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Impact of Nano-Chitosan on Proline, Antioxidant Defense Enzyme, Growth and Biochemical Parameters of Papaya Saplings (*Carica papaya* L.) Exposed to Water Stress

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ABSTRACT

The escalating problem of water deficit, along with the surging prevalence of droughts caused by the effects of worldwide climate change, poses a serious threat to plants growth and productivity. Consequently, the current investigation was performed to evaluate the effectiveness of Nano-chitosan in mitigating the adverse impacts of water stress on papaya saplings. A factorial experiment was executed during agricultural season 2023, designed using RCBD with two factors. Three levels of chitosan were used (control, normal chitosan, and nano-chitosan) by using foliar application with a dosage of 50 ppm to each of them. Four levels of irrigation periods (2, 4, 6 and 8 days) were used for water stress treatments, to simulate varying levels of water scarcity. Duncan's multiple range test was employed to analyze the statistical variances among the means at a probability of 0.05. Water stress resulted in a decline shoot and root dry weight, total leaf area, stem diameter and plant height. Leaf analysis revealed a significant decline in P %, N %, K % and total chlorophyll, while proline and peroxidase activity (POD) increased with increasing water stress levels. However, foliar spray of Nano-chitosan showed marked increased of full shoot growth parameters and total chlorophyll, N %, P % and K%. Meanwhile, the application of Nano-chitosan reduced the content of POD and proline leaves. Generally, the findings demonstrated the importance of chitosan in mitigating the harmful effect of water shortage on papaya saplings. This investigation will supply beneficial data that can be used later to develop papaya cultivation under unsuitable environmental conditions such as water scarcity.

تأثير النانو شيتوسان على البرولين و إنزيم الدفاع المضاد للأكسدة والنمو والمؤشرات البيوكيميائية لشتلات البابايا (*Carica papaya* L.) المعرضة للإجهاد المائي

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الخلاصة

إن تفاقم مشكلة نقص المياه، إلى جانب تزايد انتشار حالات الجفاف الناجمة عن آثار تغير المناخ في جميع أنحاء العالم، يشكل تهديدا خطيرا لنمو النباتات وإنتاجيتها. وبناء على ذلك، تم إجراء البحث الحالي لتقييم فعالية النانو شيتوسان في التخفيف من الآثار الضارة للإجهاد المائي على شتلات البابايا. نفذت تجربة عامليه خلال الموسم الزراعي 2023، صممت باستخدام RCBD بعاملين. تم استخدام ثلاثة مستويات من الشيتوزان (المقارنة، الشيتوزان العادي، والنانو شيتوزان) عن طريق الرش الورقي بجرعة 50 جزء في المليون لكل منهما. تم استخدام أربع مستويات من فترات الري (2، 4، 6 و8 أيام) لمعاملات الإجهاد المائي، لمحاكاة مستويات مختلفة من ندرة المياه. تم استخدام ثلاث مكررات لكل وحدة تجريبية، وتم تحليل التباينات الإحصائية بين المتوسطات باستخدام اختبار دنكن متعدد الحدود على مستوى احتمال 0.05 . أدى الإجهاد المائي إلى انخفاض الوزن الجاف للمجموع الخضري والجذري والمساحة الورقية الكلية وقطر الساق وارتفاع النبات. كشف تحليل الأوراق عن انخفاض كبير في P%، N%، K% والكلوروفيل الكلي، في حين زاد نشاط البرولين والبيروكسيديز (POD) مع زيادة مستويات الإجهاد المائي. ظهر الرش الورقي بالنانو شيتوزان زيادة ملحوظة في مؤشرات نمو المجموع الخضري والكلوروفيل الكلي، N%، P% وK%. وفي الوقت نفسه، خفض النانو شيتوزان محتوى البرولين وإنزيم الدفاع المضاد للأكسدة (POD) في أنسجة الأوراق. بشكل عام، أظهرت نتائج هذا البحث أهمية الشيتوزان في التخفيف من الآثار الضارة للإجهاد المائي على نباتات البابايا. ستوفر هذه الدراسة معلومات قيمة يمكن استخدامها مستقبلا لتحسين ممارسات زراعة نباتات البابايا في البيئات محدودة المياه.

