

METABOLIC RESPONSES TO IN VITRO IN DROUGHT-TOLERANT IN A *Cucurbita pepo* L. ELICITED BY SALICYLIC ACID AND ZINC OXIDE NANOPARTICLES

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ABSTRACT

This experiment was conducted to investigate the effect of different zinc oxide nanoparticle (ZnONPs) concentrations and salicylic acid (SA) on growth characteristics and antioxidant defence from *Cucurbita pepo* L. callus culture in response to drought stress conditions. The study included two levels of drought induced by sorbitol (0 and 20 g l⁻¹), two concentration of SA (0 and 20 mg l⁻¹), and four concentrations of ZnONPs (0, 100, 200, and 300 ppm). Drought stress by sorbitol showed negative effects on some characteristics of callus culture fresh weight (FW), dry weight (DW), and hydrogen peroxide (H₂O₂). The exogenous SA showed a positive in most characteristics except for DW, H₂O₂ and CAT. ZnONPs positively affected all study indicators on tissue culture under drought conditions. The exogenous of both SA and ZnONPs without sorbitol increased significantly in FW and DW. The SA and ZnO NPs with sorbitol increased significantly in biochemical characteristics such as H₂O₂, SOD, CAT, proline, and phenolic compounds such as coumaric acid, ferulic acid, caffeic acid, luteolin, and rutin.

Keywords: antioxidant systems, secondary metabolites, sorbitol, plant growth regulators.

ابراهيم ونعمة

مجلة العلوم الزراعية العراقية -2023: 54(5):1223-1233

الاستجابات الأيضية لمقاومة الجفاف خارج الجسم الحي في *Cucurbita pepo* L. المُحفز بواسطة حامض الساليسيليك وجسيمات أكسيد الزنك النانوية

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المستخلص

أجريت التجربة لمعرفة تأثير التراكيز المختلفة من جسيمات أكسيد الزنك النانوي وحامض الساليسيليك على صفات النمو والاستجابات الدفاعية المضادة للأكسدة في مزارع الكالس لنبات *Cucurbita pepo* L. تحت ظروف الجفاف. تضمنت الدراسة مستويين من الجفاف تم استحداثه باستعمال السوربيتول هما 0 و 20 غم لتر⁻¹، وتركيزين من حامض الساليسيليك هما 0 و 20 ملغم لتر⁻¹، وأربعة تراكيز من جسيمات الزنك النانوي 0، 100، 200، 300 جزء في المليون. أظهر إجهاد الجفاف تأثيرات سلبية على بعض الصفات مثل الوزن الطري والوزن الجاف لمزارع الكالس ومحتواها من بيروكسيد الهيدروجين. أظهرت المعاملة بحامض الساليسيليك دوراً إيجابياً في معظم الصفات باستثناء الوزن الجاف ومحتوى hydrogen peroxide وفعالية أنزيم catalase. أثرت المعاملة بجسيمات أكسيد الزنك النانوي بشكل إيجابي على جميع مؤشرات الدراسة على مزارع الكالس في ظل ظروف الجفاف. زادت المعاملة بكل من حامض الساليسيليك وأكسيد الزنك النانوي بدون السوربيتول بشكل ملحوظ في الوزن الطري والجاف للكالس. فيما زادت المعاملة بحامض الساليسيليك وأكسيد الزنك النانوي مع السوربيتول بشكل ملحوظ في الخصائص الكيميائية الحيوية مثل محتوى hydrogen peroxide وفعالية أنزيم superoxide dismutase وفعالية أنزيم catalase ومحتوى البرولين والمركبات الفينولية مثل coumaric acid و ferulic acid و caffeic acid و luteolin و rutin.

الكلمات المفتاحية: الأنظمة المضادة للأكسدة، الأيض الثانوي، السوربيتول، منظمات نمو النباتية.

