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Exogenous application of biostimulators alleviates water deficient stress on *Azadirachta indica* plants

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Key words: Brassinolide, chitosan, drought stress, neem.

Abstract: Pot experiment was conducted to evaluate the effect of chitosan or brassinolide applications on morphology and physiology parameters of *Azadirachta indica* grown under water deficient stress. The plants received different irrigation intervals, and were sprayed monthly with either chitosan or brassinolide each at concentrations of 50, 100 and 200 ppm, while the control plants were sprayed only with tap water. The results showed that water stress reduced all growth parameters, chemical constituents of pigments content, total carbohydrates, N, P, and K%, total indoles, while proline and total phenols content were increased. Instead, the plants sprayed with the higher concentrations of chitosan or brassinolide resulted in significant increase in growth parameters, pigments content, total carbohydrates, proline content, N, P and K%, total indoles while reduced total phenols content. Based on the obtained results it can be concluded that, foliar application of chitosan or brassinolide at 200 ppm can alleviate the adverse effects of water deficient stress on the growth and physiology parameters of *Azadirachta indica*.

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All relevant data are within the paper and its Supporting Information files.

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1. Introduction

Azadirachta indica A. Juss is a tropical evergreen tree that belongs to the family of Meliaceae, commonly known as neem, nimtree or Indian lilac. It is native to India, its fruits and seeds are the source of neem oil (Koul and Wahab, 2004). In addition to use of neem for landscape activates as an ornamental tree, all parts of the tree have been utilized medicinally and now being used in pharmaceutical and cosmetics industries. Neem fruits, seeds, oil, leaves, bark and roots used as antiseptics, antimicrobials, anti-inflammatory, anti-cancerous and anti-diabeticv (Islas *et al*., 2020).

Water deficiency is the major environmental factors, as a biotic stresses limiting agricultural production in most countries, especially in the arid and semi-arid regions, affecting the quality, growth and production of pants. Water stress induces various physiological, biochemical changes in ornamental plants such as a reduction in the growth parameters (Abd-Elmoneim *et al.*, 2018; Yousaf *et al.*, 2018; El-Shanhorey and Sorour,

2019; Shaltout *et al.*, 2022), reductions in total chlorophyll and carbohydrate contents (Sarker and Oba, 2018), reductions in nutrient accumulation (Singh and Singh, 2020), as well as increasing in prolin, phenols content (El-Gamal and Khamis, 2021).

Recently, study for biological methods to avert utilization of chemical products and alleviating the harmful effect of water deficient in agriculture has led to utilize of bio-stimulators. Among the various kinds of bio-stimulators are chitosan and brassinolide.

Chitosan (CHT) is a deacetylated derivative of chitin. It is a natural polymer and biodegraded by biological agents and it is environment-friendly used in agriculture (Shafiei-Masouleh, 2019). Under stressed conditions, CHT has the efficiency to mitigate the harmful effects of drought by promoting chlorophyll, carbohydrates, proline content and the capacity of antioxidant activities (Pirbalouti *et al.*, 2017; Vosoughi, *et al.*, 2018; Elansary *et al.*, 2020). The favorable influence of CHT induces photosynthetic rate, stomatal closure through ABA synthesis; stimulates antioxidant enzymes via nitric oxide and hydrogen peroxide signaling pathways, and promotes production of organic acids, sugars, amino acids and other metabolites that are required for the osmotic adjustment, stress signaling, and energy metabolism under stresses (Hidangmayum *et al*., 2019).

Brassinolide is the first brassinosteroids (BRs) isolated in plants. BRs are type of plant hormone which have a vital effect on plant growth and development. Earlier workers have elucidated that BRs can reduce the harmful effects of water deficient stress via boosting the activity of antioxidant enzymes and non-enzymatic antioxidant contents to remove the damage of reactive oxygen species (ROS) (Hosseinpour *et al.*, 2020; Cai *et al*., 2021; Omidian *et al.*, 2022) or by increasing endogenous abscisic acid (ABA) to induce stomatal closure (Bhandari and Nailwal, 2020). Additionlly, BRs can enhance the energy metabolism balance between the chloroplast and mitochondria, increase the initial activity of Rubisco, and boost the use of light energy absorbed by plants, thus enhancing photosynthetic efficiency under water deficient stress (Cai *et al*., 2021).

Although the beneficial roles of bio-stimulators on ornamental plants and their helpful effect on boosting growth and flowering parameter, there are no enough available data about their action on alleviating the harmful effect of drought on ornamental trees. Therefore, this research is aimed to evaluate the response of *Azadirachta indica* grown under water deficient stress to foliar application of chitosan or brassinolide.

2. Materials and Methods

The present study was undertaken in the experimental nursery (30-32°C temperature, 14 h light conditions and 39-61% relative humidity) of the Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza, Egypt during the two successive seasons of 2019 and 2020. The latitude, longitude and altitude of the experimental site were 30° 03’ N, 31° 13’ E and 19 m ASL.

Plant material

On 1st of March in 2019 and 2020 seasons, uniform seedling of *Azadirachta indica* plants were obtained from a commercial nursery with an average plant height of 25-30 cm, 2-3 leaves/plant and transplanted individually in 25 cm diameter plastic pots filled with clay+ sand (2:1: v/v), some physical and chemical properties of soil mixture used in the study were determined according to (Jackson, 1973), the results are presented in Table 1.

Table 1 - Some physical and chemical characteristics of the soil mixture used for growing *Azadirachta indica* (mean of two seasons)

Soil characteristics	Data
Physicical characteristics	
Soil Texture	Clay
Clay (%)	39.80
Coarse sand (%)	3.59
Fine sand (%)	21.46
Silt (%)	35.15
Chemical characteristics	
Soluble cations (meq/l)	
Ca^{++}	7.09
Mg^{++}	2.86
K^{+}	0.30
Na^{+}	6.05
Soluble anions (meq/l)	
Cl^{-}	3.53
SO_4^{-}	2.42
N (ppm)	26.57
P (ppm)	22.00
Organic matter (%)	1.61
Electrical conductivity (dS/m)	1.64
pH	7.46

pH= soil acidity.

