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Research Article

Impact of biostimulants based amino acids and irrigation frequency on agro-physiological characteristics and productivity of broccoli plants

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Abstract

Abstract scientists are concerned about the utilizing of biostimulants-based amino acids in plant feeding and mitigating negative effects of water stress. As a result, two-year field experiments were conducted at Faculty of Agriculture's experimental station at Cairo University to study the effectiveness of liquid yeast waste (CMS) and Cargo amino acids (AAs) on the agro-physiological features of broccoli plants with different irrigation frequencies (every 5, 10, and 15 days). Compared with control plants, the foliar application of AAs and CMS significantly increased plant height, photosynthesis pigments, head weight, head diameter, head height, plant yield, and quality at different irrigation frequencies. Likewise, both treatments (CMS and AAs) significantly improved ascorbic acids by 16.65% and 15.95% and increased total phenol content by 24.10% and 36.60%, respectively, compared to control plants. Accordingly, the highest productivity was achieved for broccoli grown under irrigation every five days with the CMS bio-stimulant, where it was 3111.17 kg ha^{-1}, while the lowest productivity was achieved for the control treatment in which no biostimulants were added and irrigation frequency every 15 days, and the productivity was 1376.22 kg ha^{-1}. Plants irrigated every 15 days produced the highest levels of abscisic acid (ABA) and superoxide dismutase (SOD), followed by plants irrigated every 10 days and every 5 days. Exogenous application of amino acids bio-stimulants could be suggested to improve vegetative growth, biochemical characteristics, productivity, and nutritional value as well as to mitigate negative effects of water scarcity.

Keywords: amino acids; biostimulants; *Brassica oleracea*; irrigation times; yield quantity and quality

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Introduction

Due to its great productivity as well as nutritional value, growing broccoli (*Brassica oleracea* var. italica) is becoming increasingly popular among Egyptian farmers (Acharya *et al.*, 2015; Lordwin *et al.,* 2007). Approximately 90% of it is water, 2% is dietary fibre, 3% is protein, 5% is carbs, antioxidants, phytochemicals, vitamins A, B, and C, as well as minerals (Rodrigues et al., 2013). According to Kumari *et al.* (2018), broccoli is susceptible to water stress and needs up to 4.1 mm of water per day. Due to the population's fast development, which needs increased food production and puts strain on these resources, there are growing worries about the world's threatened water and land resources (Islam and Mollah, 2014).

Egypt is considered one of the developing countries suffering from water scarcity where water availability per capita is less than 1000 m^3. In addition to consuming large amounts of water by the agricultural sector estimated about 80% of the available freshwater resources and poor irrigation efficiency result in a water shortage significantly (Bader *et al.,* 2020) which has negative impact on plant development and production (Nora *et al.,* 2012). Accordingly, improving water use efficiency by implementing sustainable management strategies and enhancing crop performance under drought is important (Haliński and Stepnowski, 2016; Sahebi *et al*., 2015; Xie *et al*., 2015).

Some crops' tolerance to a given amount of water stress varies from plant to plant, either at a specific period or over the whole growing season, but once that threshold is reached, growth and production begin to decline (Enchalew *et al*., 2016) by about 17% in agricultural production (Ahmad, 2016; AL-Shammari *et al*., 2020) and globally by about 75% of the harvested area (Kim *et al*., 2019). Furthermore, interactions between morpho-physiological, morphological, and biochemical variables influence how plants respond to and thrive under water-deficit environments (Moschen *et al.,* 2017).

Many strategies, like regulated deficit irrigation (RDI), may significantly reduce the amount of water as well as energy used in farming (Chai *et al.,* 2014), which enhances water usage efficiency as well as farmers' net profit (Nora *et al*., 2012; Wenneck *et al.,* 2021). On the other hand, multiple strategies based on the application of various natural, efficient, affordable, and inexpensive chemicals are frequently employed to improve plant tolerance to water deficiency. Plant hormones, biostimulators (such as amino acids), and other signal molecules are examples of these chemicals (El-Bassiouny *et al*., 2020; Sadak, 2016). Plants under abiotic stress employ a variety of techniques to mitigate the negative impacts, the majority of which are related to amino acid metabolism (Hildebrandt, 2018).

Amino acid application, either foliar or soil, improved plant growth and production (Spann and Little, 2010). Amino acids are essential for boosting tissue protein content and enzyme activity, both of which are required for metabolic antioxidant on-site processes (Al-Shammari *et al*., 2018; Sh Sadak *et al*., 2015). Several writers reported favourable results after using amino acids in the presence of abiotic stress. Shekari and Javanmardi (2017) L-cysteine, L-methionine, and amino acid combos were shown to improve vegetative development in broccoli (*Brassica oleracea* var. italic) transplants. Another study found that adding amino acids at 150 mg L-1 increased cabbage growth or nutritional value while mitigating harmful consequences of drought-related stress (Haghighi *et al*., 2020).

Furthermore, Mehanna *et al*. (2013) discovered that spraying canola plants with 150 ppm glutamic acid improved water use efficiency at 50% of the ETc, which improved plant response. Bader *et al.* (2020) observed the similar impact of foliar amino acid spray in improving crop development, production, and water productivity by mitigating the negative effect of water deficiency (Gonzalez *et al*., 2008). The objectives of present research were to 1) assess the influence of integrating two commercial bio-stimulants with irrigation frequency on broccoli yield and quality, and 2) characterize the impact of bio-stimulants-based amino acids and water-stress conditions on a variety of selected vegetative development indicators and physiological properties of broccoli plants.

Materials and Methods

Experimental location characteristics

Two open field experiments were performed at field station of Faculty of Agriculture, Cairo University, Giza, Egypt (30°01'10.3" N, 31°10'05.9" E, 24 m above sea level), during winter seasons of 2021-2022 and 2022-2023. The soil on the inspection site is categorized as clay loam. In accordance with Dewis and Freitas (1970) description of the pipette technique, mechanical analyses (sand, silt, and clay) were quantified. As stated by Jackson (1967), soil response (pH) was evaluated in a solution with a ratio of 1: 2.5 (soil: water). The bulk density of soil samples was assessed using cylindrical core sampler with sharp edges. Each cylinder was gently pushed into earth to desired depth in order to get an accurate measurement of the soil that was undisturbed. After being dried in an oven for 24 hours at 105 °C, the values of bulk density were reported as g cm^{-3}. (Vomocil, 1957).

The pressure membrane technique (desorption) was used to calculate field capacity (FC) and permanent wilting point (PWP) at 0.33 and 15 atm, respectively (Klute and Page, 1986) (Table 1). The chemical analysis includes high accessible potassium (K), organic nitrogen, and nitrogen, phosphate, and soil pH (Table 2). In the experimental region, irrigation water with pH of 7.2 and average electrical conductivity of 0.83 dS m^{-1} was collected from a deep well.

Table 1. Physical properties of experimental soil

Soil depth (cm)	Particle size distribution (%)			Texture	Field capacity (cm^3cm^{-3})	Wilting point ($cm^3 cm^{-3}$)	Bulk density (g cm^{-3})
	Sand	Silt	Clay				
0 - 20	25.20	38.15	36.65	Clay Loam	39.18	18.31	1.28
20 - 40	24.90	37.60	37.50	Clay Loam	39.02	19.31	1.3
40 - 60	24.50	36.80	38.70	Clay Loam	38.72	21.4	1.33

Table 2. Chemical properties of experimental soil

pH (1:2.5)	ECe (dS m^{-1})	ON (%)	N (mg kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})
7.82	0.17	0.17	41.18	58.12	512.4	5.15	22.1
7.81	2.51	0.15	36.4	51.33	498.2	4.84	19.9
7.83	2.08	0.14	29.58	50.08	473.3	4.4	19.2

Climatic conditions

Meteorological data were logged daily during both seasons, relative humidity, the amount of sunshine hours, and the maximum, lowest, and average air temperatures. For both seasons (December to February), the average maximum temperature was 17.54 °C for first growing season and 20.46 °C for second growing season. The average lowest temperature was 6.1 °C in 2021-2022 and 8.20 °C in 2022-2023, respectively (Table 3). Each year had only a trace amount of rainfall (20 mm). As a result, irrigation was primarily responsible for the soil's availability of water. The monthly mean climatic data for El-Giza's two growth seasons are shown in Table 3 as a whole.