الكلمات الافتتاحية: نباتات استوائية، العجز المائي، تقنية النانو، POD، الايونات

INTROUCTION

Papaya fruit tree **(***Carica papaya* L.) is a tropical evergreen tree. It belong to Caricaceae family. Its native habitat is South America and southern Mexico. India, Malaysia, Indonesia and Brazil are famous for the highest production of this fruit (Burns *et al.,* 2022). Papaya fruits are distinguished by their important medical benefits due to their high content of Ca, K, Ma, and several vitamins (C, A, B). It also contains carotene, ascorbic acid, riboflavin and enzymes (chymopapain and papain) which are applied in the pharmaceutical industry (Nowfia *et al.,* 2012; Vij and Prasher, 2015; Espadas *et al.,* 2019). Papaya trees are distinguished from other fruit trees by being fast growing, abundant fruit, and continuous production. They have shallow roots that are unable to absorb water from deeper layers of soil. Therefore, lack of water restricts the growth and fruiting of papaya trees (Girón-Ramírez *et al.,* 2021).

Water stress affects many physiological and biochemical processes of plants, resulting in reduced growth and yield. However, plants can promote their ability to tolerate abiotic stresses by stimulating multiple biochemical and physiological mechanisms (Abdallah *et al.,* 2018; Akyüz *et al.,* 2020). Antioxidant enzymes are one of the main defense mechanisms to resist stress. This enzymatic system is composed of antioxidant defense enzymes such as Peroxidase enzyme (POD) and non-enzymatic antioxidants such as proline, which work together to protect cells from oxidative damage and remove harmful free radicals (Mittler, 2002; Foyer and Noctor, 2005). Many previous studies have emphasized the importance of using different methods to improve the ability of plants to grow in unfavorable environmental conditions (Abdulkadhim and Hadi, 2019; Jassim and Fakhri, 2022; Mahouachi et al, 2023; Salahaddin *et al.,* 2024; Abbas and Abdulkadhim, 2024; Hama *et al.,* 2024; Hashem and Abdulkadhim, 2024). Iraq is one of the most drought-affected countries in the arabic regions. Therefore, alternative means have been discovered to mitigate the harmful effects of water shortage on plants, such as nanotechnology (Abdurrahman and Al-Jubouri, 2023; Al-Qaissi *et al.,* 2024; Alqasim and Al- Ghazal, 2024; Kamaluddin *et al.,* 2022; Ahmed, 2022). Nanomaterials, such as Nano-chitosan, have distinctive characteristics such as high surface area and small volume (Almohammedi *et al.*, 2023; Sen *et al.,* 2020). Chitosan is obtained from marine crustaceans, which are the main raw material used in the industrial production of chitosan (Alenazi *et al.,* 2024). The broad range of commercial applications in biomedicine, agriculture, chemical and food industries has led to increased interest in Chitosan (Li *et al.,* 2019). Therefore, chitosan has a high ability to mitigating environmental stresses (Kumari *et al.*, 2021; Ali, *et al.*, 2011).

latterly, papaya tree cultivation has been introduced to Iraq. There are a scarcity of studies performed on growing and caring for this plant in water scarcity conditions. Therefore, this study was executed, using chitosan and Nano chitosan as sustainable and environmentally friendly materials to enhance the growth of papaya saplings in difficult environments, including water scarcity.

