Effect of Zinc Nanoparticles in Leaves Content of Proline and Non-Enzymatic Antioxidants of Sunflower *Helianthus annuus* L. under Water Stress

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ABSTRACT:

The experiment was carried out in the municipality nursery of Najaf Municipalities Directorate during the spring season 2018 to investigate the effect of zinc nanoparticles in the non-enzymatic antioxidants of sunflower plant under water stress and plant response to reduce the number of irrigation periods in the presence of zinc nanoparticles.The Randomized Complete Blocks Design was used in this experiment, which dealt with two factors and the results were compared according to the Duncan multiple test. The first factor in this experiment was the water stress factor (irrigation every 3 days, irrigation every 6 days and irrigation every 9 days) while the second factor represented the effect of using zinc (control , ZnSO4 and zinc nanoparticles (ZnO) in 100ppm,by spraying three times for both zinc(normal and nano).Water stress caused a significant increases in antioxidants such as proline, glutathione, flavonoids, anthocyanins and ascorbic acid. The results also showed that Zinc nanoparticles caused a significant increase in all studied traits, except the leaf content of ascorbic acid ASA, in which zinc in both types significantly decreased this trait.

Keywords: sunflower,water stress, IAA,GA3, Zinc nanoparticles,Antioxidants.

تأثير الزنك النانوي في محتوى الاوراق من البرولين ومضادات الاكسدة اللاانزيمية لنبات زهرة الشمس النامي تحت الاجهاد الامائي *Helianthus annuus* L.

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الخلاصة:-

نفذت التجربة في مشتل البلدية التابع لمديرية بلديات محافظة النجف الاشرف خلال الفصل الربيعي لعام 2018 لدراسة تأثير الزنك النانوي في مضادات الأكسدة اللاأنزيمية لنبات زهرة الشمس النامي تحت الاجهاد المائي واستجابة النبات لتقليل عدد مدد الري بوجود الزنك النانوي. صممت التجربة بحسب تصميم القطاعات العشوائية الكاملة Randomized Complete Blocks Design والتي تناولت عاملين ثم قورنت النتائج بحسب اختبار دنكن متعدد الحدود. كان العامل الأول في التجربة هو عامل الإجهاد المائي (الري كل 3 أيام ، والري كل 6 أيام والري كل 9 أيام) ، بينما يمثل العامل الثاني تأثير استخدام الزنك (مجموعة السيطرة ، $ZnSO_4.7H_2O$ ، والزنك النانوي ZnO) عن طريق الرش لثلاث مرات. سبب الإجهاد المائي زيادة ملحوظة في مضادات الأكسدة مثل البرولين ، والجلوتاثيون ، والفلافونويدات ، والأنثوسيانينات ، وحامض الأسكوربيك والالفا-توكوفيرول. وأظهرت النتائج أيضًا أن الزنك النانوي عزز هو الاخر من استجابة النبات للاجهاد المائي عن طريق الزيادة الملحوظة في مضادات الاكسدة قيد الدراسة باستثناء محتوى الاوراق من حامض الأسكوربيك ASA ، حيث خفض الزنك المستعمل وبنوعيه الاعتيادي والنانوي بشكل ملحوظ هذه الصفة.

الكلمات المفتاحية: الاجهاد المائي ، زهرة الشمس، الزنك النانوي ، البرولين، مضادات الاكسدة

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Introduction:

Sunflower is grown on an area of about 25 million hectares around the world with an annual output of approximately 36 million tons (1). The yield of sunflower is influenced by many factors, including a late seeding, seeding method, plants density, them individualization, solar radiation exposure, water stress and water use efficiency (2). One of the most important environmental obstacles that can be faced by crops in general, including sunflower plant in its life cycle is water stress, which restricts its ability to survive, productivity and reproduction, which varies the response of plants depending on the conditions ,the speed of stress , physiological condition and plant type (3). Undoubtedly, one of the main causes of water scarcity is the improper management of water resources in water-scarce areas and the lack of regular and equitable distribution of water, which is one of the most important problems limiting the productivity of these crops (4).