Table 3. The meteorological data for El-Giza city during both growing seasons of 2021-2022 and 2022-2023

Year	Climate parameter	Month		
		December	January	February
2021-2022	$T_{min.}$	8.10	4.13	5.96
	T_{max}	18.18	15.88	18.55
	T_{ave}	13.14	10.01	12.25
	RH (%)	66.39	66.28	63.37
	WS (m $^{sec-1}$)	2.94	3.02	3.18
	Solar radiation (W m^{-2})	72.44	79.90	97.17
2022-2023	$T_{min.}$	10.46	7.96	6.24
	T_{max}	22.05	19.90	19.42
	T_{ave}	16.25	13.93	12.83
	RH (%)	62.47	58.36	56.17
	WS (m $^{sec-1}$)	2.86	2.77	2.85
	Solar radiation (W m^{-2})	72.58	79.36	97.17

Crop administration

In December 2021-2022, and 2022-2023, the seedlings of broccoli plants (*Brassica oleracea* var. italica) were transplanted at spacing of 0.40 m among plants and 0.50 m between rows. During the experimentation, ammonium nitrate (33%), calcium superphosphate (15.5%), and potassium sulphate (48%) at a rate of 600, 600 and 125 kg. ha^{-1}, respectively, were used as fertilizers to feed the broccoli plants. After 90 days from seedlings transplanting, healthy heads of each treatment were harvested manually using a sharp knife and transported into the laboratory for taking the measurements.

Experimental treatments

In both growth seasons, split-plot design with four replicates was utilized. The main plot was given irrigation treatments (once every five days, once every ten days, and once every fifteen days (control). The treatments included 1), Cargo amino acids (AAs) were sprayed on plant leaves at a rate of 2 g. L^{-1}, 2) Liquid yeast waste (CMS) was sprayed on plant leaves at a rate of 10 cm. L^{-1}, and 3) untreated plants (control plants, CON). Cargo amino acids and liquid yeast waste were purchased from Angel Yeast Egypt Company and Keymanda for fertilizers and chemicals industries, respectively. The chemical properties of CMS and AAs are presented in Table 4.

Table 4. Chemical composition of commercial bio-stimulant based amino acids

AAs (Cargo)	(%)	CMS (liquid yeast waste)	(%)
Aspartic acid	1.17	N	2.0
Threonine	1.69	P2O5	0.2
Serine	1.48	K2O	8.0
Glutamine acid	1.77	Ca	0.89
Glyein	1.50	S	10.04
Alanine	0.95	Mg	0.16
Isoleucine	0.81	Fe	0.003
Lysine	1.72	Zn	0.04
Phenylalanine	1.26	Mn	0.002
Histidine	1.16	Organic matter	55.0
Proline	1.70	Total amino acids	20.0
Arginine	1.90	Free amino acids	7.01
Tyrosine	0.22		
Leucine	1.62		
Valine	1.35		

Crop evapotranspiration

The Food and Agriculture Organization of the United Nations (FAO) typically recommends using reference evapotranspiration (ETo), which is based on Penman-Monteith equation (Allen, 2000) and calculated by applying daily environmental condition parameters. Allen (2000) has used the ETo with success. The meteorological data were utilized as input for ETo calculator computer code (FAO, 2009):

$$ET_o = \frac{0.408\Delta(R_n + G) + \gamma\left(\frac{900}{T + 273U_2 (e_s - e_a)}\right)}{\Delta + \gamma\ (1 + 0.34U_2)} \quad (1)$$

where ETo represents reference evapotranspiration (mm day^{-1}), Rn represents net radiation at crop surface (MJ m^{-2} day^{-1}), G represents soil heat flux density (MJ m^{-2} day^{-1}), T represents the mean daily air temperature at 2 m height (°C), U2 represents wind speed at 2 m height (ms^{-1}), and es Andean represents saturated vapor pressure deficit (kPa). The approach and procedures described in Allen (2000) are still used to calculate the crop's evapotranspiration, which already accounts for the daily water consumption of plants by collecting the water that evaporates from the soil and transpires via plant leaf stomata.

$$ET_C = ET_O\ (K_{cb} + K_e) \quad (2)$$

where Kcb represents coefficient of basal crop, Ke represents coefficient of soil evaporation, and ETo represents daily reference evapotranspiration (mm day^{-1}).

System installation

For the experimental research, a field plot of 20.25 x 10 m was used. Nine identical subplots measuring 2.25 m by 10 m were created from the field plot. In November 2021, a regulated installation of a trickle irrigation system was initiated, which included a screen filter equipped with a backflush mechanism, as well as a Venturi meter. The ridges were precisely lined up using trickle tape (Euro drip GR), which had apertures on its top edges. In the trickling system, each dripper was placed at a distance of 0.40 cm and had an application rate of 4.2 L.h^{-1}.

Assesment crteria

Water productivity (WP)

Water productivity (kg m^3) is the quotient of crop productivity (kg ha^{-1}) by the crop evapotranspiration (m^3 ha^{-1}) during its growth period, the WP was estimated using the equation of Howell *et al.* (1990).

$$WP = \frac{Y}{ET_c} \quad (3)$$

Agronomical measurements

Six plants from each treatment were chosen randomly after 80 days from transplantation to determine vegetative growth parameters of plant height, and number of leaves per plant (El-Beltagi *et al.*, 2022). Total yield of broccoli was assessed by harvesting and weighting each treatment's heads separately, and values of total yield were expressed as kilograms per hectare (kg ha^{-1}). A meter tape was used to measure the height of the head. The digital balance was used to determine the fresh weight of broccoli heads. The head diameter was estimated using digital feet, and the data were represented as cm^2. TSS of broccoli samples were tested using digital refractometer (model PAL-1, Atago, Tokyo, Japan), and TSS results were given as °Brix.

Photosynthesis pigments

Broccoli samples' fresh leaves were extracted with acetone (80%, Sigma-Aldrich Co. LLC) and left at 5 °C for an hour. The extractions were centrifuged at 3,000 x g for 15 minutes. A spectrophotometer (Helios UVG1702E, England) was used to quantify the amounts of carotenes, chlorophyll *a*, and *b* at wavelengths of 663, 647, and 470 nm, respectively. The amounts of chlorophyll or carotenoids were measured using the method described by Lichtenthaler and Wellburn (1983) and the final results were calculated using the following equations:

$$\text{Chlorophyll A} = 12.70 \times A663 - 2.79 \times A647 \quad (4)$$

$$\text{Chlorophyll B} = 20 \times A647 - 4.62 \times A663 \quad (5)$$

$$Carotenoids = \frac{((1000 \times A470 - (3.27\ Chl.\ A - 104\ Chl.B))}{229} \quad (6)$$

Antioxidant activity

The effect of various applications on broccoli antioxidant activity (AOA) was measured using the method described by Zhang *et al.* (2019). Ten grams of leaf samples were mixed in 200 mL of methanol before being filtered. The filtrate was then diluted in 25 mL of distilled water. One millilitre of the extract was combined with three millilitres of methanol and one millilitre of 2.2 diphenyl-1-picrylhydrazyl (DPPH). After 10 minutes, the mixture was homogenized in the dark at room temperature. The percentage of DPPH inhibition (DPPH I N%) was measured calorimetrically with a spectrophotometer at 517 nm and calculated using the following equation (El-Beltagi *et al.,* 2022):

$$\text{Antioxidant activity (\%)} = \frac{A_{517\,nm}\text{ of DPPH solution} - A_{517\,nm}\text{ of sample}}{A_{517\,nm}\text{ of DPPH solution}} \times 100 \quad (7)$$

Ascorbic acid and total phenolic compounds

According to AOAC (1990), ascorbic acid (AsA) was measured using titrimetric technique with 2, 6-dichlorophenol indophenol, with results of AsA expressed as mg/100 g of fresh broccoli weight. According to Singleton and Rossi (1965), the spectrophotometric technique with the Folin-Ciocalteu reagent was utilized to detect total phenolic compounds (TPC). The results of TPC in plant leaves were defined as Gallic acid mg g^1 of broccoli fresh weight (mg GAE g^{-1} fw).

