannitol/0.5 ppm GO,15: 50 mM mannitol/1.5 ppm GO, 16: 50 mM mannitol+1 ppm CaO+0.5 ppm GO, 17: 50 mM mannitol+2 ppm CaO+0.5 ppm GO, 18: 50 mM mannitol+2 ppm CaO+1.5 ppm GO, 19: 100 mM mannitol, 20: 100 mM mannitol+1 ppm CaO, 21: 100 mM mannitol+2 ppm CaO, 22: 100 mM mannitol+0.5 ppm GO, 23: 100 mM mannitol+1.5 ppm GO, 24: 100 mM mannitol+1 ppm CaO+0.5 ppm GO, 25: 100 mM mannitol+2 ppm CaO+0.5 ppm GO, 26: 100 mM mannitol+2 ppm CaO+1.5 ppm GO

M.sativa cultivars Erzurum and Konya displayed important callus formation, but the capacity for callus formation and embryogenic differentiation reduced under mannitol stress in both cultivars.

Increased levels of mannitol (50 and 100 mM) were used to induce drought stress. There was decline in callus formation percentage and embryogenic maintenance frequency with increasing levels of mannitol stress. Our results verify the findings of Abdelsalam et al. (2022) where they displayed an expected decline in development of sugarcane callus on mannitol stress medium. They also displayed that the effect of callus induction varied importantly on the genotype and mannitol concentrations. At higher concentrations, GO increased the fresh weight of callus compared to other treatments. However, the expected increase in CaO was not observed. These results displayed that *M.sativa* callus which were treated with GO alleviated severe stress and withstood mannitol better than CaO and mannitol alone. This is consistent with earlier the reports displaying that callus fresh weight is highly linked to genotypes Errabii et al. (2007) who detected a reduce in the water content and a radical effect on Na^+ concentrations in sugarcane callus under both mannitol and NaCl. Proline contents were affected in the long-term and short-term callus of two *M.sativa* cultivars both in 0.5 ppm and 1.5 ppm conditions of GO and CaO after mannitol treatments. A slightly higher proline was detected in the mannitol treatment in the Konya cultivar than Erzurum. The highest value was found in 1.5 ppm GO (2.217 mg/g FW) in Erzurum cultivar and 100 mM mannitol + 2 ppm CaO + 0.5 ppm GO (1.279 mg/g FW) in Konya cultivar (Figure 1). The stress severity development in response to mannitol treatments was at different levels; Konya cultivar exhibited a enough elimination of drought stress severity that was mostly due to a higher activity of Ca^{2+} NPs while that of the Erzurum cultivar was slowly increased, which could likely be a outcome of interactions between nanoparticule and plant growth regulators in the growth medium. These indicate that genotype of tolerance responses to stress factors can be important factor.