Experimental procedures

On 15th of March in first and second season, respectively the plants were irrigated every 3, 6, 9 and 12 days for imposing water stress. Amount of water used each time was equal to the field capacity (pot capacity) which was determined empirically as follows: three pots (25 cm) filled with about 2 kg of the soil mixture were watered thoroughly to saturation and weighed. Pots were covered with aluminum foil to prevent evaporation before they were left in a cool shaded place to drain freely for 4 hours. They were weighed again to calculate weight of water held by the soil mixture. Mean of the three pots representing the field capacity was found to be 1350 g, equivalent to 1350 cm^3 of water/pot (1.35 L /pot) (Abd-Elmoneim *et al*., 2018). Thus, 1.35 L of water were given to each pot in due time according to the irrigation schedule. This means that at the end of the experiment (after 7 months), plants irrigated every3, 6, 9 and 12 days interval were given 94.5, 47.3, 31.5 and 23.6 liters of water, respectively.

Starting from 31st March till to 15th October (in both seasons), the plants received different irrigation intervals were sprayed every 4 weeks with either chitosan (CHT, 500 mg, composed of β-(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine randomly distributed within the polymer) or brassinolide (BRs, 0.01% steroid compounds) each at concentrations of 50, 100 and 200 ppm, while the control plants sprayed only with tap water. Both CHT and BRs were obtained from Tecknogreen company, Egypt. Tween 20 as wetting agent was added to bio-solution at concentration of 1 mL L^{-1} and the plants foliage were sprayed using automatic atomizer until run off point (80 ml of bio-solution/plant).

All the plants were fertilized every month with kristalon™ (NPK 20:20:20) at a rate of 3 g/pot, manual picking of weeds, disease and pest control has also been carried out.

Layout of experimental

The layout of the experiment was randomized complete blocks design with 28 treatments [4 irrigation intervals x 7 plant bio-stimulators (including the control)] each treatment consisting of 9 pots arranged in 3 replicates, each replicate containing 84 pots (3 pots from each treatment).

The data recorded

Vegetative growth parameters. At the end of the experiment, On 30th October in both seasons (after 7 months), vegetative growth parameters were recorded, two samples of plants were randomly taken from each replicate to determine the parameters including plant height (cm, measured with a ruler from soil surface to its highest point), fresh and dry weights of shoots, roots/plant, stem diameter (mm, at 5 cm above soil surface), root length (cm), number of leaves/plant and leaf area (cm^2, measured using CI-202 Portable Laser Leaf Area Meter, CID Bio-Science, Inc., USA). Dry weight of shoots and roots /plant was evaluated by drying plant in an electric oven at 70°C until constant weight.

Chemical analysis

Chlorophyll and carotenoid contents. Chlorophyll pigments including Chl a, Chl b and carotenoid contents (mg g^{-1} FW) in leaves were determined according to Lichtenthaler and Buschmann (2005): leaves extracted in 5 ml of 95% aqueous acetone was centrifuged at 4000 g for about 10 min. The aqueous acetone supernatant was then taken for spectrophotometric measurement. A blank of acetone was taken at wavelengths of 663, 645 and 452.5 nm respectively, and data were then calculated using the following equations:

Chlorophyll a.(mg g^{-1}) = 0.0127 A663 − 0.00269A645
Chlorophyll b.(mg g^{-1})= 0.0029A663 − 0.00468A645
Carotenoids (mg g^{-1}) = 4.2 E 452.5 − 0.0264

Total carbohydrates. Total carbohydrates content in leaves (percentage of dry matter) was determined in dried samples according to Dubois *et al*. (1956). A known weight (0.1 g) of the dried samples was completely hydrolyzed with 10 ml sulphuric acid (67%) in a test tube on a boiling water bath for one hour. The solution was decolorized and the filtrate was diluted to 100 ml with distilled water. A known volume (1 ml) of the extract was taken in a test tube, to which 1 ml phenol solution (5%) was added, followed by 5 ml of concentrated sulphuric acid. The optical density of the resulting color was measured at 490 µm, using a spectrophotometer, against a blank reagent. The standard curve of glucose was used to calculate the total carbohydrates concentration in the extract.

Proline content. Proline content in fresh leaves (µ moles/g fresh matter of leaves) was determined using the method of Bates *et al*. (1973). Leaves were homogenized in 3% aqueous Sulphosalicylic acid, then centrifuged 5,000 *g* for 20 min at 4°C. The amount of 2 mL of this homogeny solution react acid-ninhdrin and 2 mL of glacial acetic acid in a tube for 1 hour at 100°C and the reaction is torn up in an ice

bath and then extracted with 4 mL of toluene. It was kept at room temperature to stabilize. Proline content was measured by spectrophotometer (UV-160A, Shimadzu, Tokyo, Japan) at 520 nm.

N, P and K content in shoot. Dried shoot samples were digested to extract nutrients and the extract was analyzed to determine concentrations of N, P and K (as percentage of D.W) which were determined according to Estefan *et al*. (2013). Nitrogen concentration was determined by using the micro-Kjeldahl method. Phosphorus was determined calorimetrically by using the chlorostannous molybdophosphoric blue colour method in sulphuric acid. Potassium was determined by using the flame photometer apparatus (CORNING M 410, Germany).

Total indole and phenol contents. Total indole and phenol contents were determined in fresh shoots (3 g) of shoots, which were crushed and extracted with 80% ethanol at 0°C for 72 hours, the ethanol being changed every 24 hours, as described by Selim *et al*. (1978).

