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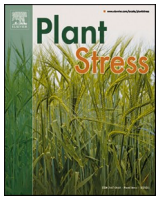

Melatonin priming could modulate primary and secondary metabolism of sunflower with better nutraceutical value and tolerance against water deficit environment

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ABSTRACT

Water deficit severely limits plant growth especially in arid and semi-arid regions. Sunflower is one of the economically important oil producing crops. It is usually grown in water limited environment due to its moderate biological tolerance towards water stress. Interestingly,sunflower growth was improved in water deficit conditions using a plant growth promoter i.e. melatonin. Seeds were primed for 12 hours with melatonin (0, 50, 75, 100 μM) and these plants were later exposed to water limited environment (100 and 50 %). Results showed that reduced water conditions negatively affected various parameters such as leaf area, plant height, leaf number, achene weight/plant, capitulum weight, leaf total soluble sugars, catalase activity, anthocyanin, quercetin, gallic acid, vanillic acid, chlorogenic acid and cinnamic acid. The seed oil content also showed reduction of unsaturated to saturated fatty acid ratio. Specifically, almost all levels of melatonin applications improved growth (leaf area, leaf fresh and dry weight, plant height), yield (achene weight/plant, number of achenes/capitulum) and leaf metabolites (total soluble proteins, total soluble sugars, proline, total phenolics, anthocyanins, quercetin, gallic acid, vanillic acid, benzoic acid, syringic acid, sinapic acid) of stressed plants. The primed sunflowers exhibited enhanced leaf catalase activity, metabolizable energy and tissue nutritive value. The following phenolic profiles were observed: caffeic acid 100, syringic acid 100, p-coumaric 100, m-coumaric 9.8 and ferulic acid 100 % increased under 100 % water irrigations. Whereas, quercetin (4.7), gallic acid (76.2), vanillic acid (34.0), benzoic acid (334.5), cinnamic (0.6), sinapic acid (22.5) exhibited proportional % reduction. The unsaturation to saturation ratio of fatty acids was also better in the melatonin (100 μM) primed plants. Unprimed plants gave 47.0, while the primed plant exhibited 58.1 unsaturated to saturated fatty acids ratio. Moving forward, the application of 100 μM melatonin delivered better antioxidant protection and nutraceutical value of sunflower plants under water deficit along with enhanced yield.

Introduction

Environmental threats, including salinity, unfavourable temperatures, and drought affect crop production globally (Munns and Gilliham, 2015;Gupta et al., 2020; Shahzad et al., 2021; Suzuki et al., 2014). Approximately 20 % of the farmlands worldwide are drought affected regularly and these water deficit episodes are increasing annually (Bakhoum et al., 2023). There are several reasons to account for the lower crop production: poor water use capacity, insufficient irrigations and massive use of fertilizers (Reisman et al., 2022; Hanafy and Sadak

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2023). Water scarcity generates osmotic stress negatively affecting many different metabolic processes in plant tissues (Gupta et al., 2020; Mukarram et al., 2021; Sadak, 2022). With reference to this context, it is predictable that till 2050, water shortage globally will cause serious holistic threats to plant growth and especially their physiology and biochemistry (Gupta et al., 2020). Among these perturbations are the foliar chlorophyll content, gas exchange and photosynthetic processes (stomatal conductances, transpirations, electron transport cycles, and carbon fixation)(Chowdhury et al., 2024; Elnaggar et al., 2024; Ma et al., 2021; Yong et al., 2010); which would vastly alter plant metabolites like sugars, proteins and fatty acids (Heng et al., 2013; Teo et al., 2011). Generally, genetic improvement for crop is one of the suggested strategies to help crops to better cope with the greater frequency of biotic and abiotic stresses (Giudice et al., 2021; Gilliham et al., 2017; Derbyshire et al., 2022; Nisa et al., 2023). However, there are number of issues linked to the use of selected transgenic crops available like delayed senescence, early maturity etc. Thus, the strategic and selective use of suitable growth promoters is considered as effective agents to restore crop growth and safeguarding food yield under normal or water limited conditions (Zhao et al., 2021; El-Bassiouny et al., 2023; Mubashir et al., 2023; Gatasheh et al., 2023).