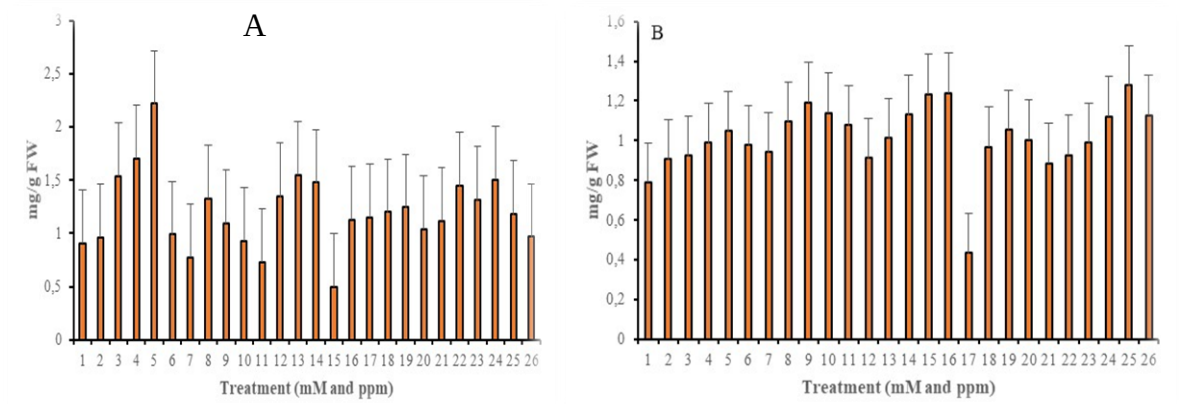

Figure 1. The contents of proline of two varieties affected by drought **(A)** The accumulation of proline of Erzurum *M. sativa* cultivar during drought stress. **(B)** The accumulation of proline of Konya *M. sativa* cultivar during drought stress1 Control, 2: 1 ppm CaO, 3: 2 ppm CaO, 4: 0.5 ppm GO, 5: 1.5 ppm GO, 6: 1 ppm CaO+0.5 ppm GO, 7: 1 ppm CaO+1.5 ppm GO, 8: 2 ppm CaO+0.5 ppm GO, 9: 2 ppm CaO+1.5 ppm GO, 10: 1/2 ppm CaO+0.5/1.5 ppm GO, 11: 50 mM mannitol, 12: 50 mM mannitol/1 ppm CaO, 13: 50 mM mannitol/2 ppm CaO, 14: 50 mM mannitol/0.5 ppm GO,15: 50 mM mannitol/1.5 ppm GO, 16: 50 mM mannitol+1 ppm CaO+0.5 ppm GO, 17: 50 mM mannitol+2 ppm CaO+0.5 ppm GO, 18: 50 mM mannitol+2 ppm CaO+1.5 ppm GO, 19: 100 mM mannitol, 20: 100 mM mannitol+1 ppm CaO, 21: 100 mM mannitol+2 ppm CaO, 22: 100 mM mannitol+0.5 ppm GO, 23: 100 mM mannitol+1.5 ppm GO, 24: 100 mM mannitol+1 ppm CaO+0.5 ppm GO, 25: 100 mM mannitol+2 ppm CaO+0.5 ppm GO, 26: 100 mM mannitol+2 ppm CaO+1.5 ppm GO

GO treatments demonstrated a high impact of drought stress that was mostly due to common physiological properties whereas CaO was gradually promoted. A high concentration of GO and CaO NPs can importantly increase the content of the proline. Therefore, protective traits of CaO and GO NPs are dependent on the type of NPs and concentration. Similar results were detected in terms of proline accumulation, which is an effective method for stressed plant tissues and cells (Elrys et al. 2020; Sitohy et al. 2020). Among two cultivars, a greatly higher MDA activity was detected

in Erzurum cultivar 1/2 ppm CaO+0.5/1.5 ppm GO (0.1849 mg/g FW) whereas Konya cultivar 1 ppm CaO (0.1742 mg/g FW) showed a lower trend in mannitol treatments *in vitro*, conditions. There was a detectable difference between nanoparticle concentration and mannitol treatments. The MDA lowest activity was found to 1/2 ppm CaO+0.5/1.5 ppm GO (0.0662 mg/g FW) in the Konya cultivar while the lowest activity was found 1.5 ppm GO (0.0516 mg/g FW) in Erzurum cultivar (Figure 2).