Statistical analysis

The means of all obtained results were subjected to two-ways analysis of variance (ANOVA) in randomized complete blocks design. Combined analysis of the two growing seasons was carried out. Means of data were compared by using Duncan's multiple range tests at 5% level Snedecor and Cochran (1989).

3. Results and Discussion

Growth parameters

Data recorded on *Azadirachta indica* plants, all growth parameters (including, plant height, fresh and dry weights of shoots and roots, stem diameter, root length, number of leaves and leaf area) were significantly affected by irrigation intervals, bio-stimulators treatment and interaction effects (Table 2). The data in Table 3 and 4 showed that within each level of the two bio-stimulators (CHT or BRs), all growth parameters were decreased significantly ($p<0.05$) with prolonged irrigation intervals daily from 3 to 6, 9 or 12 days. This reduction was steadily in most cases compared to the short intervals (3 days). The reduction of growth parameters in response to water deficient may be due to adverse effect of drought around the roots, lower soil moisture availability due to water stress, lead to reduce water and nutrients absorption by roots which in turn leading to reduction in vegetative biomass (Rouphael *et al*., 2012). This is greatly in harmony with numerous researches (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; Khatana *et al*., 2018; Toscano *et al*., 2018; El-Shanhorey and Sorour, 2019; Najihah *et al*., 2019; Tribulato *et al*., 2019; Al-Arjani *et al*., 2020; Singh and Singh, 2020; El-Gamal and Khamis, 2021; Papú *et al*., 2021; Sorour, 2021; Shaltout *et al*., 2022).

Data in same Tables also indicated that within

Table 2 - Mean square for the effect of irrigation intervals and bio-stimulators treatments and their interaction on vegetative growth parameters of *Azadirachta indica*

Traits	Source of variation				
	Treatment			Error	CV
	Irrigation intervals (A)	Bio-stimulators (B)	(A × B)		
Plant height (cm)	1281.31 ***	967.98 ***	34.50 *	19.48	4.69
Fresh weight of shoots (g/plant)	268.86 ***	731.60 ***	24.01 **	14.07	9.14
Dry weight of shoots (g/plant)	98.12 ***	279.41 **	4.87 *	3.38	9.53
Fresh weight of roots (g/plant)	225.15 ***	904.88 ***	15.76 ***	3.82	6.29
Dry weight of roots (g/plant)	47.79 ***	71.71 ***	1.83 *	3.12	13.22
Stem diameter (mm)	5.74 ***	8.85 ***	0.25 *	0.53	12.29
Root length (cm)	553.08 ***	662.22 ***	7.00 *	11.17	8.13
Number of leaves	105.61 ***	567.61 ***	7.17 *	4.32	9.65
Leaf area (cm^2)	33.01 ***	87.65 ***	0.54 *	2.83	13.57

*, **, *** significant at P≤0.05, P≤0.01, P≤0.001, respectively.

Table 3 - Plant height, fresh and dry weight of shoots and fresh and dry weight of roots of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	Plant height (cm)	Fresh weight of shoots (g/plant)	Dry weight of shoots (g/plant)	Fresh weight of roots (g/plant)	Dry weight of roots (g/plant)
3 days	Control	79.63±1.94 j-l	34.18±1.97 jk	17.10±1.21 f-h	30.99±0.94 g-i	10.97±0.41 j-m
	CHT (1)	98.58±1.97 de	42.2±1.60 d-g	21.70±0.95 cd	32.97±0.14 d-h	16.11±1.47 a-c
	CHT (2)	101.17±2.6 b-e	44.53±1.11 c-f	23.11±0.6 bc	39.51±2.20 b	16.03±0.58 bc
	CHT (3)	107.58±1.21 b	47.64±2.22 b-e	25.09±0.99 ab	41.68±1.5 b	15.40±0.26 cd
	BRs (1)	96.67±2.1 ef	46.00±4.85 b-f	23.09±0.46 bc	34.71±0.84 c-f	13.83±0.28 c-j
	BRs (2)	99.50±3.13 c-e	50.84±0.85 b	26.42±1.68 a	41.05±1.57 b	15.47±1.63 cd
	BRs (3)	117.17±0.92 a	59.72±3.34 a	27.26±1.98 a	49.11±0.82 a	19.00±2.57 a
6 days	Control	75.17±2.53 k-m	38.21±3.41 g-j	13.90±1.4 ij	27.8±0.85 ij	10.16±0.97 k-m
	CHT (1)	95.08±1.72 e-g	41.58±1.97 e-h	19.25±0.43 d-f	32.68±0.62 e-h	14.32±0.92 c-h
	CHT (2)	99.83±1.451 c-e	44.8±2.30 b-f	22.81±2.09 bc	35.90±1.70 cd	14.87±1.19 c-f
	CHT (3)	104.83±2.53 b-d	48.81±1.09 bc	22.73±1.02 bc	34.80±1.32 c-f	14.95±2.45 c-f
	BRs (1)	99.67±3.03 c-e	40.53±0.75 f-i	22.62±0.61 bc	31.77±0.75 f-h	13.42±0.65 c-j
	BRs (2)	104.33±2.05 b-d	48.27±0.94 b-d	25.49±1.01 ab	33.65±0.53 c-g	14.56±0.55 c-g
	BRs (3)	107.25±4.73 b	47.74±0.78 b-d	21.30±0.35 cd	35.67±1.55 c-e	18.60±0.38 ab
9 days	Control	74±1.59l m	32.51±2.56 j-l	13.03±0.54 j	21.16±1.44 l	8.91±1.36 lm
	CHT (1)	89.08±1.47 g-i	35.8±2.95 h-k	17.44±0.54 f-h	23.96±1.56 kl	12.29±0.74 e-k
	CHT (2)	97.08±4.12 ef	43.31±1.41 c-g	17.89±0.49 e-g	34.18±0.09 c-g	12.94±0.21 d-k
	CHT (3)	99.17±1.72 de	45.23±0.58 b-f	18.19±0.07 e-g	34.72±0.63 c-f	14.030±1.06 c-i
	BRs (1)	88.83±4.06 g-i	37.54±1.68 g-j	17.04±0.6 f-h	27.63±1.77 j	11.94±0.26 g-k
	BRs (2)	101.58±1.82 b-e	43.5±2.86 c-g	20.68±1.34 c-e	29.94±0.83 h-j	12.99±0.57 d-k
	BRs (3)	106.42±2.81 bc	44.64±1.48 c-f	16.27±1.09 f-i	36.23±0.29 c	15.18±0.40 c-e
12 days	Control	70.33±1.58 m	27.73±1.99 l	9.91±0.18 k	17.80±0.53 m	8.3±1.24 m
	CHT (1)	83.75±3.41 h-j	32.18±1.99 j-l	14.66±0.41 h-j	20.84±0.68 lm	11.18±0.65 i-m
	CHT (2)	85.33±1.6 h-j	34.27±2.02 jk	16.80±1.54 f-i	26.93±1.46 jk	11.20 ±1.32 i-l
	CHT (3)	90.17±3.03 f-h	35.48±1.42 h-k	17.26±0.41 f-h	22.68±0.59 l	11.40±0.82 i-l
	BRs (1)	79.5±0.9 j-l	30.55±0.94 kl	16.32±1.87 f-i	21.35±0.51 l	11.46±1.21 h-l
	BRs (2)	82.12±3.05 i-k	35.77±2.42 h-k	17.53±0.54 f-h	23.59±0.78 l	12.07±0.54 f-k
	BRs (3)	101.75±3.04 b-e	35.13±1.89 i-k	15.19±0.83 g-j	27.18±0.96 j	12.30±0.29 e-k