Received:27/9/2021 Accepted:23/1/2022

INTRODUCTION

Cucurbita pepo L. is considered an important plant of the Cucurbitaceae family (15). Its nutritional and pharmacological importance is due to its high content of proteins, carbohydrates, vitamins, unsaturated fatty acids, minerals and dietary fiber (20). As well as its therapeutic properties such as antioxidant, anti-inflammatory, anti-tumour, anti-bacterial, anti-intestinal parasite, anti-hypertensive, anti-hypercholesterolemic, and immunomodulatory effects. These roles are derived from its rich content of chemical compounds, including stanols, carotenoids, polyphenols, tocopherols, and sterols (11, 19). Plant tissue culture technology is the most suitable for overcoming the problems caused by drought stress, as it is characterized by accuracy, simplicity, economical and highly applicable. Consequences of implementing scientific experiments include the cultivation season and the areas allocated for agricultural land (28). As well as the possibility of producing medicinally effective compounds without adhering to the limitations imposed by conventional agriculture (43). Drought stress is one of the most dangerous abiotic stresses to which plants are exposed during their life cycle. The danger caused by the ability of drought to cause many metabolic changes in the plant, which reflect changes in the growth, physiological, biochemical and secondary metabolites characteristics of the plant (27, 44). Polysaccharides, including sorbitol, can conduct experiments related to drought stress *in vitro* through their ability to increase the osmotic pressure in tissue cultures, thus reducing water absorption by tissue cultures. This is accompanied by many metabolic changes reflected in tissue cultures physiological and then morphological effects (31). Drought is one of the causes of oxidative stress in living cells, resulting in an increase in free radicals represented by reactive oxygen species (ROS) and a disturbance in the antioxidant system. ROS damage occurs in cell membranes, which leads to damage to cellular components and cell death. Therefore, free radical scavenging should be resorted to by stimulating the antioxidant system represented by the enzymatic system such as SOD and CAT and non-enzymatic such as

polyphenols (10, 28, 38). Plant hormones such as salicylic acid play an essential role in developmental processes (3, 5, 23, 30, 39) through their influential role in protecting the plant cell from the negative impact of various stresses (4). This hormone acts as an essential signaling molecule against various abiotic stresses. This exogenous SA increases biomass and tolerance to different stresses (17, 36). Nanotechnology is a promising technology that allows materials with sizes less than 100 nanometers (41). It can be used in multiple fields, such as agriculture and industry (29). It can be applied in agriculture through its efficient use of plant nutrition and protection from the impact of various stresses. It also stimulates the vital pathways responsible for producing secondary compounds that often have defensive roles for plants (9, 25). Zinc is a micronutrient. It is key in many biochemical reactions, including stimulating vital systems and water balance inside the cell. Numerous reports have described the positive roles of ZnONPs in protecting the cell from the effects of abiotic stress and the resulting oxidative stress (40). Based on the previous, the main objective of the current study is to investigate the ability of SA and ZnONPs cause metabolic changes in *C. pepo* L. tissue cultures. The effect of this change on the biochemical and physiological characteristics and growth under normal conditions and shortage of water content.

MATERIALS AND METHODS

Seed surface sterilization: *C. pepo* L. seeds were provided from the Department of Horticulture, College of Agriculture, University of Anbar. The seeds were washed under running tap water for 30 min. The seeds were surface sterilized in 70% (v/v) ethanol for 20 sec. Then, 33% (v/v) sodium hypochlorite (NaOCl) solution for 20 min washed with sterile distilled water three consecutive times. This and all subsequent operations were performed in a laminar flow cabinet under sterile conditions (13).

***C. pepo* L. seeds germination**

The seeds Sterilized were grown in vials of 7 ml of MS media (24) free of plant growth regulators. The primary root was excised from two-week-old seedlings as the explant to

induce callus.

Callus induction

The explants of *C. pepo* L. were cut to 1 cm and cultured on MS media supplemented with several regulating hormones such as NAA, kinetin, and 2,4-D at (0.25, 0.50, and 2.00 mg l⁻¹), respectively. They were incubated at 26 ±1°C under a photoperiod of 1000 lux for 16 h.