Zinc also plays a key role in the life of plants grown under various environmental stresses, including water stress, as its distinctive role in regulating the mechanisms of opening and closing stomata through its work in maintaining the integrity of the cellular membrane of the guard cells and maintaining the level of potassium in them (5).However, because the chemical fertilizers have negative effects on the environment and the quality and quantity of food produced, sustainable agriculture is now based on environmentally friendly techniques in crop production based on successful biological and physical treatments (6).These technologies are the nanotechnology that plays an important role in improving agricultural crop management and are a pioneering revolution in enhancing plants' ability to absorb nutrients, rapid detection of plant diseases and combating viruses and bacteria that infest those crops (7) . Kaya and Higgs(8) showed that zinc plays a central role in germination and chlorophyll synthesis and at the same time reduces the harmful effect of ROS during environmental stresses and its deficiency reduces plant resistance and tolerance to these stresses.

Babaeian *et.al.*(9) observed when they studied the effect of nutrients on sunflower plants grown under water stress that foliar application by zinc has caused an increase in leaf content of chlorophyll, grain yield and its components, especially in the flowering and grain filling stages, and is essential for the sugar and enzymatic regulation of plants. In a study of Seghatoleslami and Fortani(10) on sunflower plant, they used 7 levels of zinc, 3 of which represented zinc nanoparticles in concentrations (250, 500 and 1000 ppm) and tested on stressful plants, noted that the three concentrations of zinc oxide significantly affected the biomass. Zinc plays an important role in protecting plant cells from reactive oxygen species(ROS).

From the above, the aim of the study become clear, which is the study the tolerance of sunflower plant to water stress and the effect of foliar application with zinc nanoparticles in enhancing the plant's response to environmental stresses.

Materials and Methods:

The experiment was carried out in the municipality nursery of Najaf Al-Ashraf Municipalities Directorate during the spring season 2018 to investigate the effect of zinc nanoparticles on increasing the tolerance of sunflower plant under water stress.The experiment was designed in a Complete Randomized Blocks Design two factors: water stress and nanozinc.A total of 12 pilot units represent all the consensuses.The data were analyzed by Genstat program and the means were tested according Duncan's multiple teste at 5% probability.

Calculate the amount of water:

A drip irrigation system was used to irrigate plants consisting of plastic pipes connected to liquefied faucets containing holes at the rate of 9 holes per experimental unit (1.82 m2),then the amount of water received by each board during the study period was measured by a plastic container inserted under one of the openings of the irrigation system and the water reached 30 ml in the plastic container within half a minute.The amount of irrigation water for the first plate (irrigation every three days) was 286,944 kiloliters. In the second plate (irrigation every 6 days) was 181,959 kiloliters. The third (irrigation every 9 days) was the total amount of water irrigated by the third plate is equivalent to 146.967 kiloliters.

Preparation of zinc solutions:

For preparation ZnO nanoparticles solution with concentration of 100 ppm, 100 mg dissolved in a little ethanol and then the volume is completed to 1 liter using distilled water. The normal zinc solution was prepared by dissolving 100 mg of $ZnSO_4.7H_2O$ in 1 liter of distilled water to give the concentration 100 ppm. The leaves are sprayed with the two types of zinc solutions three times: after 30 days of planting at the beginning of the flower buds, after 40 days when the flower buds are completed and the flowers were opened after 50 days of planting.

Estimation and antioxidants:

The third leaf was taken from the stem tip for five random plants from each experimental unit,after 70 days of seeding. The leaves were washed with water to remove dirt and impurities and dried using regular drying papers, Then put in the electric oven at a temperature of 60o for 72 hours until the weight is stable and then grind well by an electric mill to be turned into powder. Estimation of proline concentration in leaves was according to (11),using the spectrophotometer. Glutathione was estimated according to the method adopted by (12) and using High Performance liquid chromatographic (HPLC) technology. C18-ODS separation column (25 * 6.4mm) was used at room temperature. Ultraviolet at 360 nm wavelength, and the mobile phase was the flow of a mixture of (CH3: MeOH: Buffer) at (15:50:35), respectively, which applies at a flow rate of 0.7 ml / min.Determination of leaves flavonoids content was according to (13) by using Preparation of Alcohol Extract ,then Absorption Reading by spectrophotometer at wavelength 510 nm.The leaf content of Anthocyanins was estimated according to the method described by (14).This is called pH-differential method, which is characterized by accurate and rapid measurement of the total amount of anthocyanins depending on the difference in pH, and Anthocyanins pigments are subjected to reversals pH which significantly affect the different absorption spectra. Leaf content of α- Tocopherol was determined according to the method described by (15) using UV-visible Spectrophotometer.To estimate the amount of ascorbic acid in leaves,The method described in (16) was adopted using pigment 2,6 dichlorophenol endophenol to complete the calibration process.