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates , Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

each irrigation intervals, the plants sprayed with the higher concentrations of two tested bio-stimulators (CHT or BRs) had significant increase ($p<0.05$) in most of growth parameters compared to control plants (pants exposed to water stress and not received any bio-stimulators treatments). The data also exhibited that under the same level of the two bio-stimulators, BRs was generally superior in its effect than CHT and among the different concentrations, the highest dose (200 ppm) was the most effective one in increasing the tested growth parameters. The results are similar to those obtained by previous studies where CHT was tested (Pirbalouti *et al*., 2017; Byczyńska, 2018; El-Khateeb *et al*., 2018; Abdel-Mola and Ayyat, 2020; El-Serafy, 2020; Ashour *et al.*, 2021; Arshad *et al*., 2022), as well as BRs (El-Khateeb *et al*., 2017; Abd-Allah *et al.*, 2018; Latha and Vidya Vardhini, 2018; Mohamed, 2020; Sheng *et al.*, 2022). Moreover, other studies (Hosseinpour *et al.*, 2020; Mohammadi *et al.*, 2020; Omidian, *et al.*, 2022) stated that application of BRs has a positive effect on growth parameters of ornamental plants exposed to drought stress.

Chemical constituents

Pigments content. As shown from data listed in Table 5 irrigation intervals, bio-stimulators treatment and interaction effects had significant influence on pigments content (chlorophyll a, b, carotenoids and

Table 4 - Plant height, fresh and dry weight of shoots and fresh and dry weight of roots of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	Stem diameter (mm)	Root length (cm)	Number of leaves	Leaf area (cm^2)
3 days	Control	5.64±0.56 c-j	41.25±0.66 f-i	22.00±1.38 d-g	11.79±1.07 e-j
	CHT (1)	6.81±0.17 a-c	42.00±1.84 f-i	24.17±1.08 c-e	13.42±0.88 c-h
	CHT (2)	6.80±0.17 a-c	44.00±2.46 e-g	27.42±1.29 bc	15.54±0.69 a-c
	CHT (3)	6.91±0.02 ab	53.33±1.64 b	31.67±0.22 a	16.76±1.60 ab
	BRs (1)	6.85±0.35 ab	47.67±1.69 c-e	27.95±1.81 b	13.79±0.66 c-f
	BRs (2)	6.92±0.47 ab	50.67±1.74 b-d	33.08±1.54 a	14.88±0.47 b-d
	BRs (3)	7.10±0.49 a	59.25±1.32 a	35.00±2.81 a	18.24±1.12 a
6 days	Control	4.77±0.13 jk	30.75±1.38 l-n	17.33±0.44i-k	10.88±2.04 g-l
	CHT (1)	6.46±0.28 a-f	37.58±0.82 h-k	19.58±1.36 f-j	12.10±0.27 e-j
	CHT (2)	6.52±0.67 a-f	40.17±3.43 g-j	22.33±0.65 d-f	13.58±1.07 c-g
	CHT (3)	6.62±0.32 a-d	45.08±2.09 e-g	24.58±0.30 b-d	14.10±0.40 b-e
	BRs (1)	6.07±0.49 a-i	42.75±0.80 e-h	19.92±1.01 f-i	12.01±1.63 e-j
	BRs (2)	6.11±0.1 a-i	45.92±1.67 d-f	24.42±0.71 cd	13.52±0.51 c-h
	BRs (3)	6.53±0.22 a-e	54.17±3.83 ab	24.08±1.31 c-e	15.48±0.59 bc
9 days	Control	3.78±0.04 k	29.25±1.84 mn	14.67±1.36 kl	8.73±0.86 kl
	CHT (1)	5.33±1.14 f-j	33.83±0.88 k-m	16.33±0.33 jk	10.55±0.47 i-l
	CHT (2)	5.74±0.06 b-j	36.67±1.62 i-k	19.42±1.17 f-j	12.04±1.09 e-j
	CHT (3)	6.15±0.57 a-i	42.42±1.40 e-h	19.67±0.79 f-j	12.34±0.47 d-i
	BRs (1)	5.26±0.17 g-j	35.00±2.36 j-l	19.33±0.96 f-j	10.19±0.86 i-l
	BRs (2)	6.01±0.05 a-i	40.00±3.74 g-j	20.00±0.14 f-i	11.56±0.51 e-j
	BRs (3)	6.45±0.43 a-g	51.67±2.53 bc	20.83±0.60 e-h	13.34±0.13 c-h
12 days	Control	3.61±0.69 k	26.17±2.02 n	12.33±0.44 l	8.29±0.66 l
	CHT (1)	4.98±0.5 ij	31.08±1.08 l-n	15.00±1.23 kl	9.56±0.51 j-l
	CHT (2)	5.55±0.09 d-j	33±0.58 k-m	17.50±1.0 h-k	10.10±0.61 i-l
	CHT (3)	5.25±0.45 h-j	41.00±2.10 f-i	17.58±0.93 h-k	11.34±0.21 f-k
	BRs (1)	5.40±0.21 e-j	34.83±1.74 j-l	18.92±2.32 g-j	9.38±1.05 j-l
	BRs (2)	5.73±0.32 b-j	35.00±1.23 j-l	17.67±1.31 h-k	10.78±2.12 h-l
	BRs (3)	6.39±0.36 a-h	45.92±0.79 d-f	20.17±1.20 f-i	12.72±0.96 d-i