ZnONPs characterization and treatments

The characterization data of ZnONPs revealed

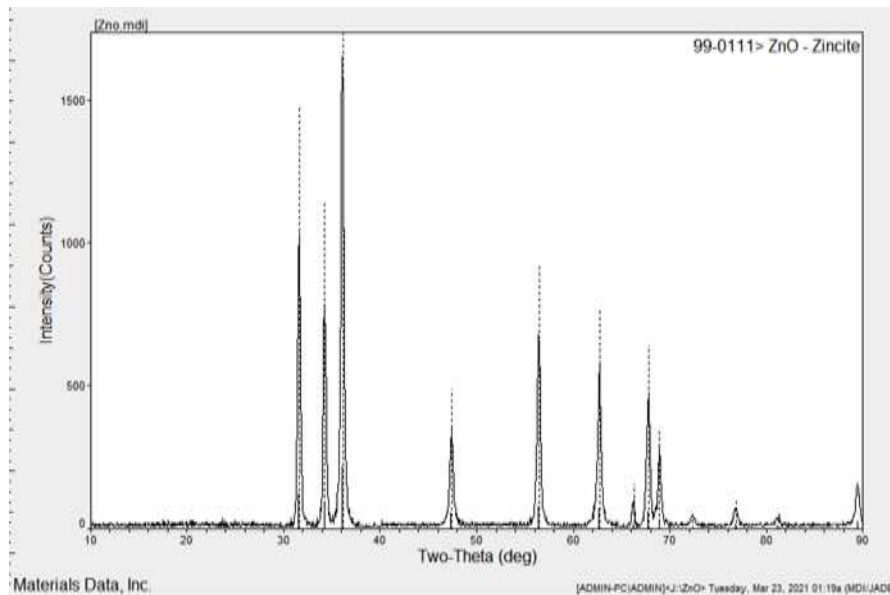


Figure 1. XRD pattern of ZnONPs

FW and DW of callus culture The fresh weight of the callus samples was calculated after four weeks of growth in the medium, as the remnants of the suspended media were removed to calculate the weight. In contrast, the callus samples were dried in an oven at 60°C for 48 h to determine the dry weight.

H₂O₂ determination

The contents of H₂O₂ were calculated based on the method of Azzedine et al. (6). In short, 100 mg of fresh callus were homogenized in trichloroacetic acid (0.1%) and centrifuged at 12,000 × g for 15 min. Then, 0.5 ml. of the supernatant was added to 0.5 ml of phosphate buffer (10mM, pH 7.0) and 1 ml of KI 1000µl (1 M). The absorbance of the solution was measured at 390 nm.

Estimation of antioxidant enzymes activities

Antioxidant enzymes were determined. Briefly, 1.0 g of fresh callus was mixed with K-phosphate buffer (50 mM, pH 7). Then, it was filtered and centrifuged (10,000 × g, 15 min, 4 °C).

SOD enzyme activity: SOD enzyme activity

the size of nanoparticles as 30 nm. The details of the ZnONPs characterization are observed in Figure 1. Callus culture were grown at 3 months in MS media that differed in their chemical content. They were treated with two concentrations of salicylic acid at 0 and 20 mg l⁻¹, and four ZnONPs at 0, 100, 200, and 300 ppm. Their effect was determined under normal and water-deficient conditions by adding 20 g of sorbitol.

was determined based on the method of Beyer and Fridovich (8). The reaction mixture consisted of some solutions. The absorbance was recorded at a wavelength of 560 nm.

CAT enzyme activity

The CAT enzyme activity was estimated based on the method of Aebi (2). The reaction mixture consists of 15 mM hydrogen peroxide with (50 mM, pH 7.0) potassium phosphate buffer solution mixed with 50 µl of enzyme extract. The reading at wavelength 240 was done every 30 s for 3 min.

Proline content

The proline content of callus culture was estimated based on the report of Bates et al. (7). Briefly, 0.2 g of dried callus per sample was mixed with sulfosalicylic acid (5 ml, 3% v/w). After the centrifugation (2,000 × g, 10 min). The supernatant of 2 ml was added to 2 ml of ninhydrin. After heating the mixture at 100 °C for 30 min, the fractions were cooled. The concentration of proline was determined using spectrophotometry at 250 nm.