Abscisic acid (ABA)

With a mortar and pestle, freeze-dried samples (6 g FW) were ground to fine powder. At 4 °C in darkness, the powder was extracted three times with methanol (80% v/v, 15 mL g^{-1} FW) supplemented with butylated hydroxytoluene DBPC as an antioxidant. According to Shalom *et al.* (2014), the methanol extract was employed following methylation to quantify Abscisic acid (ABA). The ABA was quantified utilizing an ATI Unicam Gas Liquid Chromatography system fitted with a flame ionization detector, as described by Saini *et al.* (2022). The ABA peak identification and quantification were carried out with the use of external genuine hormones and Microsoft application to determine concentrations of the detected peaks.

Bioassay of superoxide dismutase

In 5 mL of potassium phosphate buffer (100 mM, pH 7.0) with 2% (w/v) N-Vinylpyrrolidinone, 0.5% Triton X-100, and 1 mM ascorbic acid, 500 mg of the freeze-dried samples were pulverized and combined (Polle *et al.*, 1994). In accordance with Beauchamp and Fridovich (1971), the mixture was centrifuged at 12,000 × g for 20 min at a cool temperature (4 °C) and supernatants were collected to measure activity level of superoxide dismutase (SOD, EC 1.15.1.1). The activity level of SOD was assessed by the inhibition of photoreduction of nitroblue tetrazolium (NBT) by the SOD enzyme. The reaction mixture contained potassium phosphate solution and 100 µL of filtrated extract in a final volume. A control reaction was carried out without filtrated extract. After fifteen minutes of incubation, reaction mixtures, and control reactions were determined spectrophotometrically at wavelength 560 nm. One unit of SOD activity was defined as the quantity of enzyme causing inhibition of NBT.

Statistical analysis

The Factorial experiment was organized as a randomized complete block design with three replications at both seasons 2021-2022 and 2022-2023, with irrigation frequencies as the main plots and amino acids biostimulants as subplots. The obtained data were subjected to two ways of variance (ANOVA) and means were compared at the 0.05 level according to Duncan's test ($p \leq 0.05$). The Statistics 7 program was used to perform statistical analyses. An online statistical analysis and visualization software performed an analysis of Pearson's correlation and a Heatmap (Mahmoud *et al*., 2023).

Results and Discussion

Broccoli productivity and water productivity

From the general view of the results, we found that the highest productivity was achieved at irrigation frequency every 5 days, and productivity decreased as the period between irrigations increased. On the other hand, the highest productivity was achieved with the addition of the bio-stimulant CMS, followed by AAs, and finally comes the control treatment in which no amino acids are added. Accordingly, the highest productivity was achieved for broccoli grown under irrigation every five days with the CMS bio-stimulant, where it was 3111.17 kg ha^{-1}, while the lowest productivity was achieved for the control treatment in which no biostimulants were added and irrigation frequency every 15 days, and the productivity was 1376.22 kg ha^{-1} (Figure 1).

Water productivity followed the same trend as productivity. In the same context, the maximum water productivity was achieved under irrigation frequency every 5 days and CMS and it was 1.955 kg m^{-3}, while the lowest water productivity was registered with irrigation every 15 days and no biostimulants were added, where it was 0.865 kg m^{-3} (Figure 1).

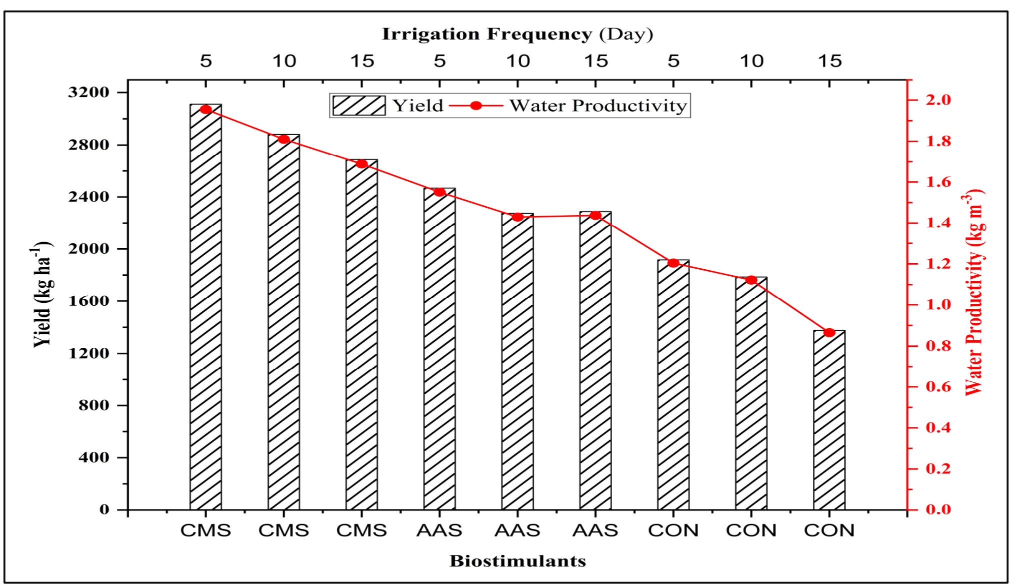

Figure 1. The broccoli productivity and water productivity under various irrigation frequency and amino acids treatments

Growth characteristics

Irrigation frequency, bio-stimulant treatments, and their interactions significantly affected growth measurements of broccoli plants , which included plant height, number of leaves, and photosynthesis pigments (Chl. a, b, and carotenoids), as shown in Tables 5, 6 and 7. The highest values of the aforementioned measurements (plant height, number of leaves, Chl. a, b, or carotenoids) were recorded in broccoli plants sprayed with biostimulants (CMS and Amino acids), and the plants were irrigated every 5 and 10 days. While the lower values were recorded in control plants and plants irrigated every 15 days (Tables 5 and 6).

Broccoli growth parameters were affected also by the interaction of irrigation frequency and biostimulants-based amino acids. Plant height, number of leaves, photosynthesis pigments (Chl. a, b, and carotenoids) of broccoli plants treated with biostimulants (CMS and Amino acids) and irrigated every 5 and 10 days were greater than untreated plants irrigated every 15 days (Table 7), at both seasons. In general, foliar application of CMS and AAs improved photosynthesis pigments and vegetative growth of the broccoli plants under different irrigation frequencies. These improvements could be more associated with amino acid molecules in applied bio-stimulates which play vital roles in plant growth and chlorophyll synthesis (Sowmya *et al.,* 2023).