MATERIAL AND METHODS

A fabric canopy experiment was conducted at Al-Mussaib Technical College, Babylon, Iraq, (Lat 32.5° North, long 44.3° East), during agricultural season 2023 on two-month-old papaya saplings. Papaya saplings were obtained from one of the private nurseries in Babylon Governorate on 15/2 2023, planted into 2 kg - plastic bags. Saplings were attentively replanted into plastic pots weighing 15 kg. The pots were filled with a soil mixture consisting of 3: sand with 1: peat moss. Saplings service operations such as weed removal, fungicide, addition of a balanced NPK fertilizer, were performed throughout the duration of the study. The factorial investigation was implemented utilizing a randomized complete block designs on 72 Papaya saplings. The study involved two factors, 12 treatments, and 36 experimental units (2 saplings per experimental unit). The experimental units were replicated three times. The study involved two factors (4*3). The first factor was water stress at four levels of irrigation intervals (2, 4, 6 and 8 days). The second factor was spray of chitosan at three levels (control, normal chitosan, and Nano-chitosan). Normal chitosan and Nano-chitosan were applied at a dose of 50 ppm each. The first spraying with normal chitosan was done on March 20. After one day, Nano-chitosan was sprayed.

1- Shoot and root Parameters:

1- The increase rate of plant height (cm): It was determined from soil surface to the top of the sapling utilizing a tape measure, in twice. The first time was before treatments on 15/3/2023 and the second time on 1/11/2023.The rate of increase in plant height was calculated by the difference between the two readings.
2- The increase rate of stem diameter (mm): It was determined at a 5 cm elevation above the soil surface, with measurements taken twice utilizing a Vernier Caliper. The first time was before the start of the experiment and the second time was after the experiment ended. The increase rate of stem diameter was calculated by the difference between the two readings.
3- The total leaf area per plant (cm^2 $plant^{-1}$): At first, the average number of leaves in each experimental unit was calculated by dividing the number of leaves by the number of plants. Then, the area of a single leaf was calculated using a Scan Leaf Area meter by taking four full-width leaves (from the sixth node to the ninth node at the growing tip) and printing them on A4 white paper. The device's lens was passed over each leaf to calculate its area, then the average area of a single leaf was extracted. Finally, the total leaf area was calculated by multiplying the area of a single leaf × the average of leaves number for every plant.
4- Shoots and root dry weight (g $plant^{-1}$): The shoot parts were separated from the root parts of each experimental unit. Each of them was placed separately in perforated paper bags within an electric oven set at 65°C for a duration of 72 hours. Upon reaching a stable weight, measurements were taken using a sensitive balance.

2- Biochemical Parameters:

1- Nitrogen (%): Based on Novozamsky *et al.*, 1974, Micro- kjeldahl apparatus has been utilized to determine the nitrogen% in leaves.

2- Phosphorous (%): Estimated by chromatography utilizing a spectrophotometer at 622 nm (John, 1970).

3- Potassium (%): It was determined in the leaves using a flame photometer in the digested sample (Horneck and Hanson, 1998).

4- Chlorophyll content (mg g^{-1} fresh weight): It was determined in accordance with the procedures described by Knudson et al. (1977). The measurements were taken through the utilization of the subsequent mathematical equations:

Chlorophyll (a) (mg g^{-1} mw) = (13.70) × (A665) – (5.76) × (A649)

Chlorophyll (b) (mg g^{-1} mw) = (25.80) × (A649) – (7.60) × (A665)

Total Chlorophyll (mg g^{-1} mw) = Chl. (a) + Chl. (b)

Where A = represents the wavelength.

5- Proline (mg g^{-1} dry weight): It was estimated based on Bates *et al.* (1973).

6- Peroxidase enzyme activity (POD) (unit g^{-1} fresh weight): It was measured based on Nezihh (1985).

Statistical Analysis

Data analysis was conducted with the use of the statistical program (SPSS 25.0) software. Duncan's multiple range test was employed to analyze the statistical variances among the means at a probability of 0.05 (Al-Khafaji and Al-Khamisi, 2012).

RESULTS AND DISSCUSION

Shoot and root indices (Table 1) declined significantly with decrease in water availability. Increasing the irrigation intervals to 8 days recorded the lowest average of shoots and root dry weight, total leaf area, stem diameter and plant height. These averages were 17.86 g $plant^{-1,}$ 10.46 g $plant^{-1}$, 1872.3 cm^2 $plant^{-1}$, 7.90 mm, 26.75 cm, respectively, compare with 2-day period.