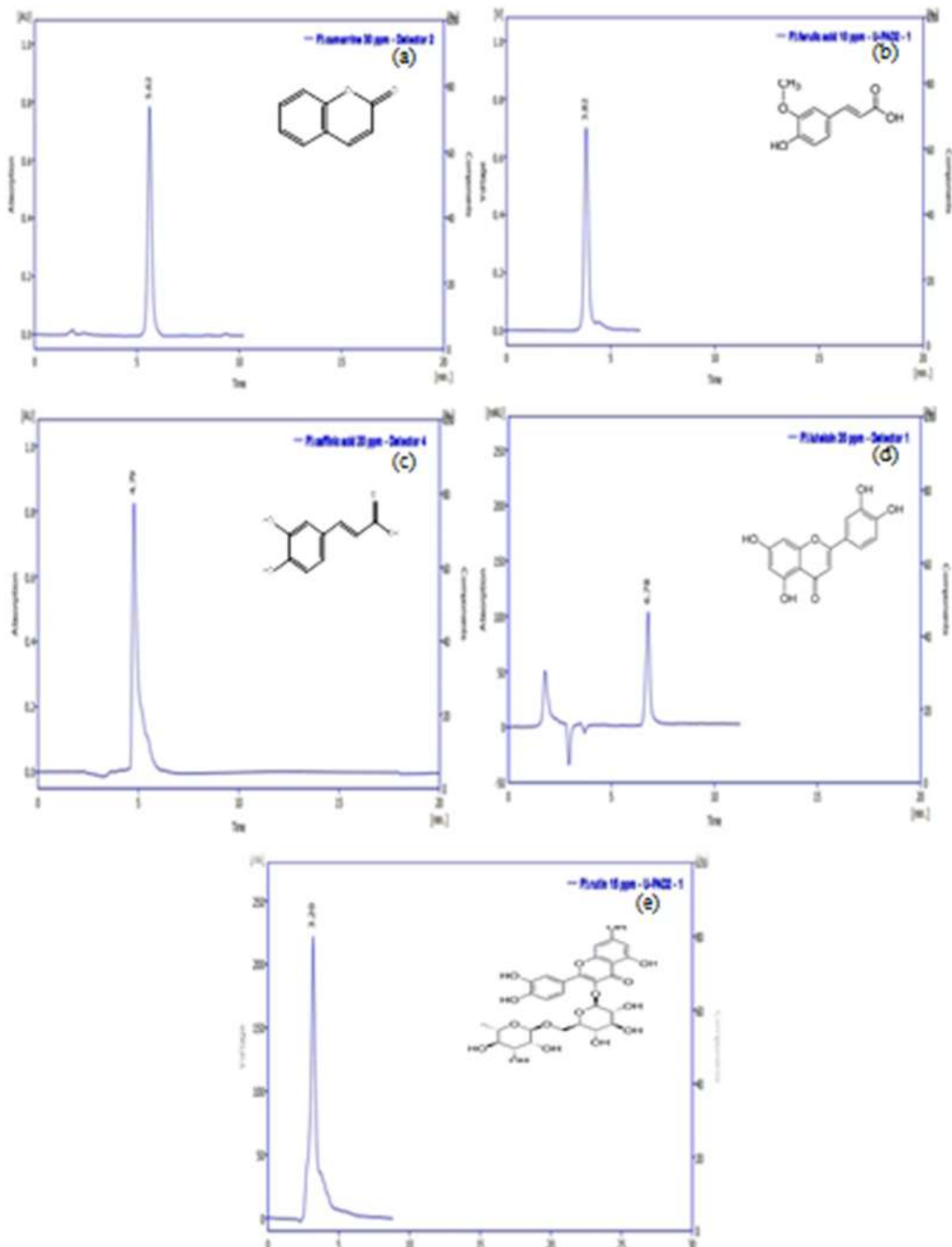


Figure 2. The standard curve of phenolic compounds includes (a) coumaric acid, (b) ferulic acid, (c) caffeic acid, (d) luteolin (e), and (e) rutin.

Extraction and HPLC analysis of phenolic compounds: *C. pepo* L. extract was prepared by soaking 2.0 g of dried callus powder at 40 °C in 15 ml of chloroform and 10 ml of hexane and stirring for 10 h. Then the extract was placed in an ultrasonic cracker for 20 min at 45°C. Then 25 mL of methanol is added and transferred to a separating funnel (26). The polar organic layer of methanol was collected

and moved to a rotary evaporator to obtain a dry extract. Samples were analyzed according to the method (22) using the high-performance liquid chromatography (HPLC) Model (SYKAM) Germany. The mobile phase was (methanol: distilled water: formic acid) (70:25:5), the column was C18-ODS (25 cm×4.6 mm). The UV reagent was 280 nm at a flow rate of 1.3 ml.min⁻¹. The content of the

polyphenols shown in Figure 2 was estimated based on the formula described by Mradu et al., (22).

Statistical analyses

Statistical analysis was performed according to a complete randomized design (CRD) with three replicates. Data were subjected to data analysis using analysis of variance (Three-way ANOVA) using the Genstat software. The value of the least significant difference (LSD) between the means was calculated based on the probability level of $p < 0.05$.

RESULTS AND DISCUSSION

Biomass accumulation and biochemical characteristics: The data indicate a change in the biomass and biochemical characteristics of callus culture due to treatment with sorbitol, SA, and ZnONPs. The FW and DW results showed that the concentration of 0 mg l⁻¹ of SA and ZnONPs 300 ppm free of sorbitol had higher mean FW and DW values, reaching 593.3 and 40.92 mg, respectively. It increased significantly ($p < 0.05$) from all SA and ZnONPs treatments. The lowest mean values of FW and DW at 20 g l⁻¹ of sorbitol, 20 mg l⁻¹ of SA, and 100 ppm of ZnONPs were 310.0 and 21.38 mg, respectively (Fig. 3a,b). The decrease of biomass accumulation of callus culture in a *C. pepo* L. is because of the osmotic pressure caused by sorbitol (35). Whereas the treatment of calli with SA and ZnONPs increased the tolerance of calli to the stress caused by sorbitol, which led to resistance to oxidative stress and thus an increase in biomass accumulation. These results are similar to those that showed an increase in the weight of callus culture due to SA-treated (1). The level of oxidative stress was determined in callus culture by calculating the H₂O₂ content as an indicator. The H₂O₂ content differed significantly ($P < 0.05$). Treatment with SA, ZnONPs and sorbitol-free