Mohammadipour and Souri (2019) stated that amino acids also participate as a fundamental component for protein biosynthesis, which is related to plant growth such as root and stem development as well as increase in area and number of plant leaves. Furthermore, amino acids such as serine, alanine or lysine are involved in chlorophyll and carotenoid biosynthesis. Several researches have revealed that the application of amino acids may have impact on plant development since these compounds can activate physiological processes in plants such as protein synthesis, carbohydrate metabolism, and hormone precursors.

In this respect, it has been stated that foliar supplementation of AAs improves vegetative growth, quantity and quality in *Solanum tuberosum* (Mobini *et al.,* 2014), garlic (El-Shabasi *et al.,* 2005) and bean (Abdel-Mawgoud *et al.,* 2011). In another publication, the foliar application of amino acid mixtures to broccoli seedlings significantly increased growth of the roots and shoots (Shekari and Javanmardi, 2017). In a similarly, Mobini *et al.* (2014) stated that foliar spraying with three different AAs enhanced growth, yield or bulb quality of onion plants.

Table 5. Effect of biostimulants on plant growth and photosynthesis pigments

Biostimulant	Plant height (cm)		Number of Leaves		Chl. *a* (mg g^{-1} fw)		Chl. b (mg g^{-1} fw)		Total Chl. (mg g^{-1} fw)		Carotenoids (mg g^{-1} fw)	
	F	S	F	S	F	S	F	S	F	S	F	S
CMS	61.89 a	58.44 a	21.0 a	20.22 a	6.94 a	7.38 a	3.25 a	2.60 a	9.14 a	9.98 a	0.902 a	1.350 a
AAs	61.22 a	58.33 a	22.11a	21.33a	7.57 a	7.31 a	2.77 a	2.98 a	9.46 a	10.29 a	0.77 a	1.160 a
CON	57.33 b	53.00 b	18.56 b	17.89b	5.08 b	5.72 b	1.66 b	1.63 b	6.53 b	7.34 b	0.40 a	1.027 a

Significant differences exist between means with different letters in the same column at a significance level of $P<0.05$ (Duncan multiple test, n=3), Chl. = chlorophyll, F = first growing season, S = second growing season, fw= fresh weight, AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

Table 6. Effect of irrigation frequency on plant growth and photosynthesis pigments

Irrigation frequency (Day)	Plant height (cm)		Number of leaves		Chl. *a* (mg g^{-1} fw)		Chl. b (mg g^{-1} fw)		Total Chl. (mg g^{-1} fw)		Carotenoids (mg g^{-1} fw)	
	F	S	F	S	F	S	F	S	F	S	F	S
5	66.44 a	63.11 a	24.44 a	23.89 a	8.126 a	8.27 a	3.10 a	2.86 a	10.40 a	11.1 a	0.910 a	1.289 a
10	59.11 b	57.22 b	19.67 b	20.33 b	6.912 a	7.57 a	2.52ab	2.67 a	8.76 b	10.2 a	0.797 a	1.387 a
15	54.89 c	53.44 c	16.56 c	17.22 c	4.55 b	4.57 b	2.08 b	1.68 b	5.96 c	6.25 b	0.36 b	0.86 b

Significant differences exist between means with different letters in the same column at a significance level of $P<0.05$ (Duncan multiple test, n=3). Chl. = chlorophyll, F = first growing season, S = second growing season.

Table 7. Effect of irrigation frequency, biostimulants, and their interaction on plant growth measurements.

Treatments (Biostimulats / Day)		Plant height (cm)		Number of Leaves		Chl. a (mg g^{-1} fw)		Chl. b (mg g^{-1} fw)		Total Chl. (mg g^{-1} fw)		Carotenoids (mg g^{-1} fw)	
		F	S	F	S	F	S	F	S	F	S	F	S
CMS	5	68.67 a	65.0 a	25.67 ab	24 ab	7.48 ab	8.41 a	3.78 a	2.9 bc	11.03 a	11.30 a	1.68 a	1.49 ab
	10	60.0 bcd	57.33 bc	19.67 cd	20 cd	8.56 ab	8.96 a	3.23 abc	3.09 b	10.74 a	12.06 a	0.796 bc	1.610 a
	15	57.0 b-e	53.00 c	17.67 cd	16.67 e	4.76 de	4.76 bc	2.75 a-d	1.8 ef	5.64 ef	6.58 cd	0.23 d	0.94 bc
AAs	5	68.33 a	63.00 a	26.67 a	25.33 a	9.17 a	8.33 a	3.44 ab	3.96 a	12.06 a	12.20 a	0.94 b	0.93 bc
	10	61.0 bc	57.33 bc	21.67 bc	21.00 c	7.35 abc	7.62 ab	2.49 bcd	2.9 bcd	9.08 b	10.5 ab	0.81 bc	1.35 ab
	15	54.33 de	54.67 c	18.0 cd	17.67 de	6.18 bcd	5.98 abc	2.40 bcd	2.1 cde	7.24 cd	8.10 bc	0.56 bcd	1.18 ab
CON	5	62.33 b	61.33 a	21.0 cd	22.33 bc	7.72 ab	8.04 a	2.07 cd	1.74 ef	8.11 bc	9.78 ab	0.14 d	1.44 ab
	10	56.33 c-e	57.00 bc	7.67 cd	20.00 cd	4.82 cde	6.13 abc	1.82 de	2.04 de	6.48 de	8.17 bc	0.78 bc	1.19 ab
	15	47.33 e	48.67 c	17.01 d	17.33 e	2.72 e	2.98 c	1.083 e	1.117 f	5.017 f	4.093 d	0.29 bcd	0.45 c

Significant differences exist between means with different letters in the same column at a significance level of $P<0.05$ (Duncan multiple test, n=3). Chl. = chlorophyll, F = first growing season, S = second growing season, fw= fresh weight, AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

Broccoli yield and its quality

Similar trends were observed in yield components and their quality. Yield, head weight, head diameter, head height, and TSS content were affected by bio-stimulants based amino acid, irrigation frequency, and all of their interactions (Tables 8-10). Head weight, head diameter, head height, and TSS content due to spraying leaf with CMS and AAs were similar and greater than in untreated plants (Table 8).

In addition, the maximum values of head weight, head diameter, head height, and TSS content were recorded in broccoli plants watered every 5 days followed by the plants irrigated every 10 days, and lowest values were noted in plants irrigated every 15 days (Table 9).

Data in Table 10 show that the highest values of yield, head weight, head diameter, head height, and TSS content were noted in broccoli plants sprayed with bio-stimulants based amino acid and irrigated every 5 days and 10 days. While lowest values were noted in untreated plants irrigated every 15 days, at both seasons.

Table 8. Effect of biostimulants based amino acids on broccoli yield and its quality

Biostimulants	Yield (kg ha^{-1})		Head height (cm)		Head weight (Kg)		Head diameter (cm)		TSS (^{O}Brix)	
	F	S	F	S	F	S	F	S	F	S
CMS	2809.58 a	2904.82 a	1297 a	11.67 a	0.607 a	0.599 a	20.61 a	19.44 a	5.51 ab	5.50 a
AAs	2329.41 b	2468.30 a	11.41 ab	10.56 b	0.492 b	0.475 b	20.27 a	19.00 a	5.70 a	5.53 a
CON	1699.24 c	1836.54 b	10.08 b	9.05 c	0.355 c	0.341 c	16.53 b	16.28 b	5.20 b	5.00 a

Significant differences exist between means with different letters in the same column at a significance level of $P<0.05$ (Duncan multiple test, n=3). F = first growing season, S = second growing season. AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

Table 9. Effect of irrigation frequency on broccoli yield and its quality

Irrigation frequency (Day)	Yield (kg ha^{-1})		Head height (cm)		Head weight (Kg)		Head diameter (cm)		TSS (^{O}Brix)	
	F	S	F	S	F	S	F	S	F	S
5	1969.09 a	2176.23 a	13.53 a	12.33 a	0.524 a	0.533 a	22.76 a	20.72 a	5.76 a	5.73 a
10	1773.85 b	1904.80 b	11.14 b	10.61 b	0.485 ab	0.472 ab	18.92 b	18.33 b	5.63 a	5.53 a
15	1354.79 c	1428.60 c	9.77 c	8.33 c	0.444 b	0.419 b	15.73 c	15.67 c	5.02 b	5.20 b

Significant differences exist between means with different letters in the same column at a significance level of P<0.05 (Duncan multiple test, n=3). F = first growing season, S = second growing season.