These findings support the hypothesis that drought-tolerant plants close their stomata at the appropriate time, which serves to reduce transpiration and water loss in the leaves, and maintenance of the cell turgor (Martin-StPaul *et al.,* 2017). Severe drought leads to the closure of stomata in leaves and prevents the synthesis and accumulation of proteins and solutes (Gomes *et al.,* 2023; Li *et al.,* 2020). It is known that stomata movement affects many physiological processes by regulating photosynthesis and intracellular water levels. A decrease in total leaf area can be an indication of reduced photosynthesis in leaves, which also causes a decrease in chlorophyll content (Zahra, *et al.*, 2023). Moreover, water deficiency caused a noticeable decline in plant growth through a decrease in the studied physiological parameters. The decrease in shoots and root dry weight, stem diameter and plant height indicates a limitation in the plant's roots to allocate resources needed for plant growth in the root zone. Many researchers have observed a decrease in shoot and root parameters under water deficiency (Mahouachi *et al.*, 2023; Bunya-atichart *et al.*, 2014).

Table 1: Impact of Nano-Chitosan on Shoots and Roots Parameters of Papaya Saplings Exposed to Water Stress

Chitosan	**Water stress**	**Plant height (cm)**	**Stem diameter (mm)**	**Total leaf area ($cm^2 plant^{-1}$)**	**Shoot dry weight (g $plant^{-1}$)**	**root dry weight (g $plant^{-1}$)**
0	2 day	32.66 abc	7.73 cd	2296.2 cd	29.20 b	23.77 bc
	4 day	29.50 cde	11.72 a	2056.5 d	25.13 cd	16.99 de
	6 day	27.75 cdef	9.38 bc	2167.7 cd	20.60 ef	10.22 g
	8 day	26.75 defg	7.90 cd	1872.3 d	17.86 f	10.46 g
normal	2 day	36.33 ab	9.62 bc	3343.9 ab	30.91 b	24.59 b
	4 day	37.50 a	8.15 cd	3357.2 ab	27.78 bc	20.33 cd
	6 day	21.66 g	8.71 bcd	2688.6 cd	19.95 ef	15.30 ef
	8 day	31.50 bcd	10.61 ab	2143.9 cd	21.09 ef	11.82 fg
Nano	2 day	27.83 cdef	8.47 bcd	4074.8 a	34.62 a	28.96 a
	4 day	24.50 efg	7.69 cd	3156.4 b	28.68 bc	24.62 b
	6 day	35.66 ab	8.89 bcd	2922.2 bc	23.90 de	16.91 de
	8 day	22.83 fg	7.08 d	2056.9 d	20.40 ef	14.58 ef
chitosan	0	29.16 B	9.18 A	2098.17 B	23.20 B	15.36 C
	normal	31.75 A	9.27 A	2883.4 A	24.93 B	18.01 B
	Nano	27.70 B	7. 90 B	3052.57 A	26.90 A	21.26 A
Water stress	2 day	32.27 A	8.60 A	3238.3 A	31.62 A	25.77 A
	4 day	30.50 AB	9.18 A	2856.7 AB	27.20 B	20.64 B
	6 day	28.36 BC	8.99 A	2592.8 B	21.48 C	14.14 C
	8 day	27.02 C	8.53 A	2024.3 C	19.78 C	12.29 C

Different letters within the same column indicate statistically significant differences at $p \leq 0.05$, according to Duncan's multi-range test

Foliar spray of normal and nano-chitosan increased shoot and root indices by alleviating the negative effects of water deficit. Nano-chitosan was more efficient in enhancing shoots and roots growth indices (total leaf area, shoots and root dry weight). These averages were 3052.57 cm2 $plant^{-1}$, 26.93 g $plant^{-1}$, and 21.26 g $plant^{-1}$, while normal chitosan was more efficient in plant height and stem diameter (31.75 cm and 9.27 mm) respectively, compared to control.