resulted in a significant increase in H₂O₂ of 10.06 $\mu\text{mol g}^{-1}$. In contrast, the lowest oxidative stress appeared when combining 20 g l⁻¹ sorbitol, 0 mg l⁻¹ SA and 300 ppm ZnONPs reached 4.394 $\mu\text{mol g}^{-1}$. The results showed that sorbitol treatment significantly increased H₂O₂ content in calli compared to untreated callus. This increase may be due to the role of sorbitol in inducing drought stress in treated tissues through the generation of free radicals. ROS causes damage to cell membranes due to its interaction with lipids in those membranes, resulting in damage to important cellular components (14). The present results are similar to previous studies showing the role of SA and ZnONPs (12, 46). The results showed that treatment with the combination containing sorbitol with a high concentration of both SA and ZnONPs showed that the highest significant level was 3.46 $\mu\text{mol g}^{-1}$. In contrast, the lowest proline content established the combination containing the comparison level of SA and ZnONPs products was 1.47 $\mu\text{mol g}^{-1}$ grown in media not containing sorbitol. Proline production is one of the defence mechanisms by which cells face dehydration stress to protect them from oxidative stress damage. This is due to its role in alleviating dehydration through osmotic modifications and thus increasing cell adaptation. This result was similar to previous studies showing the role of treatment with SA or ZnONPs in inducing proline production as a defence mechanism for cells of different plant species (12, 21, 32, 42).

Antioxidant enzymes activities

The SOD and CAT are the most important antioxidant enzymes through their role in efficiently protecting the cell from ROS damage and are used to detoxify H₂O₂. *The results indicated the interaction between high levels of SA and ZnONPs in increasing*