Results:

Proline:-

The results in Table (1) indicated the significant differences between the irrigated plants with different irrigation periods in the leaf content of the proline, which increased with the increasing of water stress severity. Plant irrigated every 9 days significantly excelled in this trait and recorded the highest value of 5.29 ml.mg-1 , while plants irrigated every 3 days recorded the lowest value was 4.183 ml.mg-1. From the study of the effect of zinc nanoparticles in the same results, it was observed that the zinc nanoparticles caused a superiority in the leaf content of proline, and the plants treated with nanozinc had the highest significant value of proline reached 5.486 $ml.mg^{-1}$.

The combination between irrigation intervals and foliar application of nanozinc showed that irrigated plants every 9 days treated with nanozinc had higher proline content and had the highest significant value among other treatments, which was 6.141 $ml.mg^{-1}$.

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Table(1) Effect of zinc nanoparticles in sunflower leaf content of proline (ml.mg-1)under water stress .

Irrigation times	Zinc treatments (Zn)			Effect of Irrigation times
	control	Normal zinc	Nanozinc	
Every 3 days	3.633 b	4.048 b	4.870 ab	4.183 c
Every 6 days	3.785 b	4.700 ab	5.448 ab	4.644 b
Every 9 days	4.648 ab	5.088 ab	6.141 a	5.292 a
Effect of Nanozinc	4.022 c	4.612 b	5.486 a	

***** Means followed by the same letter in each treatment are not significantly different according to the Duncan polynomial test at a probability level of 5%.

Glutathione (GSH):-
We can see from Table (2) the effect of water stress in leaf content of GSH. We observed significant increase in the level of GSH by increasing the severity of water stress. The irrigated plants every 9 days recorded the highest significant value of GSH was 22.558 ppm, compared with the lowest value of 12.269 ppm recorded by irrigated plants every 3 days. The positive effect of nanozinc is also evident from the results in the same table.Zinc nanoperticles treated plants recorded the highest value of GSH was 18.925 ppm with significant differences with the treatment of normal zinc, and the control group which recorded the lowest significant value was 13.821 ppm.The results of the table also showed that the irrigated plants every 9 days and treated with zincnanoparticles were significantly superior and gave the highest value in the leaf content of GSH which was 25.148 ppm.

Table(2) Effect of zinc nanoparticles in sunflower leaf content of glutathione (ppm)under water stress .

Irrigation times	Zinc treatments (Zn)			Effect of Irrigation times
	control	Normal zinc	Nanozinc	
Every 3 days	9.643 e	11.897 de	15.265 cd	12.269 c
Every 6 days	12.332 de	14.174 de	16.363 cd	14.290 b
Every 9 days	19.485 bc	23.042 ab	25.148 a	22.558 a
Effect of Nanozinc	13.821 c	16.371	18.925 a	

***** Means followed by the same letter in each treatment are not significantly different according to the Duncan polynomial test at a probability level of 5%.

Total Flavonoides Content (TFC):-
The highest level of TFC was observed in Table (3). The irrigated plants every 9 days achieved the highest significant value in this trait which was 34.096 ppm, whereas the irrigated plants every 3 days recorded a decrease in TFC in leaf which was 23,385 ppm. Regarding the comparison of plants among them with the effect of nanozinc, we found that the nanozinc significantly exceeded the rest of the treatments and gave the highest value of TFC was 31.558 ppm.

Combination between irrigation intervals and zinc nanoparticles showed that the irrigated plants every 9 days and treated with zincnanoparticles were significantly superior and gave the highest value in the leaf content of TFC which was 37.743 ppm.

Table(3) Effect of zinc nanoparticles in sunflower leaf content of flavonoids (ppm)under water stress .