Among the many known growth promoters (biostimulants, speciality chemicals) to strengthen plants, melatonin is gaining prominence as an effective agent to alleviate the negative effects of water deficit (EL Sabagh et al., 2022; Ishfaq et al., 2024; Sadak, 2016b; Saleem et al., 2023; Sani et al., 2023; Wong et al., 2020). It is an amphiphilic low molecular weight hormone (Shafi et al., 2021) with it's the formula N-acetyl-5-methoxytryptamine. The hormone was first reported in bovine glands in 1958 (Chowdhury et al., 2008). It stimulates many biological functions such as sleep/awake cycle, immune system, emotional behavior and seasonal reproduction (Lesicka et al., 2023). But for agronomist and plant scientists, it gained much importance after its role identified in vascular plants in 1995 (Sadak, 2016; Altaf et al., 2021). Melatonin has diverse contributions in plant metabolism ranging from growth stimulation, rapid seed germination, flowering and rooting (Hernández-Ruiz et al 2021;Sadak and Ramadan, 2021) and indole acetic acid mediated growth (Sadak and Bakry, 2020; Yang et al., 2021). Melatonin is a growth promoting molecule in plants and generates many valuable actions, most importantly physiological responses that include seed germination, growth/yield, photosynthesis and the directive of the diverse metabolic pathways (carbohydrates, lipids, nitrogen compounds, sulphur, and phosphorus cycles). With regard to secondary metabolism, it encourages the biosynthesis of phenols, anthocyanins flavonoids, carotenoids, and several terpenoids. Both exogenous and endogenous melatonin confers stress tolerance against drought, salinity, ultraviolet radiation and heavy metals. It improves the activity of scavenging enzymes that leads to eliminate reactive oxygen species (Arnao et al., 2021).

Sunflower (*Helianthus annuus* L.) is widely consumed all over the world for its oil quality (Sher et al., 2022), nutritional and economic values (Dawood et al., 2012 and Nguyen et al., 2021). Sunflower leaves are known as remedy for skin issues, inflammation (Sher et al., 2022) cancer (David et al., 2022), diabetes, high cholesterol (Rabail et al., 2021). Although, sunflower is moderately drought tolerant crop but still experiences yield loss in limited water conditions (Sadak et al., 2012). It was hypothesized that additional application of a growth regulator (melatonin) may result in better sunflower yield in water scarce areas.

Some published resports are available about the ameliorative role of melatonin priming during water limitation (Zhao et al., 2021). To deepen our understanding about the role of melatonin during water deficit conditions, a metabolomics study of melatonin primed sunflower plants in water deficit conditions with two specific objectives, was planned. First, we aimed to determine the appropriate melatonin levels for priming; and secondly, to study the effects of exogenously applied melatonin on morphological, physiological and biochemical parameters under limited water conditions.

Materials and methods

Seed source and sowing

The experiment was performed in Botanical Garden of Government College University, Faisalabad, Pakistan. Sunflower variety FH-129 was used for experimental study and seeds were collected from Ayub Agricultural Research Institute, Faisalabad, Pakistan. Experiment one was done under 100 percent irrigation for melatonin dose optimization. Sunflower seeds (non-primed and hydro-primed) were used to compare with melatonin (50, 75 and 100 μM) primed plants. Time of priming was set as 12 hours. Seeds were paper dried before sowing. First harvesting (vegetative stage) was done after 30 days of germination and data was recorded for different parameters. All three levels of melatonin (50, 75 and 100 μM) were used for second experiment at two irrigation levels (100 and 50 %). At seed bed preparation, potassium (K) and phosphorous (P) were used as triple super phosphate and Sulphate of potash (K_2SO_4). Further, recommended doses of fertilizer (150-60-60 NPK kg ha^{-1}) was also applied. One third part of nitrogen as urea was added to soil at time of sowing and remaining two parts were used at vegetative stage and flowering stage. All cultural practices such as weed management, hoeing, irrigation and plant protection etc. were kept normal during whole experiment. The growth and biochemical attributes were studied at vegetative stage, 30 and 60 days old plants were harvested (first experiment) and then third harvesting at maturity was collected. When the back of the capitulum turned yellow and bract were brown harvest was taken and dried for 4 to 5 days Five capitulum were selected for the determination of different yield components. After measuring the capitulum weight, achenes were separated. No of achenes per capitulum were counted and 100 achenes weight was measured. The individual parameters were quantified by averaging the five readings.