Table 10. Effect of interaction between irrigation frequency and biostimulants on broccoli yield and its quality

Treatments (Biostimulants /Day)		Yield (kg ha^{-1})		Head height (cm)		Head weight (Kg)		Head diameter (cm)		TSS (°Brix)	
		F	S	F	S	F	S	F	S	F	S
CMS	5	3111.17 a	3309.59 a	15.33 a	14 a	0.653 a	0.665 a	24.57 a	22.33 a	5.73 ab	6.06 a
	10	2881.01 ab	3285.78 a	12.83 ab	12 bc	0.605 ab	0.586 b	20.317 b	20.33 ab	5.56 ab	5.33 bc
	15	2687.36 b	2904.82 b	10.73 bc	9 de	0.564 bc	0.548 b	17.10 cd	15.67 cd	5.23b	5.10 c
AAs	5	2468.30 bc	2595.29 bc	14.87 a	13 ab	0.518 cd	0.533bc	24.73 a	21.67 a	6.03 a	5.76 ab
	10	2274.65 c	2381.00 cd	9.76 c	10.5 cd	0.477 de	0.459cd	20.40 b	19.00 b	5.70 ab	5.70 ab
	15	2287.35 c	2428.62 cd	9.60 c	8.16 e	0.480 d	0.432 d	15.17 de	16.33 cd	5.36 ab	5.13 c
CON	5	1915.91 d	2176.23 d	10.40bc	10 d	0.402 e	0.401 de	18.97 bc	18.17 bc	5.53 ab	5.36 bc
	10	1785.75e	1904.80 e	10.83 bc	9.3 de	0.375 ef	0.343 ef	16.20 de	15.67 cd	5.63 ab	5.56 abc
	15	1376.22f	1428.60 f	9.00d	7.83 f	0.289 f	0.278 f	14.43 e	15.0 d	4.46 c	5.00 d

Significant differences exist between means with different letters in the same column at a significance level of *P*<0.05 (Duncan multiple test, n=3). F = first growing season, S = second growing season. AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

Improvement in yield, head weight, head diameter, head height, and TSS content due to foliar spraying with amino acids biostimulants could be attributed to stimulation of root growth, increased proliferation of root hairs which enhanced nutrient uptake and water absorption (Sadak *et al.,* 2023).

Several studies confirmed that a positive impact of foliar spraying of amino acid on plants, such as increased crop productivity due to enhancement of chlorophyll content and photosynthetic efficiency leading to the accumulation of polysaccharides, proteins and other assimilates in edible parts (Sh Sadak *et al*., 2015). These results were in agreement with the findings observed by Koukounaras *et al.* (2013) who found that the productivity and fruit quality of tomato plants significantly increased when sprayed with amino acid mixtures on plants. In the same concern, Aly *et al*. (2019) provided that the foliar application amino acids increased growth parameters of hot pepper compared to control in both first and second seasons. Also, increased phenolic and flavonoid contents as well as the antioxidant activity of hot pepper fruits. Likewise, Sh Sadak *et al*. (2015) observed that faba bean plants treated with amino acids showed higher accumulation of dry biomasses, chlorophylls, total carbohydrates, and polysaccharides as well as higher crop productivity than control plants.

Ascorbic acid, total phenol content, and total antioxidants

As shown in Tables 11-13, the ascorbic acid and total phenol content are influenced by irrigation frequency, biostimulants, and their interactions. Untreated plants irrigated every 15 days recorded the highest content of ascorbic acids (AsA) and the lowest value of total phenol content (TPC) compared to irrigated plants every 5 and 10 days (Table 11). On the contrary, the foliar spraying with amino acids biostimulants significantly upgraded the concentrations of leaf AsA and TPC in broccoli plants more than in control plants (untreated plants), as shown in Table 12.

The total antioxidant content of treated and untreated plants is only affected by biostimulants and their interactions (Table 13). The highest value of total antioxidants was observed in plants treated with amino acids than all the other treatments. The maximum content of total antioxidants was obtained in broccoli plants sprayed with bio-stimulants under different irrigation times and minimum contents were noted in untreated plants.

The enhancement of AsA, TPC, and total antioxidant content in sprayed plants with biostimulants-based amino acids might be correlated to the improvement of the photosynthesis system in plants which leads to the accumulation of secondary metabolites. Furthermore, several authors confirmed that foliar application with amino acid bio-stimulant plays an important role in antioxidant metabolism in plants (Hildebrandt *et al.*, 2015; Weiland *et al.*, 2015; El-Beltagi *et al.*, 2023; Ramadan *et al.*, 2023).

The accumulation of ascorbic acid (AsA), under the foliar implementation of amino acid bio-stimulants, could be attributed to the high production of total non-structural carbohydrates (TNC). This is because TNC (D-glucose) is considered precursor for the biosynthesis of AsA in plants, with more accumulation of TNC more AsA would be produced in L-galactose pathways (De Tullio and Arrigoni, 2004; Hancock and Viola, 2005).

Table 11. Effect of irrigation frequency on ascorbic acid (AsA), total phenol content (TPC), and total antioxidants

Irrigation frequency (Day)	AsA (mg 100 g^{-1} fw)		Total antioxidant (%)		TPC (mg GAE 100 g^{-1} fw)	
	F	S	F	S	F	S
5	478.4 b	479.5 c	29.6 a	29.75 a	189.2 a	180.7 a
10	515.7 b	525.6 b	31.52 a	30.06 a	159.7 b	162.2 a
15	578.5 a	593.9 a	29.90 a	29.46 a	132.9 c	134.8 b

Significant differences exist between means with different letters in the same column at a significance level of $P<0.05$ (Duncan multiple test, n=6). F = first growing season, S = second growing season.

Table 12. Effect biostimulants based amino acids on ascorbic acid (AsA), total phenol content (TPC), and total antioxidants

Biostimulants	AsA (mg 100g^{-1} fw)		Total antioxidant (%)		TPC (mg GAE 100 g^{-1} fw)	
	F	S	F	S	F	S
CMS	556.5 a	579.6 a	26.32 b	26.16 b	164.2 b	160.4 b
AAs	552.1 a	536.7 b	35.73 a	34.61 a	195.8 a	192.8 a
CON	464.0 b	482.7 c	29.07 b	28.50 b	121.8 c	124.5 c

Significant differences exist between means with different letters in the same column at a significance level of $P<0.05$ (Duncan multiple test, n=3). F = first growing season, S = second growing season, fw= fresh weight, AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

Table 13. Effect of interaction between irrigation frequency and biostimulants on ascorbic acid (AsA), total phenol content (TPC), and total antioxidants