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates , Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

total chlorophyll). Data in Table 6 showed that within each level of the two bio-stimulators (CHT or BRs), chlorophyll a, b, carotenoids and total chlorophyll were reduced gradually (in most cases) in repose to prolonged irrigation intervals. Photosynthetic pigments play a vital role in photosynthetic process. Under drought stress, stomata functioning is changed that affect photosynthesis and CO_2 uptake, thus resulting various chlorophyll contents level through the entire growth period of the plant (Khatana *et al*., 2018). Similar reductions in pigments content as a result of water deficient stress were reported by various studies (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; Khatana *et al*., 2018; Sarker and Oba, 2018; El-Shanhorey and Sorour, 2019; Al-Arjani *et al*., 2020; Singh and Singh, 2020; El-Gamal and Khamis, 2021; Papú *et al*., 2021; Sorour, 2021; Shaltout *et al*., 2022).

The data in Table 6 also revealed that within each irrigation intervals, the highest concentrations of CHT or BRs caused significant increase in pigments content compared to control plants. The data also clarified that BRs was better in its effect than CHT particularly the highest concentration (200 ppm) since recorded the highest values of the tested traits. These results are in agreement with findings of prior authors who reported that application of CHT resulted in increase in pigments content (Byczyńska, 2018; El-Khateeb *et al*., 2018; El-Serafy, 2020; Elansary *et al*., 2020; Abdel-Mola and Ayyat, 2020; Gerami *et al*.,

Table 5 - Mean square for the effect of irrigation intervals and bio-stimulators treatments and their interaction on some chemical constituents of *Azadirachta indica*

Traits	Source of Variation				
	Treatment			Error	CV
	Irrigation intervals (A)	bio-stimulators (B)	(A × B)		
Chlorophylls A content (mg/g f.w)	22.90 ***	8.91 ***	0.32 *	0.93	16.99
Chlorophylls B content (mg/g f.w)	2.72 ***	1.35 **	0.21 *	0.39	24.19
Carotenoids content (mg/g f.w)	5.30 ***	5.94 ***	0.21 *	0.64	20.35
Total chlorophyll (mg/g f.w)	39.85 ***	16.72 ***	0.56 *	1.74	15.96
Total carbohydrates (%) in leaves	206.87 ***	156.23 ***	6.81 ***	1.05	4.09
Proline (µ moles/g fresh matter)	25.67 ***	9.65 ***	0.82 ***	0.11	7.60
N% in shoot	1.23 ***	2.59 ***	0.17 ***	0.01	5.03
P% in shoot	0.18 ***	0.46 ***	0.05 ***	0.001	9.21
K% in shoot	0.34 ***	0.85 ***	0.05 ***	0.01	6.29
Total indoles (mg/100 g DW)	1310.44 ***	4091.18 ***	101.34 ***	2.75	2.45
Total Phenols (mg/100 g DW)	514.11 ***	3724.62 ***	116.61 ***	3.44	2.94

*, **, *** significant at P≤0.05, P≤0.01, P≤0.001, respectively.