Photosynthetic pigments. Leaf chlorophyll content was estimated by the method of Arnon, (1949). Leaf carotenoids determination was done with protocol of Davis, (1976). Absorbances of the leaf extracts (sample with 80 % acetone) was recorded at 645, 663 and 480 nm.

Total phenolics, flavonoids and total protein contents. Leaf total soluble phenolics were calculated by following Julkunen-Tiitto (1985) using Folin Ciocalteu's reagent. Sample extract was used as prescribed in protocol. After 90 minutes of incubation, the absorbance was noted at 765 nm using spectrophotometer. Leaf total flavonoid component was investigated by following detailed procedure of Marinova et al. (2005) and for total soluble protein contents; Bradford (1976) method was applied. Reaction mixture was set and absorbance of the sample was noted by spectrophotometer at 595 nm.

Reducing sugars contents and total soluble sugars. Leaf reducing sugars components were investigated as mentioned by Miller, (1972). Reaction mixture was prepared and vortexed, then absorbance was taken at 540 nm. Total soluble sugars were determined with the method of Yoshida et al., (1971). After vortexing, the reaction mixture was heated at 95°C for 15 min. Absorbance of samples was measured at 625 nm.

Total Anthocyanin content. The quantitative analysis of anthocyanin was determined spectrophotometrically by the method of Nozzolillo, (1978). Acidified methanolic leaf extract (3 mL) was taken and absorbance was recorded by using spectrophotometer at 536 and 600 nm.

Determination of % oil content. Percent oil content was determined by using AOAC (1996). In 50 mL capacity plastic test tube, 30 mL n-hexane and three-gram plant sample were used. Shaking was done (100 rpm) for 24 h. Later samples were centrifuged and supernatant was collected. After two successive repetitions, extractions were made on the residue by vortex and centrifugation. Clear extract from all three collections were preserved for oil estimation. The residue was dried as fat free sample difference of weight after extraction was recorded as fat content.

Nutritional value and Metabolizable energy. This value was determined with protocol of Indrayan et al., (2005) as Nutritive value=

4x%age of protein + 9x%age of fat + 4x%age of carbohydrates

Metabolizable energy of samples was calculated with following expressions (WHO 1985).

$$\text{Energy(kJ)} = (4\text{kcal} / \text{gxgproteinx4.186}) + (4\text{kcal} / \text{gxgfatx4.186}) + (9\text{kcal} / \text{gxgcarbohydratex4.186})$$

Growth and yield. Plant growth in terms of root length, shoot length, root/shoot fresh weight, root/shoot dry weight, leaf fresh weight and dry weight and yield attributes like cake weight and achenes weight were determined in gram via electronic balance. Achenes per cake were counted and noted. For growth and yield five plants were used for single replicate.

Free proline. Proline was estimated by using method of Bates et al., (1973). Frozen leaves samples (0.2 g) were homogenized in 5 ml 3 % sulpho-salicylic acid using a mortar and pestle and finally centrifuged supernatant was collected. One mL sample, ninhydrin and glacial acetic acid were taken respectively. The solution was kept in water bath for one hour at 100°C then shifted to ice bath. Two mL toluene was mixed and kept on room temperature for 30 minutes until two layers formed. At 520 nm the absorbance was measured. The same sequence was run with blank (2 mL toluene). Formula for calculation is as under

$$\text{Proline}(\mu\text{moles} / \text{gfresh} - \text{weight}) = \frac{(\mu\text{gproline/mLxmLtoluene})}{(115.5\mu\text{g/mole})/\text{gsample}/5)}$$