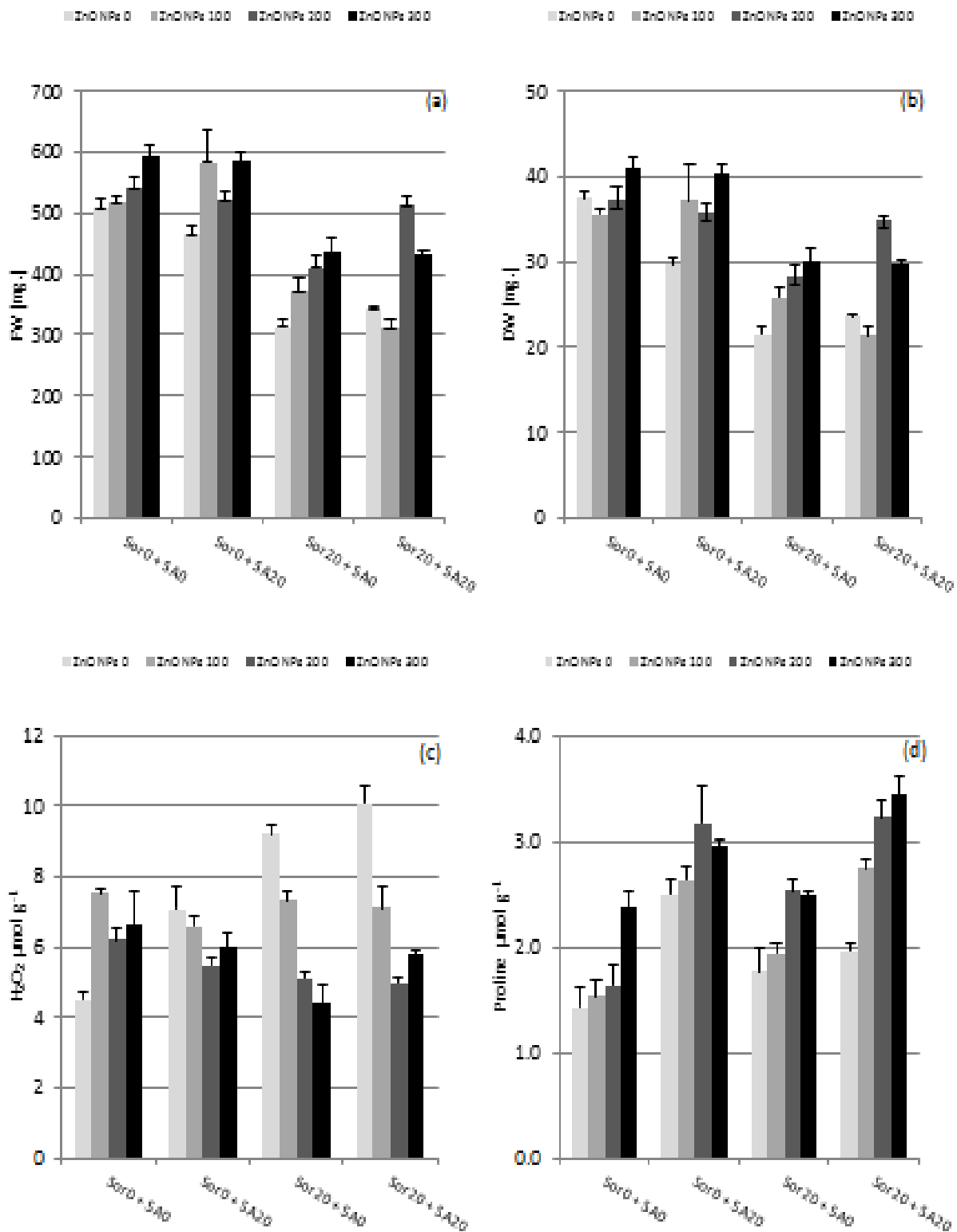


Figure 3. FW (a), DW (b), H₂O₂ content (c), and proline content (d) in a *C. pepo* L. callus culture after 28 days in MS media containing Sorbitol, SA and ZnONPs under normal conditions. Vertical bars show mean ± S.E.

The activity of the enzyme SOD is due to its role in generating the expression of the gene responsible for the induction of enzymes antioxidant, such as SOD, which protects the cell from the effect of oxidative stress. Yordanova and Popova (45) found that with

the exogenous treatment of wheat with SA, the SOD activity also increased the results, similar to the study. In previous studies, *Brassica juncea* treated under various concentrations of ZnONPs increased antioxidant enzymes, including SOD (32). The results indicated SA

and ZnONPs at the combination of 0 mg l⁻¹ SA and 200 ppm ZnONPs with sorbitol showed the highest level of CAT was 22.74 U mg⁻¹ protein min⁻¹ compared with other treatments. The lowest significant activity was at control treatments of 8.91 U mg⁻¹ protein min⁻¹ (Fig. 4b). The CAT is one of the essential antioxidant defence enzymes which plays an indispensable role in detoxifying H₂O₂ by converting the ROS to H₂O and O₂. The data showed that treatment with sorbitol could lead

to the induction of the cells' self-defence systems, especially with the presence of nanomaterials, which plays a prominent role in increasing the activity of the CAT enzyme, which inhibits the generation of reactive oxygen species. These results are similar to previous studies that showed the ability to induce CAT activity by exogenous SA (37). At the same time, reports indicated the ability of ZnONPs to influence the activity of CAT (16).