Table 6 - Pigments content of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	chlorophyll a (mg/g FW)	chlorophyll b (mg/g FW)	Carotenoids (mg/g FW)	Total chlorophyll (mg/g FW)
3 days	Control	3.88±0.23 h-j	2.05±0.07 d-f	2.95±0.05 i-k	5.93±0.15 h-j
	CHT (1)	5.76±0.44 c-g	2.40±0.49 c-f	4.78±0.59 a-e	8.16±0.89 d-g
	CHT (2)	5.86±0.81 c-f	2.84±0.30 b-d	4.02±0.66 b-j	8.70±1.10 c-g
	CHT (3)	7.22±0.40 bc	2.80±0.25 b-d	4.64±0.10 a-f	10.02±0.62 b-d
	BRs (1)	5.79±0.04 c-g	2.96±0.30 b-d	4.41±0.17 b-g	8.75±0.34 c-g
	BRs (2)	6.73±0.91 cd	3.48±0.69 ab	5.33±0.96 ab	10.22±1.53 b-d
	BRs (3)	9.56±1.08 a	3.99±0.22 a	5.94±0.73 a	13.55±0.90 a
6 days	Control	4.27±0.16 g-j	1.71±0.14 ef	2.98±0.07 h-k	5.99±0.24 h-j
	CHT (1)	5.18±0.48 d-h	2.62±0.20 b-e	4.22±0.28 b-i	7.80±0.32 e-h
	CHT (2)	5.47±0.62 d-g	2.98±0.41 a-d	3.54±0.18 e-k	8.44±0.41 c-g
	CHT (3)	6.39±0.94 c-e	2.86±0.39 b-d	4.36±0.38 b-g	9.25±1.32 b-e
	BRs (1)	5.69±0.13 c-g	2.54±0.15 b-e	4.07±0.69 b-j	8.23±0.15 d-g
	BRs (2)	6.48±0.50 c-e	2.81±0.39 b-d	4.29±0.65 b-h	9.29±0.48 b-e
	BRs (3)	8.54±0.82 ab	2.86±0.29 b-d	5.03±0.39 a-c	11.40±1.08 ab
9 days	Control	3.58±0.28 ij	1.62±0.12 ef	2.87±0.11 jk	5.20±0.36 ij
	CHT (1)	4.49±0.42 f-j	2.52±0.10 b-f	3.73±0.65 c-k	7.01±0.33 f-i
	CHT (2)	5.02±0.33 e-i	2.55±0.29 b-e	3.42±0.45 f-k	7.57±0.05 e-h
	CHT (3)	5.88±0.78 c-f	2.27±0.19 d-f	4.13±0.20 b-j	8.16±0.89 d-g
	BRs (1)	5.08±0.37 e-i	2.45±0.43 c-f	3.95±0.19 c-j	7.54±0.76 e-h
	BRs (2)	6.45±0.29 c-e	2.63±0.21 b-e	3.78±0.24 c-k	9.08±0.34 c-f
	BRs (3)	7.22±0.50 bc	2.82±0.45 b-d	4.88±0.44 a-d	10.05±0.91 b-d
12 days	Control	3.05±0.13 j	1.51±0.04 f	2.47±0.17 k	4.56±0.17 j
	CHT (1)	4.34±0.25 f-j	2.51±0.57 b-f	3.41±0.62 f-k	6.85±0.60 g-i
	CHT (2)	4.48±0.54 f-j	2.36±0.55 c-f	3.09±0.46 g-k	6.84±0.69 g-i
	CHT (3)	5.51±0.72 d-g	2.03±0.22 d-f	3.12±1.06 g-k	7.54±0.92 e-h
	BRs (1)	4.54±0.19 f-j	2.08±0.11 d-f	3.29±0.21 g-k	6.62±0.15 g-j
	BRs (2)	5.45±0.53 d-h	2.86±0.45 b-d	3.67±0.44 d-k	8.31±0.95 d-g
	BRs (3)	7.12±0.69 bc	3.36±0.66 a-c	4.06±0.29 b-j	10.49±1.33 bc

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates , Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

2020; Ashour *et al.*, 2021; Arshad *et al*., 2022; Samany *et al*., 2022; Attaran Dowom *et al*., 2022). While, the valuable enhance in tested components due to BRs treatments are in harmony with another reports (El-Khateeb *et al*., 2017; Abd-Allah *et al.*, 2018; Rezaei *et al.*, 2018; Mohamed, 2020; Pacholczak *et al*., 2021). Furthermore, previous workes (Hemmati *et al.*, 2018; Mohammadi *et al.*, 2020; Mojaradi *et al*., 2020; Zafari *et al*., 2020; Omidian *et al.*, 2022) showed that application of BRs has a favorable effect on photosynthetic pigments of plants subjected to drought stress.

The positive effect of BRs in increase the pigments content may be due to application of BRs increases the photosynthetic rate of plants by increasing the RuBisCo activity and other main enzymes included in the Calvin cycle, BRs also enhance the uptake of CO_2 which increase the stomatal conductance (Vikram *et al.*, 2022).

Total carbohydrates (% of dry matter)

As shown in figure 1 A, the data indicated that within each level of CHT or BRs total carbohydrates were reduced in parallel with increasing irrigation intervals from 3 to 6, 9 or 12 days. The reductions in total carbohydrates percentage due to water deficient stress are in agreement with findings of other researchers (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; Sarker and Oba, 2018; El-Shanhorey and Sorour, 2019; Sorour, 2021). The water deficit may elevate the production of reactive oxygen species under drought stress which lead to oxidative stress and harm to chloroplasts structure and chlorophyll loses. Reducing chlorophylls contents and photosynthetic activity could indirectly cause a reduction in carbohydrates content. Moreover, water deficient assists translocation of absisic acid via xylem vessels to the shoot of stressed plants for stomatal closure which may be resulted in reduction of net photosynthesis and carbohydrate accumulation (Baccari *et al*., 2020).

Results in the same figure also showed that within each irrigation intervals, spraying the plants with any concentration of CHT or BRs resulted in significant increase in total carbohydrates compared to control plants. Under the same level of the two tested bio-stimulators, CHT was generally better in its effect than BRs for increasing total carbohydrates. The lowest value (15.99 %) was obtained from plants irrigated with the longest intervals (12 days) and not received any bio-stimulators treatments, whereas the highest mean value (38.64%) was resulted from plants irrigated with the shortest intervals (3 days)

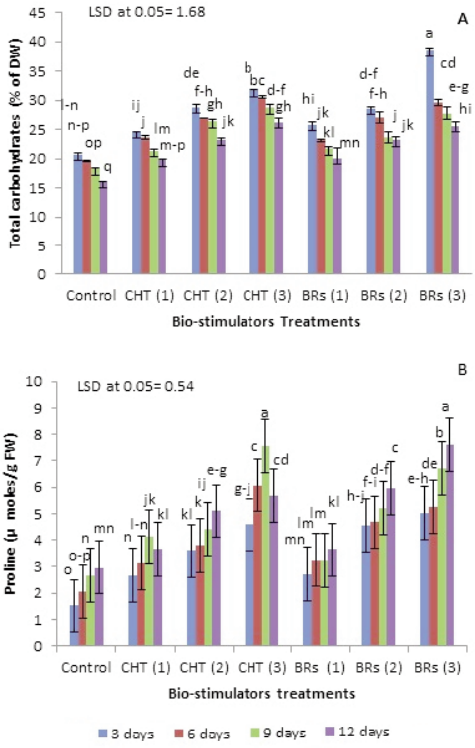

Fig. 1 - Total carbohydrates (% DW) (A), proline content (B) as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons). Column with different letters indicate a significant difference at 5% level. Vertical bars indicate to standard error (SE) of three replicates.