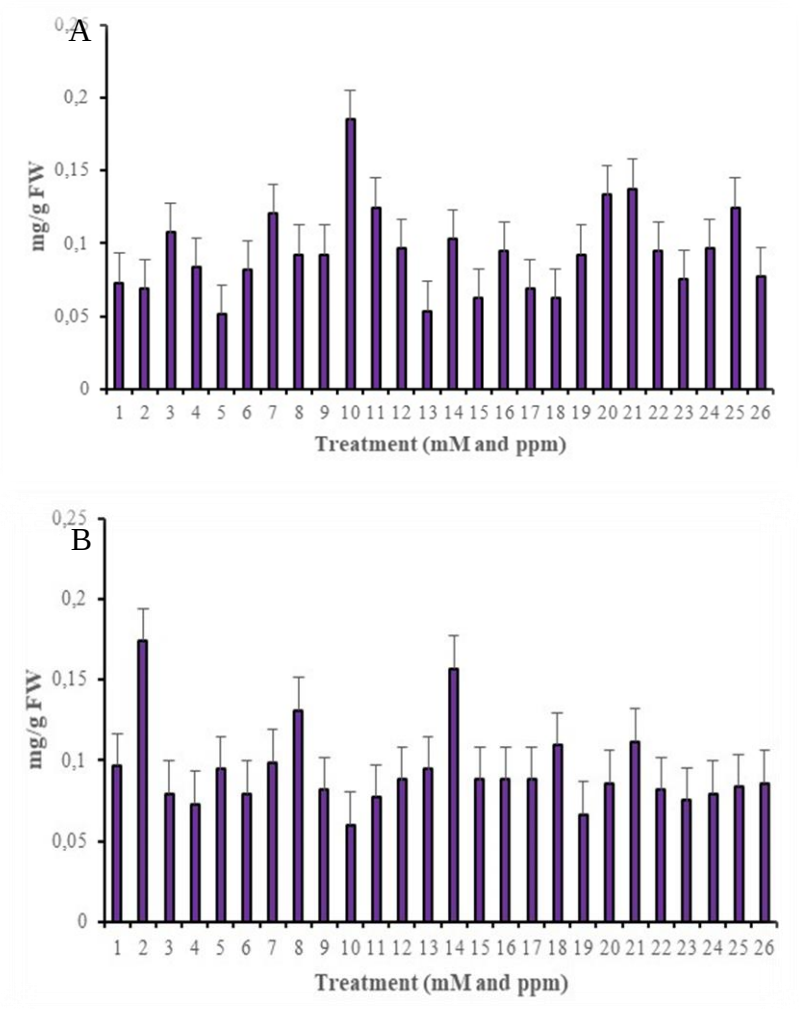

Figure 2. The contents of MDA of two varieties affected by drought **(A)** The accumulation of MDA of Erzurum *M. sativa* cultivar during drought stress. **(B)** The accumulation of MDA of Konya *M. sativa* cultivar during drought stress1 Control, 2: 1 ppm CaO, 3: 2 ppm CaO, 4: 0.5 ppm GO, 5: 1.5 ppm GO, 6: 1 ppm CaO+0.5 ppm GO, 7: 1 ppm CaO+1.5 ppm GO, 8: 2 ppm CaO+0.5 ppm GO, 9: 2 ppm CaO+1.5 ppm GO, 10: 1/2 ppm CaO+0.5/1.5 ppm GO, 11: 50 mM mannitol, 12: 50 mM mannitol/1 ppm CaO, 13: 50 mM mannitol/2 ppm CaO, 14: 50 mM mannitol/0.5 ppm GO,15: 50 mM mannitol/1.5 ppm GO, 16: 50 mM mannitol+1 ppm CaO+0.5 ppm GO, 17: 50 mM mannitol+2 ppm CaO+0.5 ppm GO, 18: 50 mM mannitol+2 ppm CaO+1.5 ppm GO, 19: 100 mM mannitol, 20: 100 mM mannitol+1 ppm CaO, 21: 100 mM mannitol+2 ppm CaO, 22: 100 mM mannitol+0.5 ppm GO, 23: 100 mM mannitol+1.5 ppm GO, 24: 100 mM mannitol+1 ppm CaO+0.5 ppm GO, 25: 100 mM mannitol+2 ppm CaO+0.5 ppm GO, 26: 100 mM mannitol+2 ppm CaO+1.5 ppm GO

MDA content is known to be a marker of oxidative stress. In *M.sativa* callus, It was importantly increased under mannitol stress treatments and the impacts of nanoparticles on MDA content are well related with the drought resistance ability. A similar result of MDA activity of triticale callus with CaO nanoparticle treatments under salt stress was also reported (Yazıcılar et al. 2021).

The amount of H_2O_2 in two tested cultivars was significantly affected by mannitol stress. Erzurum 100 mM mannitol+0.5 ppm GO (0.1017 mg/g FW) was higher than Konya 100 mM mannitol+1 ppm CaO+0.5 ppm GO (0.0690 mg/g FW). The lowest H_2O_2 amount was found in only treatments of 100 mM mannitol (0.0567 mg/g FW) in Erzurum cultivar, the lowest membrane damage

was found in concentration of 100 mM mannitol+2 ppm CaO+0.5 ppm GO (0.0543 mg/g FW) in Konya cultivar (Figure 3).