Catalase (CAT)

Fresh frozen leaves were taken (0.2 g) and 50 mM 5mL of cooled phosphate buffers (pH 7.8) were grinded using a tissue grinder mortar and pestle it was placed over ice bath for the direction of antioxidant enzyme extraction. The mixture solution was centrifuged at 4°C at 14000 x g for 10 min. The supernatant was stored in eppendorf tubes at -20°C and was utilized for antioxidant activities glance on CAT. Catalase activity was determined by calculating the conversion rate of H_2O_2 to H_2O and oxygen molecules, by protocol explained by Chance & Maehly, (1955). The reaction mixture was prepared by using 300µl 30 % H_2O_2 mixed in 200mL phosphate buffer (pH 7.0). Later, reaction solution was added to it (3 mL) as well as 0.1 mL of supernatant was also added. Then the reaction was started by the addition of enzyme extract. A change in absorbance of the reaction solution was interpreted for every 60s at 240 nm. CAT activity was described as an absorbance change of 0.01units per min.

$$\text{CAT} = (\Delta\text{A240xVI})/(\text{WxVsP0.01xI})$$

HPLC based phenolic profiling. The internal standards were used: Quercetin, Gallic acid, Caffeic acid, Benzoic acid, vanillic acid, Cinnamic acid, Syringic acid, p-coumaric acid, m-coumaric acid, ferulic acid, sinapic acid and chlorogenic. Phenolics were extracted with minor modifications in method of Stalikas, (2007). Powdered leaves were extracted in 100 % methanol (1:10) and was filtered via 0.45um cellulose acetate filter (EMD Millipore, Billerica, MA, USA). An aqueous suspension of the extract was then produced with double distilled water and pH was adjusted (pH = 2) with 6 M HCl and the mixture was kept in 100°C for 3 h. All of the standards and extracts were filtered through a 0.451 m syringe membrane filter (Type Millipore) and sonicated for 15 min in a Micro clean 109 bath prior to analyze by HPLC. Phenolic compounds were analyzed using Gradient HPLC (LC-10AT, SCTL, Shimadzu, Japan). Elution was done for 60 min with a flow rate of 1mL/min in a gradient system of two mobile phases A (H_2O_2: AA-94:6, pH 2.27), B (ACN100 %).

GC-based fatty acid profiling. Fatty acid methyl esters (FAMEs) were organized by the IUPAC, 1987 standard method 2.301 and determined on a Perkin Elmer gas chromatograph model Clarus 500fitted with aRt-2340 NB (RESTEK, Corp., 800-356-1638, USA) methyl-lignocerate-coated (film thickness 0.20 µM), polar capillary column (60 m x 0.25 mm) and an FID detector. At a flow rate of 67.4 Psi, oxygen free nitrogen was used as a carrier gas. Other conditions were kept as: initial oven temperature, 80°C; final temperature, 210°C ramp rate, 3°C/min; injector temperature 210°C; detector temperature, 220°C and FAMEs were determined by analyzing their relative and absolute retention times with authentic standards bought from Sigma-Aldrich (Buchs, Switzerland). Preparation of fatty acid methyl esters (FAMEs) by Official method. The IUPAC, 1987 standard protocol was followed for the preparation of FAMEs, which carried out by derivatization of samples into fatty acid methyl esters of triglycerides by saponification of the glycoside's liberation, and esterification by methanol of the fatty acids. The oil sample (0.2 g) was weighed into 100 mL round neck round bottom flask. One pellet of KOH and 30 mL of methanol were then mixed into the flask and the flask was refluxed for 25 min until the droplets of fat disappeared. The reaction mixture was cooled down, it was gently shifted to a separating funnel and small amount of n-hexane was mixed. The separating funnel was stirred gently by rotating many times and the upper layer of hexane was separated, and washed many times with 10 mL of deionized water. This hexane solution was dried over anhydrous sodium sulfate, filtered and used for GC analysis. The dry and solvent free methyl esters were preserved in a sealed sample tube in a deep freezer and used for further analysis.