and sprayed with BRs at 200 ppm. The results of increasing total carbohydrates due to CHT treatments are the same as the results of previous researches (Zohreh *et al*., 2017; Shafiei-Masouleh, 2019; Elansary *et al*., 2020; Ashour *et al*., 2021; Salachna and Pietrak, 2021). While, the beneficial increase in total carbohydrates due to BRs treatments are in agreement with earlier studies (Mohamed, 2020; Pacholczak *et al*., 2021). Additionally, previous workers (Hemmati *et al.*, 2018; Mojaradi *et al*., 2020; Cai *et al*., 2021) indicated that application of BRs has a useful effect on carbohydrates accumulation of plants subjected to drought stress.

Proline content

Data in figure 1 B elucidated that in most cases under bio-stimulators treatments, proline content was increased progressively with prolonging irrigation intervals. Proline, an amino acid, plays a highly useful role in plants subjected to different stress conditions. Besides acting as an excellent osmolyte, proline plays three main roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat *et al*., 2012). Similar

results were reported by many studies (Ashour and El-Attar, 2017; El-Shanhorey and Sorour, 2019; Al-Arjani *et al*., 2020; El-Gamal and Khamis, 2021; Papú *et al*., 2021; Sorour, 2021; Samany *et al*., 2022; Shaltout *et al*., 2022).

The data also demonstrated that under each irrigation intervals, spraying any concentration of CHT or BRs resulted in significant higher values of proline content compared to control plants and BRs was superior in its effect than CHT. The highest mean value (7.61 µ moles/g FW) was registered from plants irrigated with the longest intervals (12 days) and sprayed with BRs at 200 ppm, while the lowest value (1.52 µ moles/g FW) was produced from plants irrigated with the shortest intervals (3 days) and not received any bio-stimulators treatments. The results of increasing proline content due to application of CHT treatments has been reported by earlier authors (Zohreh *et al*., 2017; Elansary *et al*., 2020; Ashour *et al*., 2021; Attaran Dowom *et al*., 2022). Whereas, the obvious increases in proline content due to application of BRs on ornamental water stressed plants are in good accordance with those elicited by prior authors (Zafari *et al*., 2020; Hosseinpour *et al.*, 2020; Mohammadi *et al.*, 2020; Omidian *et al.*, 2022). The accumulation of proline due to application of BRs may be due to BRs enhanced gene expression of biosynthetic genes (Sharma *et al*., 2019).

N, P and K (% of dry matter)

As shown in Table 5, N, P and K % were significantly affected by irrigation intervals, bio-stimulators treatment and interaction effects. The data in Table 7 indicated that within each level of the two bio-stimu-

Table 7 - N, P and K% of *Azadirachta indica* as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons)

Irrigation intervals	Bio-stimulators	N%	P%	K%
3 days	Control	1.28±0.03 lm	0.16±0.01 j-m	1.17±0.01 l-n
	CHT (1)	1.62±0.10 g-i	0.28±0.01 ef	1.42±0.01 f-i
	CHT (2)	1.95±0.04 d	0.32±0.01 d	1.51±0.02 ef
	CHT (3)	2.23±0.01 c	0.86±0.02 a	1.51±0.02 ef
	BRs (1)	1.55±0.01 h-j	0.30±0.01 de	1.75±0.05 bc
	BRs (2)	2.13±0.58 c	0.58±0.01 b	1.92±0.05 a
	BRs (3)	2.98±0.01 a	0.90±0.03 a	1.97±0.04 a
6 days	Control	1.31±0.01 kl	0.17±0.01 i-l	1.14±0.05 mn
	CHT (1)	1.59±0.11 g-i	0.28±0.03 ef	1.29±0.01 i-l
	CHT (2)	1.66±0.01 f-h	0.34±0.01 d	1.45±0.02 e-h
	CHT (3)	2.46±0.03 b	0.43±0.04 c	1.50±0.05 e-g
	BRs (1)	1.71±0.04 fg	0.20±0.01 h-j	1.59±0.02 de
	BRs (2)	2.22±0.02 c	0.30±0.01 de	1.68±0.14 cd
	BRs (3)	2.20±0.06 c	0.46±0.02 c	1.89±0.14 ab
9 days	Control	1.16±0.02 m-p	0.13±0.01 mn	1.12±0.01 mn
	CHT (1)	1.44±0.01 jk	0.18±0.01 i-l	1.18±0.01 l-n
	CHT (2)	1.42±0.02 jk	0.18±0.01 i-k	1.30±0.03 i-l
	CHT (3)	1.90±0.10 de	0.23±0.01 gh	1.36±0.07 g-j
	BRs (1)	1.49±0.01 ij	0.19±0.01 h-j	1.45±0.01 e-h
	BRs (2)	1.79±0.01 ef	0.20±0.01 g-i	1.29±0.01 i-l
	BRs (3)	1.94±0.11 d	0.30±0.01 de	1.33±0.01 h-k
12 days	Control	1.06±0.02 op	0.10±0.01 n	1.07±0.01 n
	CHT (1)	1.15±0.02 m-p	0.13±0.01 mn	1.11±0.02 mn
	CHT (2)	1.18±0.02 l-p	0.14±0.01 lm	1.10±0.02 mn
	CHT (3)	1.25±0.12 l-n	0.19±0.01 h-j	1.19±0.04 k-n
	BRs (1)	1.12±0.04 n-p	0.14±0.01 lm	1.23±0.10 j-m
	BRs (2)	1.25±0.02 l-n	0.15±0.01 k-m	1.22±0.01 k-m
	BRs (3)	1.27±0.04 lm	0.24±0.03 fg	1.21±0.02 k-n