Irrigation times	Zinc treatments (Zn)			Effect of Irrigation times
	control	Normal zinc	Nanozinc	
Every 3 days	21.300 f	23.353 ef	25.473 def	23.385 c
Every 6 days	22.630 f	28.643 cde	31.460 bc	27.578 b
Every 9 days	29.303 cd	35.243 ab	37.743 a	34.096 a
Effect of Nanozinc	24.311 c	29.062 b	31.558 a	

***** Means followed by the same letter in each treatment are not significantly different according to the Duncan polynomial test at a probability level of 5%.

Total Anthocyanine Content (TAC):-

Results in table (4) shown that the TAC increased with the length of the irrigation periods.Plants irrigated every 9 days achieved a significant superiority in TAC and recorded the highest value of TAC was 24.627 mg.L^{-1}, while plants irrigated every 3 days recorded the lowest value of TAC was 20.191 mg.L^{-1}. The level of TAC in the leaves was positively influenced by the use of zinc nanoparticles . Plants treated with zinc nanoparticles had the highest significant value of TAC and recorded 24.299 mg .L^{-1}, while the control group had the lowest value of TAC and recorded 19,421 mg . L^{-1}.

As for the combination between the irrigation periods and the zinc nanoparticles, it is clear from the same table that the highest value of leaf TAC appeared in irrigated plants every 9 days and treated with zinc nanoparticles which amounted to 26.960 mg.L^{-1}

Table(4) Effect of growth regulators and zinc nanoparticles in sunflower leaf content of anthocyanin (mg.L^{-1})under water stress .

Irrigation times	Zinc treatments (Zn)			Effect of Irrigation times
	control	Normal zinc	Nanozinc	
Every 3 days	18.285 b	20.066 b	22.228 ab	20.191 c
Every 6 days	18.490 b	22.633 ab	23.708 ab	21.600 b
Every 9 days	21.488 ab	25.427 ab	26.960 a	24.627 a
Effect of Nanozinc	19.421 c	22.709 b	24.299 a	

***** Means followed by the same letter in each treatment are not significantly different according to the Duncan polynomial test at a probability level of 5%.

Ascorbic Acid(ASA):-

The results of the statistical analysis in Table (5) showed that there was a significant effect of the length of irrigation period in the level of ASA in the leaves. Irrigated plants every 9 days recorded a significant superiority in this trait and gave the highest value of ASA was47.483mg.gm^{-1} , while irrigated plants every 3 days recorded the lowest value was 28.704 mg.gm^{-1}. The use of zinc nanoparticles and normal zinc had a negative effect on leaf content of ASA compared with non-treated plants. Plants in control achieved significant superiority and recorded the highest value was 41.146 mg.gm-1, while plants treated with nanozinc recorded the lowest value of 34.608 mg.gm-1. Combination between irrigation periods and the use of zinc nanoparticles explained that the treatment of irrigated plants every 9 days and non-treated with zinc significantly exceeded and gave the highest value of ASA was 53.013 mg.gm^{-1},compared to irrigated plants every 3 days and treated with nano zinc which recorded the lowest value was 27.955 mg.gm^{-1}.

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Table(5)Effect of zinc nanoparticles in sunflower leaf content of ascorbic acid ($mg.gm^{-1}$) under water stress .

Irrigation times	Zinc treatments (Zn)			Effect of Irrigation times
	control	Normal zinc	Nanozinc	
Every 3 days	31.008 ef	27.150 f	27.955 f	28.704 c
Every 6 days	39.418 bcd	35.700 cde	33.618 def	36.245 b
Every 9 days	53.013 a	44.935 b	42.250 bc	47.483 a
Effect of Nanozinc	41.146 a	35.928 b	34.608 b	

***** Means followed by the same letter in each treatment are not significantly different according to the Duncan polynomial test at a probability level of 5%.

α- Tocopherol :-

The results in Table (6) explained that there were a significant difference between irrigated plants every 3 or 6 days and irrigated plants every 9 days in leaf content of α -tocopherol which increased with increasing the severity of water stress. The irrigated plants every 9 days recorded the highest value of α –tocopherol was 71.125 $mg.gm^{-1}$. compared with Irrigated plants every 3 days which recorded the lowest value of α –tocopherol was 61.977 $mg.gm^{-1}$. From the same table ,there was no significant difference between the use of zinc nanoparticles and the normal zinc in leaf α -tocopherol content, but both achieved a significant difference from the control group.Nanozinc treated plants recorded the highest value of α –tocopherol was 67,151 $mg.gm^{-1}$. while the control group recorded the lowest value was 61,801 $mg.gm^{-1}$.