Data concluded that normal and nano-chitosan is applied to water-stressed plants by foliar spraying to enhance of Shoots and root parameters. Foliar application of nano-chitosan may be effective in mitigating stress conditions, and/or has a prompt response to drought. Moreover, nano-chitosan seems to maintain photosynthetic efficiency, stomata conductance and alleviate oxidative stress under abiotic stresses conditions. Meanwhile, nano-chitosan affects of diverse biochemical and physiological factors (Sohby *et al.*, 2023, and Liu *et al.*, 2023). Nano chitosan also has several features such as large dispersion, small volume and high surface area, thus presenting beneficial uses in agriculture, the food industry and biomedicine (Alenazi *et al.*, 2024: Kumari *et al.,* 2021). The application of nano-chitosan on crops species' growth compared to traditional chitosan has been the focus of several research studies (Durmuş and Ünlü, 2024; Arshad *et al.,* 2024; Alenazi *et al.,* 2024).

It is clear from Table 2 that water shortage resulted in a significant reduction in ion uptake. Increasing the irrigation intervals to 8 days was more effect in decrease N%, P% and K% 1.78, 0.51 and 1.59% respectively. Analysis of the leaves exhibit a notable reduction in total chlorophyll contents 3.99 mg g^{-1} F.W. . While proline and peroxidase enzyme (POD) gradually increased with watering periods spaced. Irrigation periods of 8 days were most effective in increasing proline and POD 147.66 mg g^{-1} D.W., 11.19 unit mg^{-1} F.W. , compare with 2-day irrigation period.

In several plants, drought impacts induce cell wall changes and membrane instability, as well as reduce photosynthetic activity, water potential and leaf water relations (Brito *et al.*, 2019). These changes decrease the ionic content (N P K) of the leaves, which points the plant's inability to uptake ions present in the root zone. Moreover, the reduction in the total leaf area indicates a decrease in photosynthetic capacity, which was also supported by the decline in chlorophyll content (Zahra *et al.*, 2023, Ruas *et al.,* 2022). The increase in proline and peroxidase levels indicated to the plant's response to water stress to maintain cellular homeostasis. Accumulation of proline in the cytoplasm has been notarized as a method of protecting plant cells from dehydration in many plants (Moloi and Van der Merwe , 2021). Increased proline concentrations have been shown to be associated with maintenance of cell elasticity, protein and membrane structure, and physiological processes (Hayat *et al.,* 2012). Proline relieves oxidative stress and restrict free radicals inside cells, while peroxidase enzyme scavenges reactive oxygen species (Laxa *et. al.*, 2019). These results explain the response of

Table 2: Impact of Nano-Chitosan on ions uptake, Total chlorophyll, Proline and POD enzyme of Papaya Saplings Exposed to Water Stress

chitosan	Water stress	N %	P %	K %	Total chl. (mg g^{-1} F.W.)	Proline (mg g^{-1} D.W.)	POD (unit mg^{-1} F.W.)
0	2 day	1.86abcd	0.56 abc	1.79 bc	3.92 b	109.33 def	9.73 cd
	4 day	1.85 bcd	0.53abcde	1.74cd	4.13 b	115.33cdef	9.90 cd
	6 day	1.79 e	0.49 de	1.69de	4.14 ab	130.67 bcd	11.38 bc
	8 day	1.78 e	0.49 de	1.50 g	3.83 b	159.00 a	13.72 a
normal	2 day	1.91 ab	0.57 ab	1.83 ab	4.48 ab	113.67cdef	11.62 bc
	4 day	1.87 abc	0.54abcde	1.78 bc	4.30 ab	120.33 cde	12.61 ab
	6 day	1.81 de	0.52 bcde	1.72 d	3.80 b	128.00 cde	10.71 bcd
	8 day	1.78 e	0.51 cde	1.62 f	3.96 b	148.00 ab	10.15 cd
Nano	2 day	1.92 a	0.57 a	1.85 a	4.85 a	93.33 f	10.47 bcd
	4 day	1.88 abc	0.54 abcd	1.79 bc	4.32 ab	104.33 ef	9.08 d
	6 day	1.82 cde	0.53abcde	1.73 d	4.47 ab	118.00cdef	10.89 bcd
	8 day	1.79 e	0.53abcde	1.65 ef	4.17 ab	136.00 abc	9.69 cd
chitosan	0	1.82 B	0.52 B	1.68 B	4.00 B	128.58 A	11.18 A
	normal	1.84 AB	0.53 AB	1.74 A	4.14 B	127.50 A	11.27 A
	Nano	1.85 A	0.54 A	1.75 A	4.45 A	112.91 B	10.03 B
Water stress	2 day	1.90 A	0.56 A	1.82 A	4.42 A	105.44 C	10.61 A
	4 day	1.87 A	0.54 B	1.77 B	4.25 AB	113.33 B C	10.53 A
	6 day	1.80 B	0.51 B	1. 71 C	4.14 AB	125.55 B	10.99 A
	8 day	1. 78 B	0.51 B	1. 59 D	3.99B	147.66 A	11.19 A