Treatments (Biostimulants /Day)		AsA (mg. 100g^{-1} fw)		Total antioxidant (%)		TPC (mg GAE 100 g^{-1} fw)	
		F	S	F	S	F	S
CMS	5	481.7 b	501.5 b	25.02 d	25.13c	154.3 d	143 cd
	10	541.1 bc	508.1 b	24.43d	25.41c	170.3 bc	170 bc
	15	716 a	659.9 a	29.03 bcd	28.41 c	168 c	168.2 bcd
AAs	5	455.3 d	461.9 de	33.04 ab	32.3 abc	273.3a	259.8 a
	10	567.5 b	593.9 a	37.05 a	39.1a	178.3 b	181.1 b
	15	587.3 b	600.5 a	33.7 ab	35.79 ab	135.7 ef	137.6 cd
CON	5	501.5 c	441.8 e	31.18 abc	31.64 bc	140 e	139.4 cd
	10	468.2 d	465.1 d	28.68 bcd	30.07 bc	130.3 f	135.6 d
	15	478.4 d	475.1 c	25.62 cd	25.51 c	95 g	98.5 e

Significant differences exist between means with different letters in the same column at a significance level of $P<0.05$ (Duncan multiple test, n=3). F = first growing season, S = second growing season, fw= fresh weight, AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

Ardebili *et al.* (2012) stated that foliar spraying with amino acids induced activity of phenylalanine ammonia-lyase (PAL) and improved the accumulation of phenol content in *Aloe vera* L plants. Watts et al. (2006) confirmed that L-tyrosine ammonia-lyase was also contributed in biosynthesis of phenolic compounds via shikimic pathway.

Abscisic acid and superoxide dismutase

It was observed that the treatment with amino acid showed the maximum values of abscisic acid (ABA), and superoxide dismutase (SOD) followed by the plants treated with AAs while the lowest values were control (Figure 2). However, AAs significantly improved the content of ABA and SOD in leaves by 45.26%, and 19.92% in first season while it was 45.04, and 23.66% in second season, respectively, in comparison to the untreated plants (control).

Whereas, irrigation frequency also affects the abscisic acid and superoxide dismutase. The maximum level of abscisic acid, and superoxide dismutase was recorded in broccoli plants irrigated every 15 days followed by plants irrigated every 10 days and 5 days. Furthermore, abscisic acid and superoxide dismutase are affected by the interaction between amino acids biostimulants and irrigation (Figure 3).

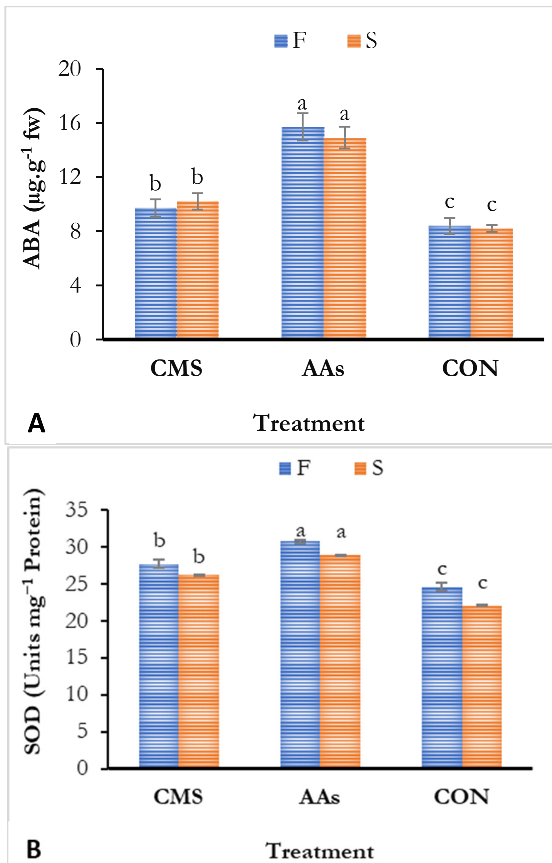

Figure 2. Effect of biostimulants based amino acids on abscisic acid (ABA-A) and superoxide dismutase (SOD-B)

Means with same subscript letters in the following columns are not significantly different at (Duncan multiple test, $P<0.05$). n=3, Bars indicated to standard error (±SE). F = first growing season, S = second growing season, fw= fresh weight, AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

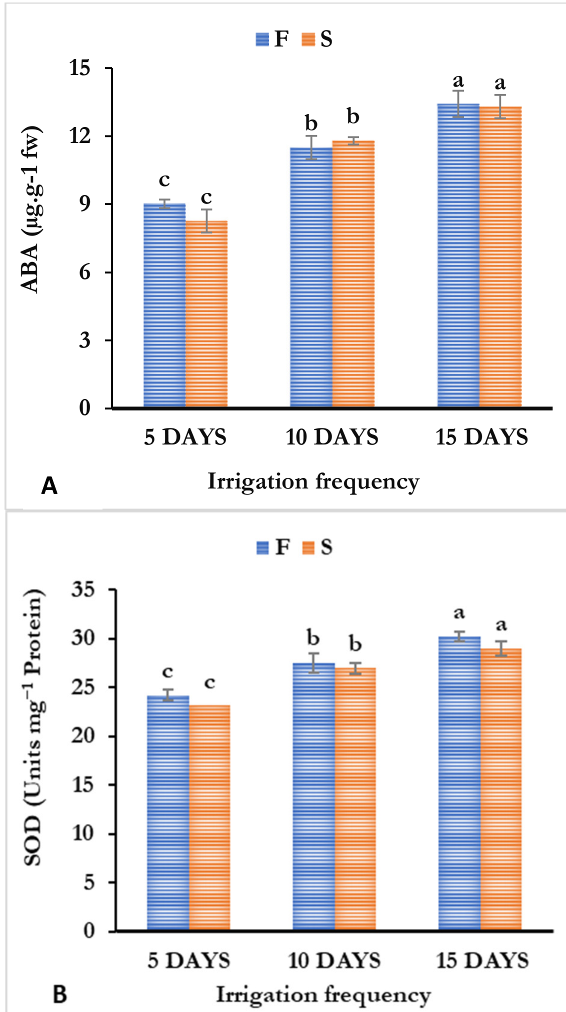

Figure 3. Effect of biostimulants and irrigation frequency on abscisic acid (ABA-A) and superoxide dismutase

(SOD-B Means with same subscript letters in the following columns are not significantly different at (Duncan multiple test, $P<0.05$). n=3, Bars indicated to standard error (±SE). F = first growing season, S = second growing season and fw= fresh weight.

The higher values of abscisic acid (ABA), and superoxide dismutase (SOD) were observed in broccoli plants sprayed with amino acids biostimulants at different irrigation frequencies in comparison to control plants (Figure 4). However, ABA and SOD significantly increased in leaves of broccoli plants irrigated every 15 days by 32.78%, and 19.80% in first season and 37.89, and 20.28% in second season, respectively, compared with plants irrigated every 5 days (control).

An increase in levels of ABA and SOD in broccoli plants irrigated every 15 days could be associated with accumulation of reactive oxygen species (ROS) in plant cells, in addition to altering membrane flexibility due to water shortage. The SOD and ABA levels are higher in drought susceptible cultivars but slowly deteriorated in drought-tolerant cultivars. Abdelaziz *et al.* (2021) reported that water stress induced accumulation of ABA and SOD in cucumber plants.