Statistical analysis

Data collected was subjected to analysis of variance technique (ANOVA), two way ANOVA by using computer software Costat CoHrot 6.4. The mean values were compared by least significant difference (LSD).

Results

Experiment 1 (phase 1)

At the vegetative stage, different morphological attributes (root fresh/dry weight, Shoot fresh/dry weight, shoot length, leaf area and leaf number) were significantly ($P \leq 0.001$) enhanced in plants pretreated with melatonin (50, 75 and 100µM) as compared to untreated and hydro-primed plants (Table 1). Whereas, for root length effect of priming treatment remained ineffective (Table 1). Plants grown with or without melatonin priming (50, 75 and 100µM) showed similar values for photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) (Table 1).

Total soluble sugars, reducing sugars, nutritional value and metabolizable energy ($P \leq 0.001$) were notably higher in melatonin (50, 75 and 100 µM) primed plants at vegetative stage. While soluble sugars and leaf % oil contents remained similar to non-primed and hydro-primed plants. However, significant increase was observed for anthocyanin accumulation of melatonin in melatonin primed plants at this stage (Table 1)

Melatonin priming (50, 75 and 100 µM) exhibited different responses with respect to treatment and its levels for secondary metabolite accumulation. Total phenolic content in leaves was raised by all levels of melatonin treatment. Anthocyanin accumulation was increased by 50µM pre-seed treatment only. Values of flavonoids were found non-significant (Table 1) overall.

Table 1
Growth and biochemical study of sunflower plants grown from melatonin priming (50,75 and 100 μM) at vegetative stage (30 days plants).

Melatonin treatments	Root Fresh weight	Root Dry weight	Shoot Freshweight	Shoot Dry weight	Root Length	Shoot Length	Leaf Area	No. of Leaves	Chlorophyll a	Chlorophyllb	Total Chlorophyll
No-priming	0.60^{b}	0.06^{bc}	7.25^{bcd}	0.765c	5.23^{ab}	18.6^{bc}	31.5^{cd}	11^{b}	7.25^{b}	2.87^{ab}	9.57^{ab}
Hydro-priming	0.54^{b}	0.05^{bc}	3.17^{bcd}	0.336c	6.63^{ab}	13.5^{bc}	43.6^{cd}	11.3^{b}	6.32^{b}	3.04^{ab}	9.57^{ab}
50μM	3.6^{a}	0.59^{a}	26.3^{a}	2.41a	6.06^{ab}	41.5^{a}	57.6^{ab}	14.3^{a}	0.96^{b}	1.47^{ab}	2.52^{ab}
75μM	1.53^{a}	0.34^{a}	24.5^{a}	2.23a	5.66^{ab}	32.3^{ab}	37.8^{abc}	10.6^{b}	0.65^{b}	1.04^{ab}	2.12^{ab}
100μM	1.90^{a}	0.41^{a}	24.4^{a}	2.30a	6.00^{ab}	35.0^{ab}	69.73^{ab}	21.6^{a}	8.45^{ab}	3.68^{ab}	6.84^{ab}