Different letters within the same column indicate statistically significant differences at $p \leq 0.05$, according to Duncan's multi-range test

plants to water scarcity by studying plant physiological processes, emphasizing the importance of conducting further studies to illustrate the mechanisms associated with these responses. Previous experimental studies have indicated the adverse effects of water scarcity on the biochemical and physiological mechanisms of papaya and other plants (Ruas *et al.,* 2022; Abdulkadhim and Mortada, 2022; Magdalita *et al.*, 202l; Espadas *et al.*, 2019). Foliar application of Nano and normal-chitosan reduced the harmful effects of water shortage by increasing the uptake of ions into leaf tissues. Nano-chitosan had more influential in promoting leaf content of N%, P% & K% (1.85, 0.54, 1.75%) respectively. Moreover, Nano-chitosan appeared a notable impact on total chlorophyll content (4.46 mg g^{-1} FW.). Whereas, the leaf contents of proline and peroxidase (POD) gradually decreased when using normal and Nano-chitosan. Nano-chitosan was the most effective in reducing proline and POD 112.91mg g^{-1} D.W., 10.03unit mg^{-1} F.W. respectively, compared to control.

Nano-chitosan was more effectiveness in enhancing total chlorophyll, N, P, and K content, by mitigating oxidative stress and improving photosynthetic efficiency under water stress conditions. During the current investigation, water deficiency enhanced proline levels and the activity of the antioxidant enzyme peroxidase, while nano-chitosan treatment reduced this activity. The primary function of enzymatic antioxidants and proline in plants is to combat a wide range of abiotic stressors. Proline and peroxidase neutralize free radicals and ROS (Sohby *et al.*, 2023; Baccari *et al.,* 2020; Boguszewska & Zagdańska, 2012).The reduction in proline and peroxidase levels is due to the ability of Nano-chitosan to improve plant metabolism, protect cell membranes from photo-oxidation. In addition to its regulating of many metabolic processes, activate gibberellin and auxin pathways and increase the nutrients absorption (Safikhan *et al.*, 2018). These data agree with the findings of prior investigations that concluded utilizing various strategies to help plants resist abiotic stresses (Abdulkadhim & Hussein, 2023; Fadhil and Abdulkadhim, 2020; Abdulkadhim, 2019).

CONCLUSION

The current investigation demonstrated that water shortages lead to a reduction in shoot and root parameters, ions uptake and total chlorophyll. While proline and POD were enhanced in the leaves of papaya saplings under water stress. However, foliar application of nano-chitosan mitigated the influence of water deficiency in all parameters studied, particularly following an extended period of stress (8 days). Data concluded that application of nano-chitosan to plants subjected to water stress led to a notable raise on shoots and root parameters, total chlorophyll, N, P, and K content. While nano-chitosan reduced proline, and POD enzyme. Therefore, the foliar application of nano-chitosan appears to be advantageous in in promoting the adaptation of papaya saplings to water shortages. The investigation aims to supply useful insights into the possible advantages of using nano-chitosan as a growth promoter under unfavorable environmental conditions.

CONFLICT OF INTEREST

There are no conflicts of interest associated with this manuscript.

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