Combination between the effect of irrigation intervals with the effect of zinc nanoparticles also showed that the irrigated plants every 9 days and treated with nanozinc recorded the highest significant value in leaf content of α-Tocopherol was 72.215 $mg.gm^{-1}$. Non-treated Plant with zinc (both types) gave the lowest value of α- Tocopherol was 56.988 $mg.gm^{-1}$.

Table(5) Effect of zinc nanoparticles in sunflower leaf content of α-Tocopherol ($mg.gm^{-1}$)under water stress .

Irrigation times	Zinc treatments (Zn)			Effect of Irrigation times
	control	Normal zinc	Nanozinc	
Every 3 days	56.988 e	63.488 cd	65.455 bc	61.977 b
Every 6 days	58.283 de	63.248 cd	63.783 cd	62.630 b
Every 9 days	70.133 ab	71.028 ab	72.215 a	71.125 c
Effect of Nanozinc	61.801 b	65.921 a	67.151 a	

***** Means followed by the same letter in each treatment are not significantly different according to the Duncan polynomial test at a probability level of 5%.

Discussion :

From this study, we note that there was a clear superiority in the antioxidant levels in the leaves due to moisture stress. This results may be due to that the accumulation of proline in the sunflower plant growing under water stress is an important indicator in the stressful plants tolerance, especially in small and young leaves. Proline accumulation was found to be associated with low water potential, which enhances its role in osmotic adjustment within plants and stabilization of cellular structures and stability such as biological membranes and proteins. On the other hand, it has a role in scavenging free radicals and regulating oxidative stress of

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cells, thus reducing the severity of stress (17),It should also be noted in this context that under stress conditions, the accumulation of proline in plants is increased as a result of stimulating the activity of proline biosynthesis enzymes such as Ornithine aminotransferase and pyrroline-5-carboxylate reductase with a pronounced decrease in the inhibition of proline degradation enzymes such as proline oxidase and proline dehydrogenase (18). These results agree with (19, 20 ; 21) on sunflower.

Also under stress conditions, the leaves increase the content of other antioxidants that work together to protect chloroplasts from oxidative damage in stressful plants. Chloroplasts are an important center for the functional connection of many antioxidants such as tocopherol, ascorbate and glutathione (22). Tocopherol in chloroplasts, for example, protects lipids and other membrane components and thus protects the structure of the PSII from oxidation due to stress, so the accumulation of tocopherol is an important physiological process in stress conditions, such as water stress, salinity and other (23) , addition to its ability to interact with Polyunsaturated acyl groups of free radicals and thus helps eliminate oxidative damage of reactive oxygen species ROS (24).

The central importance of zinc in the survival and adaptation of plants under environmental stress conditions and its apparent effect on plant water relations (5) may be the cause of the increase caused by zinc nanoparticles in the proline and antioxidant content of leaves, as zinc improves the response to water stress. During the enhancement of the activity of various enzymes, including that it mediates the action of enzymes involved in the manufacture of amino acids, including proline and antioxidants in the sunflower plant (25). As well as the exogenous application of micronutrients enhances the performance of sunflower plant against water stress by improving its antioxidants, phenols and enzymes associated with its manufacture (26) as well as its role in raising the level of GSH and ASA in leaves (27). However, zinc nanoparticles had a stronger effect than normal zinc, as nanofertilizers are more efficient in terms of reducing the negative effects of high doses of regular fertilizers and provide additional drought tolerance (28). These results were similar to that in studies of (25) on sunflower plant; (29) on rosemary and (30) on carrots .

Conclusion:

Water stress caused a significant increase in leaf content of proline and all antioxidants. The positive effect of the use of zinc nanoparticles at a concentration of 100 ppm was observed in all studied traits, as well as the role played by normal zinc in improving traits under study compared to untreated plants, with the exception of ascorbic acid, in which zinc caused a significant decrease in its content in leaf.

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