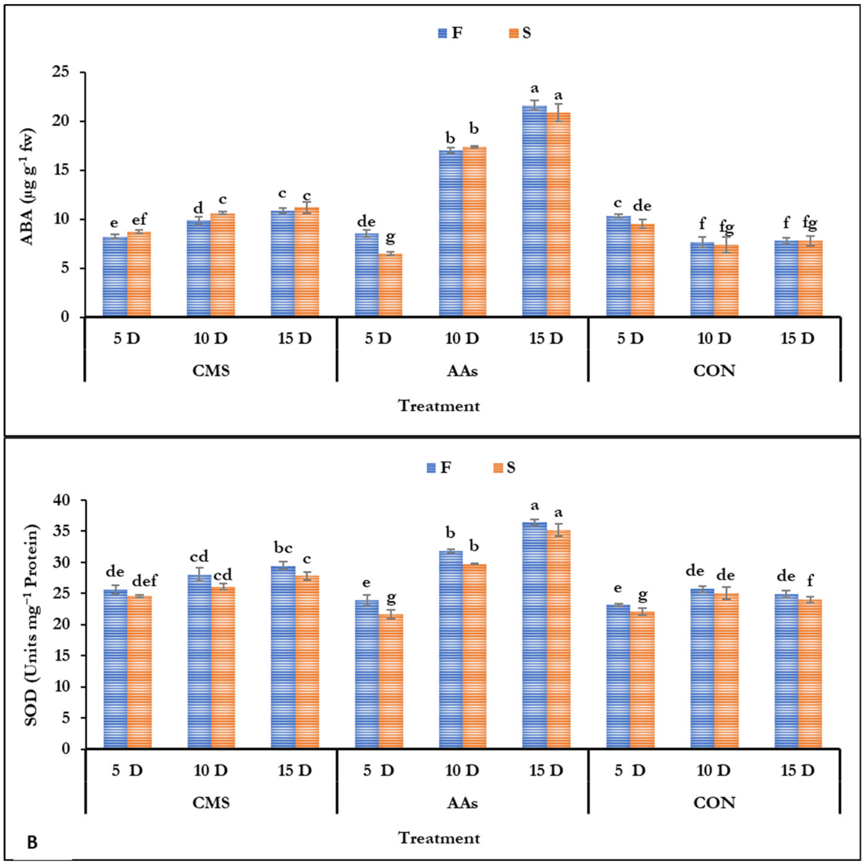

Figure 4. Effect of interaction between irrigation frequency and biostimulants on abscisic acid (ABA-A) and superoxide dismutase (SOD-B)

Means with same subscript letters in the following columns are not significantly different at (Duncan multiple test, $P<0.05$). n=3, Bars indicated to standard error (±SE). D= Days, F = first growing season, S = second growing season, fw= fresh weight, AAs= Cargo amino acids, CMS= liquid yeast waste and CON= control plants (untreated plants).

Abscisic acid is a well-characterized signalling phytohormones that reduces stomatal opening and water loss from the water-stressed plants (Mega *et al.,* 2019), along with stress-induced ROS accumulation. A similar trend was observed by Aly and Latif (2011) who cited that ABA reduces transpiration water loss by promoting stomatal closure and inhibiting stomatal opening by multiple cascades of cellular-biochemical events. The SOD enzymes act as antioxidant role by scavenging superoxide anion free radical to H_2O_2 or O_2, thus diminishing the injury caused by ROS through the breakdown of superoxide radicals that are produced by oxidative stress (Caverzan *et al.,* 2016). Similar findings were observed by Abdelaziz *et al.* (2021) and Mahmoud *et al.* (2023). They stated that the levels of ABA and SOD significantly increased under stressful conditions such as drought and salinity stresses.

Moreover, SOD and ABA levels increased due to the application of the biostimulants-based amino acid under drought stress. this could be attributed to role of amino acids in improving antioxidant metabolism and phytohormone precursors (Mohamed *et al.,* 2009; Weiland *et al.,* 2015). Previous researches have shown that foliar implementation of amino acids biostimulants on cabbage and *Aloe vera* leaves considerably upsurges antioxidant activities such as CAT, POD, SOD, and APX (El-Beltagi *et al.,* 2010; Ardebili *et al.,* 2012; Haghighi *et al.*, 2020). Similarly, the activity levels of SOD, CAT, POD, and APX were increased by increasing the application of amino acids under normal and stressful conditions, particularly in cabbage plants (Haghighi *et al.,* 2022). It has been confirmed that antioxidant enzymes play principal role in reducing free radicals; as a result, their stimulation can increase the plant's tolerance to a biotic stresses (Caverzan *et al.*, 2016). Our findings supported the results of other studies (Haghighi *et al.,* 2022, Ardebili *et al.,* 2012). The hypothesis is that amino acids can enhance antioxidant enzyme capacity.

Correlation study

Heatmap correlation and Pearson's correlation display the variations in Agro-physiological assets of broccoli plants sprayed with bio-stimulants and grown under different irrigation frequencies (Figures 5 and 6). The Heatmap depends on 14 measurements evidently classified them into three groups (A, B, and C), while CMS, AAs, and CON treatments were inserted under groups A, B, and C; respectively (Figure 5).

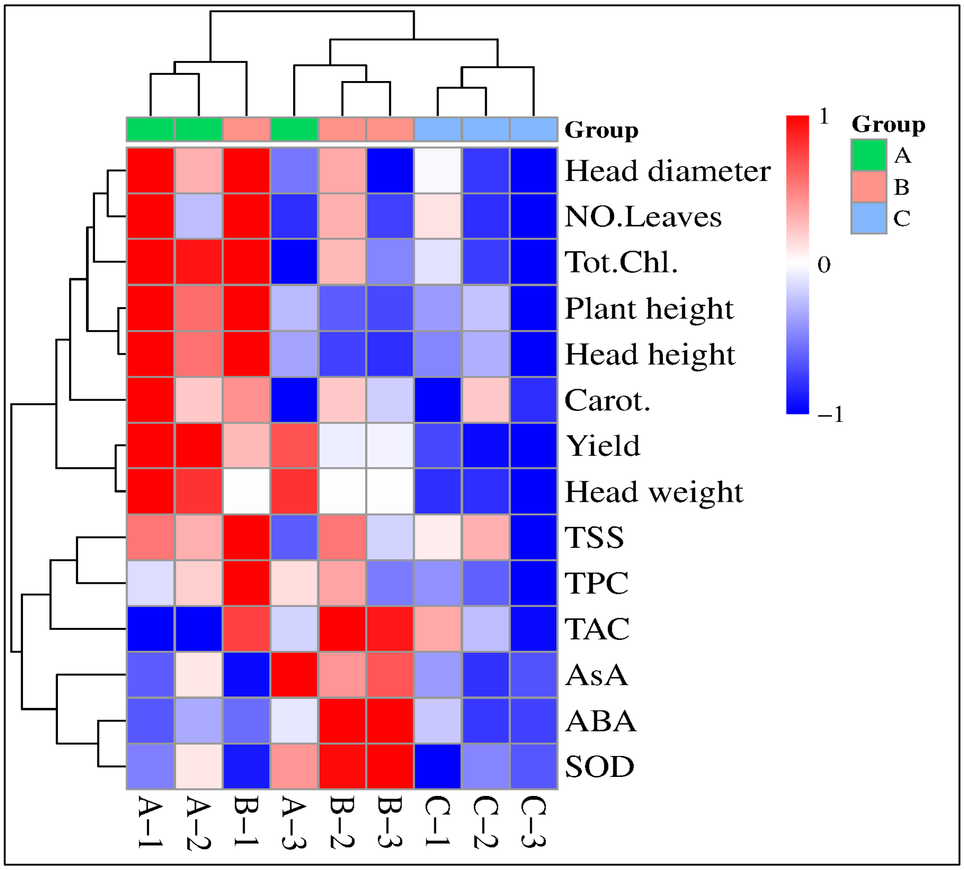

Figure 5. Heatmap shows changes in agro-physiological properties of broccoli plants treated with biostimulants-based amino acids and irrigation frequency

Abbreviations: Tot. Chl, Total chlorophyll content; Carot, carotenoid content; AsA, ascorbic acid; TPC, Total phenol content; TAC, total antioxidants content; ABA, Abscisic acid; SOD, superoxide dismutase; and TSS, Total soluble solids.