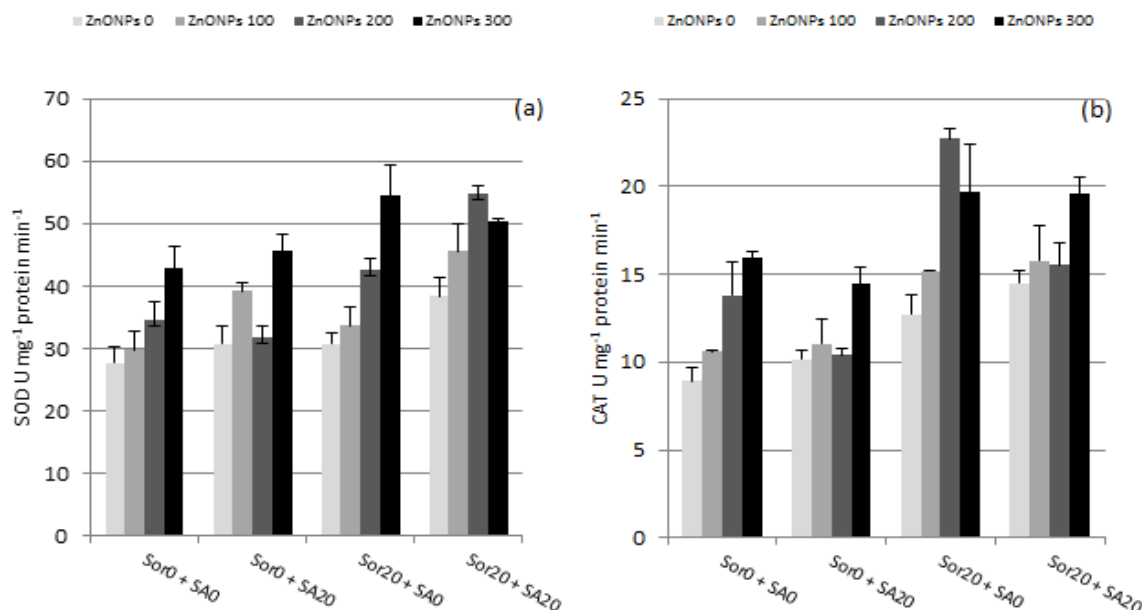


Figure 4. SOD activity (a), and CAT activity (b) in a *C. pepo* L. callus culture after 28 days in MS media containing Sorbitol, SA and ZnONPs under normal conditions. Vertical bars show mean \pm S.E

Antioxidant non-enzyme activity

Polyphenols are classified as non-enzymatic antioxidants, which can scavenge free radicals and protect cells from the effects of oxidative stress. The results demonstrated a prominent role of ZnONPs in improving coumaric acid production by increasing nanomaterial concentration. The concentration of 300 ppm in the presence of sorbitol and SA achieved the highest production of the compound, reaching 349.85 $\mu\text{g ml}^{-1}$, an increase of about 13.62-, 1.48- and 1.34- fold for the nanomaterial concentrations of 0, 100 and 200 ppm, respectively. Also, the combination of sorbitol and SA didn't increase coumaric acid production without treatment with the nanomaterial (Fig. 5a). As well as, the level of effect of ZnONPs was significant in ferulic acid production. Especially with a 20 g l⁻¹ sorbitol concentration and 20 mg l⁻¹ SA. Which didn't significantly affect the

production of this compound except in the presence of nano-zinc oxide to reach the highest level of production 337.84 $\mu\text{g ml}^{-1}$ at the high concentration of all study factors (Fig. 5b). The production of caffeic acid was significantly affected by the study factors. The triple combination (Sorbitol 20 +SA 20 + ZnONPs 300) had a significant effect in increasing the production of the compound to 401.53 $\mu\text{g ml}^{-1}$, superior to the rest of the concentrations of ZnONPs 0, 100, and 200 ppm in which the production of the compound with the same concentrations of sorbitol and SA reached 26.61, 257.84, 315.38 $\mu\text{g ml}^{-1}$, respectively (Fig. 5c). Also, luteolin levels increased under different concentrations of ZnONPs. They were significantly higher in all samples treated with sorbitol and SA, reaching in (Sorbitol 20 + SA 20 + ZnONPs 300) 335.07 $\mu\text{g ml}^{-1}$ compared to those treated with sorbitol or SA alone (Fig. 5d). The study

factors significantly affected in production of rutin compound. The SA-pretreated in sorbitol-supplemented media was $53.3 \mu\text{g ml}^{-1}$. The highest rutin levels occurred when tissue cultures of *C. pepo* L. were treated with 300 ppm concentrations of ZnONPs by $369.22 \mu\text{g ml}^{-1}$ outperforming the fewer concentrations by about 6.9-, 1.4- and 1.3-fold, respectively (Fig. 5e). The enzymatic antioxidants in Figure 4 indicate that the exposure of callus cultures to stress factors such as sorbitol and salicylic didn't cause a change in secondary

metabolites. The nanomaterial induced changes in secondary metabolism. Thus, it caused an increase in the production of these compounds, including phenolic compounds known to be non-enzymatic antioxidants. Other authors, such as Iziy et al., (18), Regni et al., (33), and Salih et al., (34), have achieved similar results related to the role of exogenous of ZnONPs in increasing the phenolic compounds in a *Portulaca oleracea*, *Olea europaea*, and *Juniperus procera* respectively.