CHT (1) = chitosan at 50 ppm, CHT (2) = chitosan at 100 ppm, CHT (3) = chitosan 200 ppm, BRs (1) = Brassinolide at 50 ppm, BRs (2) = Brassinolide at 100 ppm, BRs (3) = Brassinolide at 200 ppm. Each value represents the mean ± standard error of three replicates. Means in a column with different letters indicate a significant difference for each variable at 5% level using Duncan multiple rang test.

lators (CHT or BRs), prolonging irrigation intervals generally decreased three nutrients (N, P and K %). Similar reduction has been obtained by other reports (Ashour and El-Attar, 2017; Abd-Elmoneim *et al*., 2018; El-Shanhorey and Sorour, 2019; Singh and Singh, 2020; El-Gamal and Khamis, 2021).

The unfavorable effect of water deficient on the uptake and accumulation of the three nutrients in plant may be due to water deficient stress caused by extending the irrigation intervals resulted in low soil moisture content which affects the elements solubility and their absorbing efficiency by plants which in turn leading to reduce their accumulation in plant tissues. Additionally, limited transpiration rates and impaired active transport and membrane permeability lead to reduce nutrient uptake by the roots and accumulation in the shoots (Farooq *et al*., 2009).

The data in the same Table disclosed that within each level of irrigation frequency, in most cases three nutrients were significantly higher in the plants sprayed with any concentrations of two tested bio-stimulators (CHT or BRs) than those recorded with control plants. Under the same level of the two tested bio-stimulators, BRs appeared to be more effective than CHT and among BRs concentrations; the highest dose (200 ppm) was the most effective one. The results of increasing N, P or K% due to application of CHT confirmed the reports of previous study (Abd-El-Hady, 2020; Salachna and Pietrak, 2021). While, the increase due to application of BRs are similar to those obtained by prior workers (Mohamed, 2020).

Total indoles and phenols content

Results in figure 2 A showed that with each level of CHT or BRs, increasing irrigation intervals caused steady reduction in content of total indoles**.** Within each irrigation intervals, application of any concentrations of CHT or BRs resulted in significant increase in total indoles compared to control plants**.** Additionally, CHT appeared to be more effective than BRs and the highest concentration (200 ppm) was the most effective one.

Results in figure 2 B indicated that total phenols content showed different trend in response to water deficient, under each level of CHT or BRs, content of total phenols was increased linearly with increasing irrigation intervals from 3 to 6, 9 or 12 days. The present increase in total phenols content as result of water deficient are similar to those obtained by (Sarker and Oba, 2018; El-Gamal and Khamis, 2021; Papú *et al*., 2021).

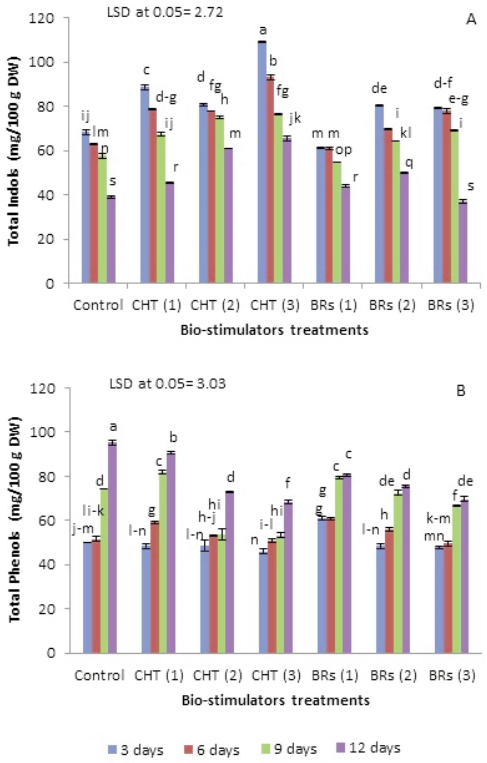

Fig. 2 - Total indoles (A), total phenols (B) as affected by the interactions between irrigation intervals and bio-stimulators treatments (mean of two seasons). Column with different letters indicate a significant difference at 5% level. Vertical bars indicate to standard error (SE) of three replicates.

The data also revealed that within each irrigation intervals, application of CHT or BRs at the highest concentration (200 ppm) reduced the mean values compared to control. Although previous studies revealed increase in total phenols content due to CHT treatments (Pirbalouti *et al*., 2017; Vosoughi *et al*., 2018; El-Serafy, 2020; Elansary *et al*., 2020; Arshad *et al*., 2022; Attaran Dowom *et al*., 2022) or due to BRs treatments (Amraee *et al*., 2020; Mohammadi *et al*., 2020). However, under the present study total phenols content was reduced in response to application of CHT or BRs which support the results of Ashour *et al*. (2021) who found that application of CHT decreased total phenols content.

4. Conclusions

Water deficient stress had a harmful effect on growth parameters, pigments content, total carbohydrates and nutrient uptake while, increased proline

and total phenols content. Foliar application of chitosan or brassinolide at the higher concentrations (200 ppm) increased growth parameters, pigments content, total carbohydrates, proline content and nutrient uptake. Based on the obtained results it can be concluded that, foliar application of chitosan or brassinolide at 200 ppm can alleviate the adverse effects of water deficient stress on the growth and physiology parameters of *Azadirachta indica*.

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