Correspondingly, each group (A, B, and C) is divided into 3 subgroups containing irrigation frequency (1, 2 and 3). whereas, the subgroups/ treatments A-1, A-2, A-3, B-1, B-2, B-3, C-1, C-2, and C-3 indicates to CMS-5, CMS-10, CMS-15, AAs-5, AAs-10, AAs-15, CON-5, CON-10 and CON-15; respectively. The red colour points out positive impact, and blue colour indicates negative impact. Heatmap correlation also points out that CMS-5 (A-1), and AAs-5 (B-1) treatments have a positive impact on studied parameters (plant height, No. of leaves, Total Chl., head height, head weight, head diameter, TSS, and yield) except for AsA, ABA, and SOD; on the contrary, CON-15 (C-3) treatment has more negative effects for all studied measurements (Figure 6).

Likewise, the positive and negative correlation between the examined parameters was determined using Pearson's correlation analysis, as presented in Figure 6. Pearson's correlation analysis showed that total yield positively linked to plant height, total chlorophyll (Tot. Chl.), total phenol content (TFC), head weight, and head diameter. In contrast, leaf carotenoids content is negatively related to No. of leaves, Tot. Chl., and head height (Figure 6).

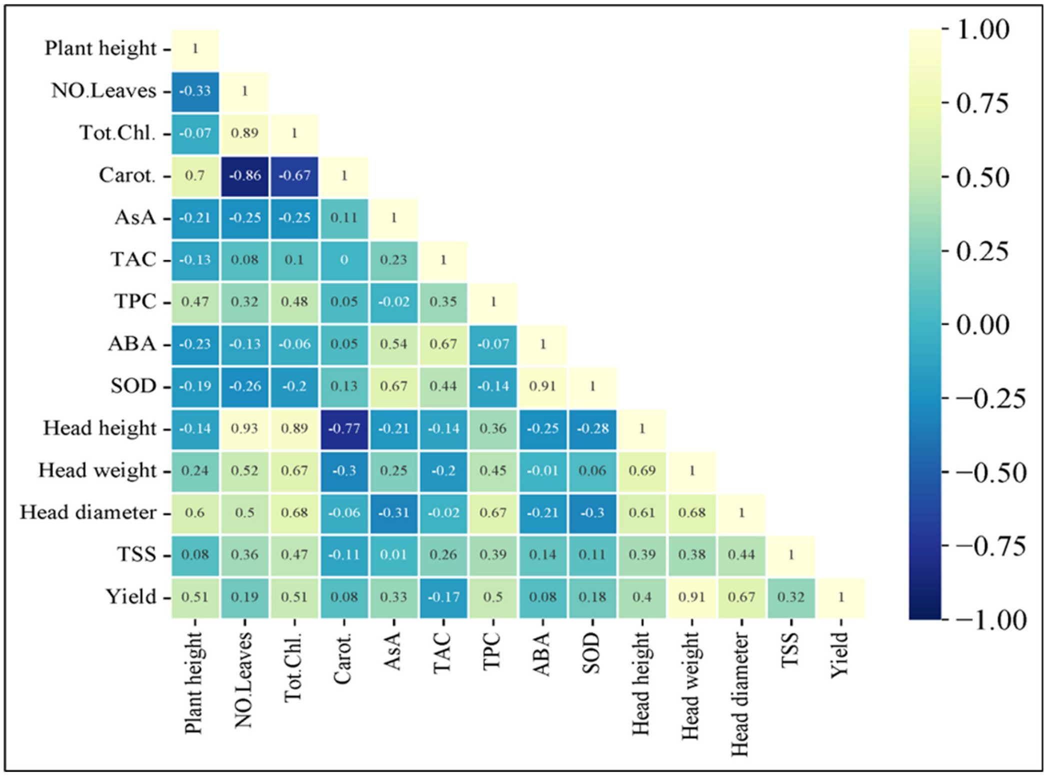

Figure 6. Pearson's correlation analysis between agro-physiochemical traits of broccoli plants treated with biostimulants-based amino acids and irrigation frequency

Abbreviations: Tot. Chl, Total chlorophyll content; Carot, carotenoid content; AsA, ascorbic acid; TAC, total antioxidants content; TPC, total phenol content; ABA; Abscisic acid; SOD, superoxide dismutase; and TSS, total soluble solids.

Based on the above, the results of the present study confirmed that the improvement in growth, and production of broccoli plants is significantly associated with the exogenous application of amino acids bio-stimulants under normal and stressful conditions, especially water deficit conditions. This could be related to the ability of these bio-stimulants to modulate the chemical and physiological processes inside the plants through the increasing phytohormones and activation of enzymes engaged with chlorophyll formation, nutrient assimilation, carbon metabolism, and antioxidant biosynthesis, (Mahmoud *et al*., 2023)

Conclusions

The current study confirmed that foliar application of bio-stimulants based amino acids improves water productivity, plant growth or productivity as well as enhances biochemical compounds such as total phenol content, ascorbic acid, total antioxidant content, ABA, and superoxide dismutase (SOD), at different irrigation times (Figure 7). Therefore, it can be used amino acids biostimulants as foliar applications to support broccoli growth and enhance farming quality. These improvements in broccoli plants could be associated with enhancing the physiological process and signalling action of the amino acids. Therefore, future experiments are needed to assess the effective of these amino acids on genetic transcription of various measurements, including phytohormones production nutrient uptake, and metabolism of antioxidants compounds. In this way, it can be understood well the role of these bio-stimulants-based amino acids in broccoli plants.

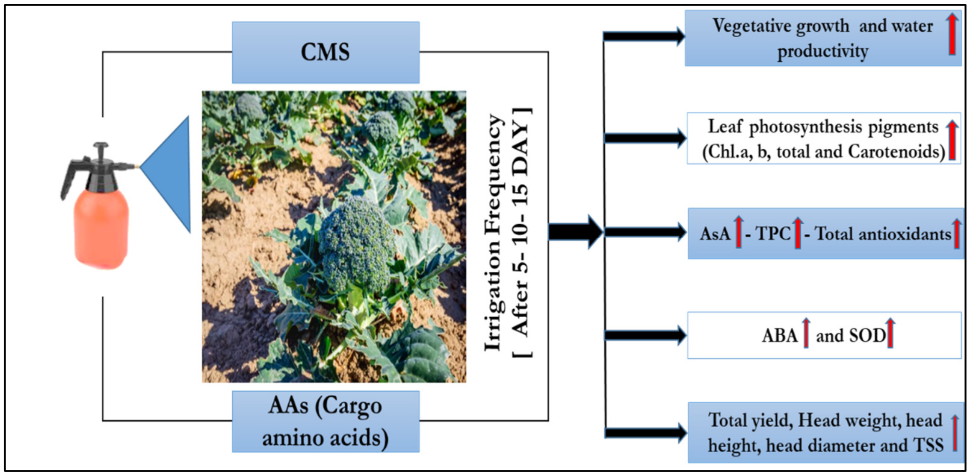

Figure 7. Simplified conclusion for the suggested impact of bio-stimulants based amino acids (CMS and AAs) and irrigation frequency on agro-physiological and biochemical of broccoli plants

Authors' Contributions

Ghada, HSEB, EAA and MEA collected, analyzed research data and wrote draft manuscript; Ghada. SMK, and SGA designed research and provided suggestions regarding data analysis; EAA generated figures in main manuscript; Ghada, HSEB, SNK, SA, MEA and EAA edited draft and final manuscript with suggested changes; Project administration: HSEB and EAA; Supervision: HSEB and EAA; Funding acquisition: HSEB.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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