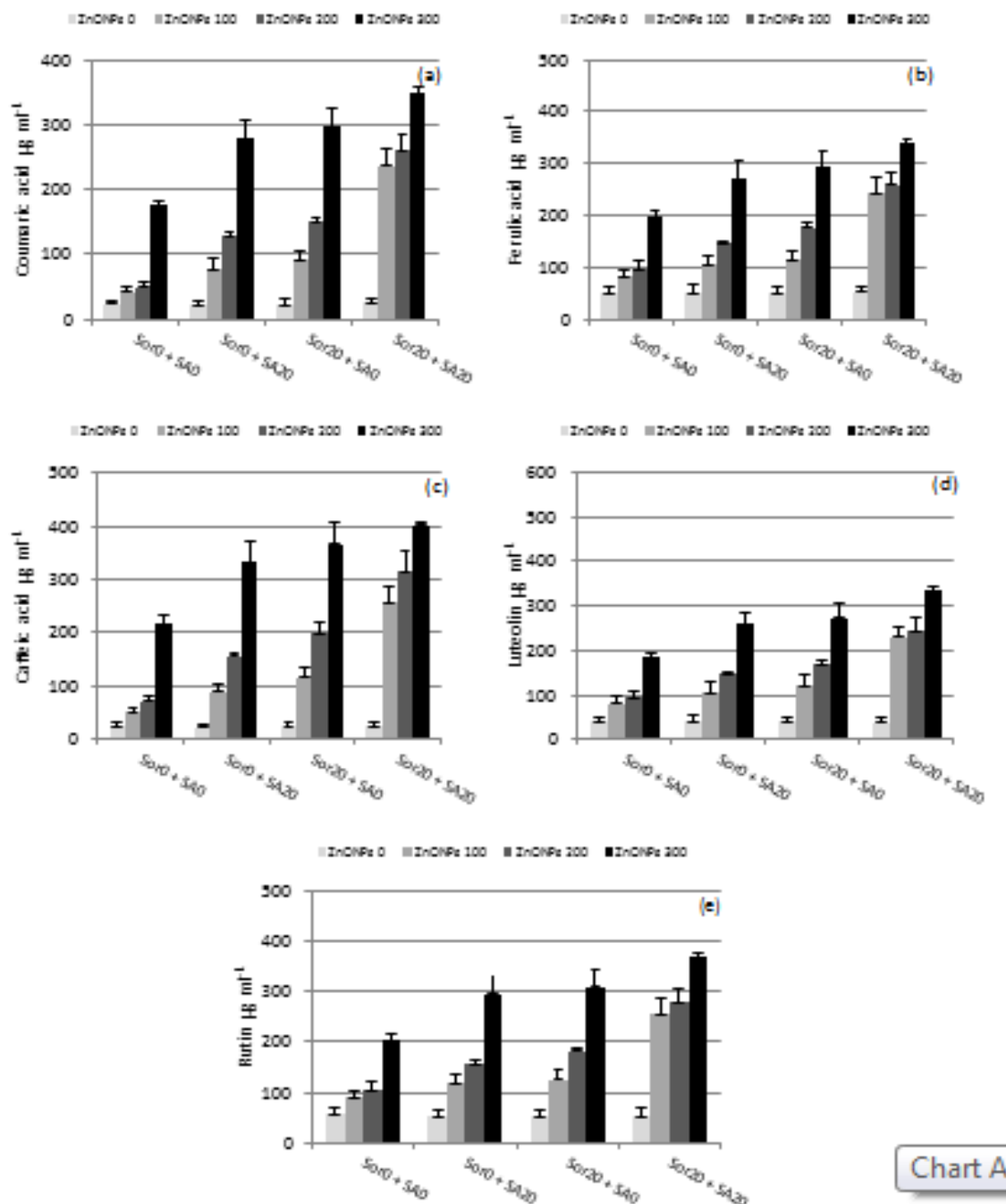


Figure 5. Coumaric acid (a), ferulic acid (b), caffeic (c), luteolin (d), and rutin (e) accumulation in a *C. pepo* L. callus culture after 28 day in MS media contains Sorbitol, SA and ZnONPs under normal conditions. Vertical bars show mean \pm S.E

CONCLUSION

The effect of sorbitol as a stress factor and exogenous of SA and ZnONPs on calli cultures of *C. pepo* L. callus was studied to determine their ability to induce oxidative stress and thus increase the production of secondary metabolites. The sorbitol showed an increase in free radicals (H_2O_2) and proline content production, which led to the strengthening of the defence systems of the cell by increasing the production of antioxidant enzymes such as SOD and CAT, and non-enzymatic antioxidants, including coumaric acid, ferulic acid, caffeic acid, luteolin and rutin. Simultaneously, SA and ZnONPs were considered maintenance factors for plant cells from oxidative stress. They up-regulated the ROS level and increased calli cultures' biomass accumulation. Due to the low costs, this approach has a significant role in producing medically effective compounds. The callus culture is in contact with the surface medium, which ensures the direct effect of elicitation.

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