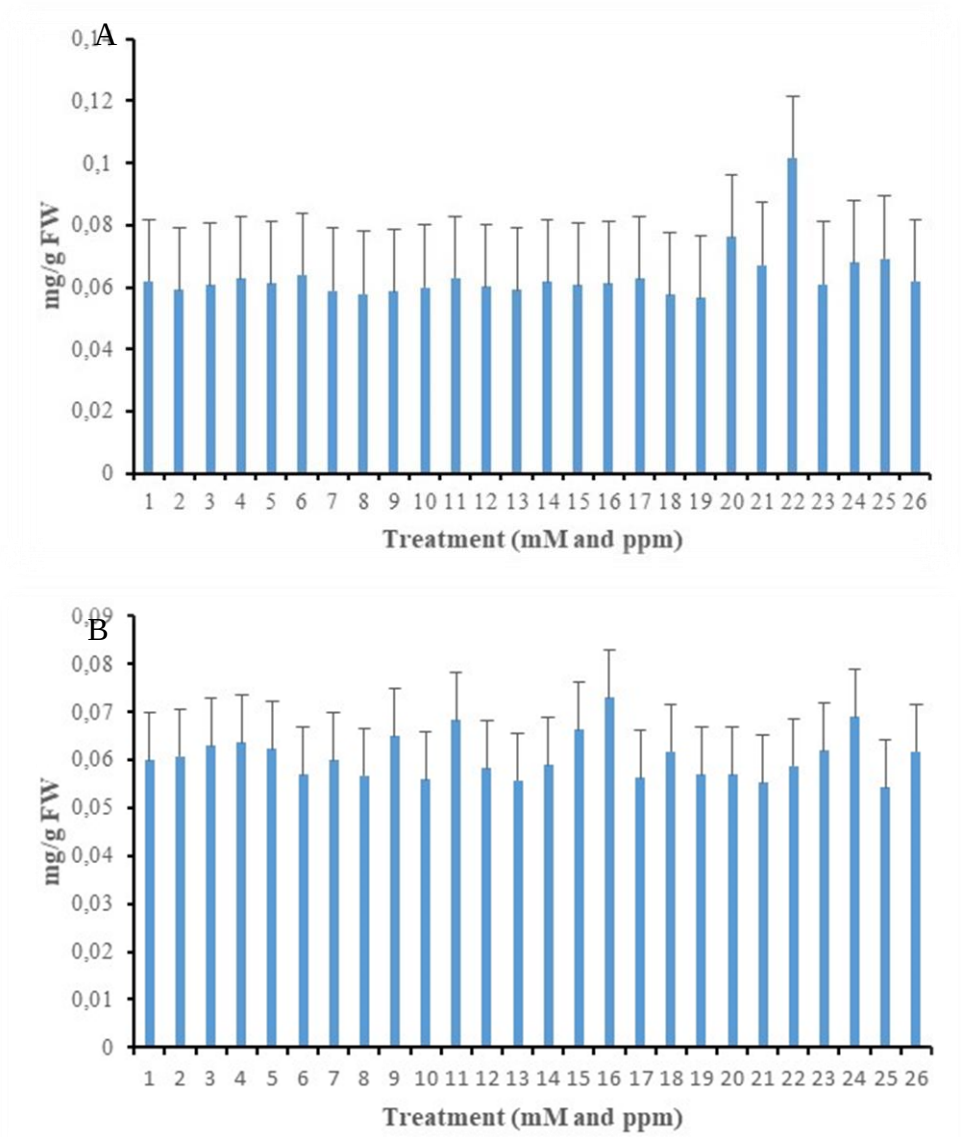

Figure 3. Changes of H_2O_2 level in two *M. sativa* varieties after drought treatment **(A)** The accumulation of H_2O_2 of Erzurum *M. sativa* cultivar during drought stress. **(B)** The accumulation of H_2O_2 of Konya *M. sativa* cultivar during drought stress

1: Control, 2: 1 ppm CaO, 3: 2 ppm CaO, 4: 0.5 ppm GO, 5: 1.5 ppm GO, 6: 1 ppm CaO+0.5 ppm GO, 7: 1 ppm CaO+1.5 ppm GO, 8: 2 ppm CaO+0.5 ppm CaO+1.5 ppm GO, 10: 1/2 ppm CaO+0.5/1.5 ppm GO, 11: 50 mM mannitol, 12: 50 mM mannitol/1 ppm CaO, 13: 50 mM mannitol/2 ppm Ca mannitol/0.5 ppm GO,15: 50 mM mannitol/1.5 ppm GO, 16: 50 mM mannitol+1 ppm CaO+0.5 ppm GO, 17: 50 mM mannitol+2 ppm CaO+0.5 ppm G mannitol+2 ppm CaO+1.5 ppm GO, 19: 100 mM mannitol, 20: 100 mM mannitol+1 ppm CaO, 21: 100 mM mannitol+2 ppm CaO, 22: 100 mM mannitol 23: 100 mM mannitol+1.5 ppm GO, 24: 100 mM mannitol+1 ppm CaO+0.5 ppm GO, 25: 100 mM mannitol+2 ppm CaO+0.5 ppm GO, 26: 100 mM m CaO+1.5 ppm GO

GO NPs was more quickly H_2O_2 accumulated by mannitol than in CaO, the GO NPs hormone interaction increased as a result of mannitol severity since the ionic stress stimulated by the exogenous mannitol treatments. The H_2O_2 values in the GO-supplied callus are remarkably greater than in the control- and CaO supplied callus under mannitol stress conditions. This is proved that the GO-supplied callus absorbed water efficiently and prevented water loss. The impact of higher levels of GO-NPs has been reported in agreement with the findings obtained in stem, leaves and root growth of plant species, including soybean, by Wu et al. (2016) and Zhao et al. (2015).

CONCLUSION

We detected that GO and CaO NPs *M.sativa* callus demonstrated higher tolerance to 0.5 and 1.5 ppm than control *M.sativa* callus and that the analysis of proline, MDA and H_2O_2 displayed that activities in callus is probable an active process induced by subject to mannitol stress. The treatment GO NPs and CaO callus developed seem to benefit in tolerant drought stress. Moreover; callus fresh weight and dry weight seem to be basic for callus growth and development under drought stress. In future, drought resistant alfalfa plants can be developed using especially GO NPs under *in vitro* conditions.

Conflict of Interest

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

Author's Contributions

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

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