Melatonin treatments	Carotenoids	Soluble Proteins	Soluble Sugars	Reducing. Sugars	Non-reducing. Sugars	Leaf oil	Nutritive value	Metabolizable energy	Total phenolics	Flavonoids	Anthocyanin
No-priming	4.04^{ab}	0.07^{c}	0.32^{ab}	0.04^{cd}	2.13^{a}	7.88^{abc}	9419.5^{d}	813.4^{cd}	0.27^{cd}	0.443^{cd}	0.7^{b}
Hydro-priming	4.37^{ab}	0.09^{c}	0.26^{ab}	0.04^{cd}	2.13^{a}	7.44^{abc}	10028.2^{d}	820.7^{cd}	0.34^{cd}	0.329^{cd}	0.72^{b}
50μM	15.37^{ab}	0.47^{a}	0.19^{ab}	0.07^{ab}	0.09^{bc}	12.8^{ab}	13122.3^{bc}	1077.51^{ab}	2.04^{ab}	0.534^{abcd}	1.04^{a}
75μM	1.81^{ab}	0.58^{a}	0.23^{ab}	0.10^{ab}	0.12^{b}	14.4^{ab}	15062.0^{ab}	1005.2^{ab}	1.5^{ab}	0.433^{accd}	0.88^{b}
100μM	2.11^{ab}	0.56^{a}	0.25^{ab}	0.12^{ab}	0.124^{b}	19.6^{ab}	15794.5^{ab}	1060.84^{ab}	1.53^{ab}	0.370^{abcd}	0.80^{b}

Means with same letters, for each trait, do not differ significantly from each other and with different letters differ significantly at $P \leq 0.05$

Table 2
Growth and yield of sunflower plants grown from melatonin priming (50, 75 and 100 μM), grown with two irrigation conditions (100 and 50 %) at flowering and maturity stage.

Stress level	Melatonin treatments	Leaf area	Leaf number	Leaf fresh weight	Leaf dry weight	Plant height	Capitulum weight	% oil yield	Achene number/ capitulum	50 achenes weight	Achene weight /plant
100 % irrigation	No-priming	242.3^{ab}	19.0^{cd}	2.00^{d}	0.34^{e}	99.66^{bc}	129.3^{a}	14.8^{a}	193^{d}	2.54^{a}	16.66^{d}
	Hydro-priming	242.3^{ab}	20^{cd}	2.5^{d}	0.32^{e}	100^{bc}	130^{a}	13.7^{a}	192^{d}	2.60^{a}	14.00^{bc}
	50μM	321.8^{a}	21^{c}	4.6^{cd}	1.03^{d}	148.3^{a}	64^{bc}	20.2^{a}	766^{ab}	2.38^{a}	32.6^{a}
	75μM	287.5^{ab}	26.6^{b}	13.07^{a}	2.75^{a}	155.0^{ab}	67^{bc}	17.6^{a}	917^{a}	2.57^{a}	34.6^{a}
	100μM	193.5^{bc}	33.3^{a}	13^{a}	3.03^{a}	137.3^{a}	79.6^{b}	16.8^{a}	766^{ab}	2.61^{a}	35^{a}
50 % irrigation	No-priming	139.6^{c}	14^{d}	3.32^{d}	0.54^{e}	72.6^{c}	36.0^{cd}	15.2^{a}	531^{c}	2.03^{a}	11^{e}
	Hydro-priming	139.6^{c}	15^{d}	3.30^{d}	0.57^{e}	71.3^{c}	42.6^{cd}	15.1^{a}	498^{c}	2.03^{a}	15.6^{e}
	50μM	162.1^{c}	20.3^{c}	6.40^{bc}	1.6^{c}	137^{a}	40.6^{cd}	14.8^{a}	767^{ab}	2.03^{a}	21.0^{c}
	75μM	306.6^{a}	20.6^{c}	10.8^{a}	2.07^{a}	140^{a}	62.4^{bc}	12.4^{a}	684^{b}	2.15^{a}	26.3^{b}
	100μM	253.3^{ab}	24.3^{bc}	7.33^{b}	1.3^{cd}	122^{ab}	71.6^{bc}	8.53^{a}	938^{a}	2.09^{a}	32.0^{a}

Means with same letters, for each trait, do not differ significantly and with different letters differ significanty from each other at $P \leq 0.05$.

None of the treatment level melatonin priming (50, 75 and 100 μM) induced negative effect upon growth and metabolism of sunflower so all the levels were subjected to experiment 2 for further study under water limitations.

Experiment 1 (phase 2)

Analysis showed that there was a small effect of reduced water supply upon leaf number, plant height, leaf fresh and dry weight with the only exception for leaf area that increased under limited water

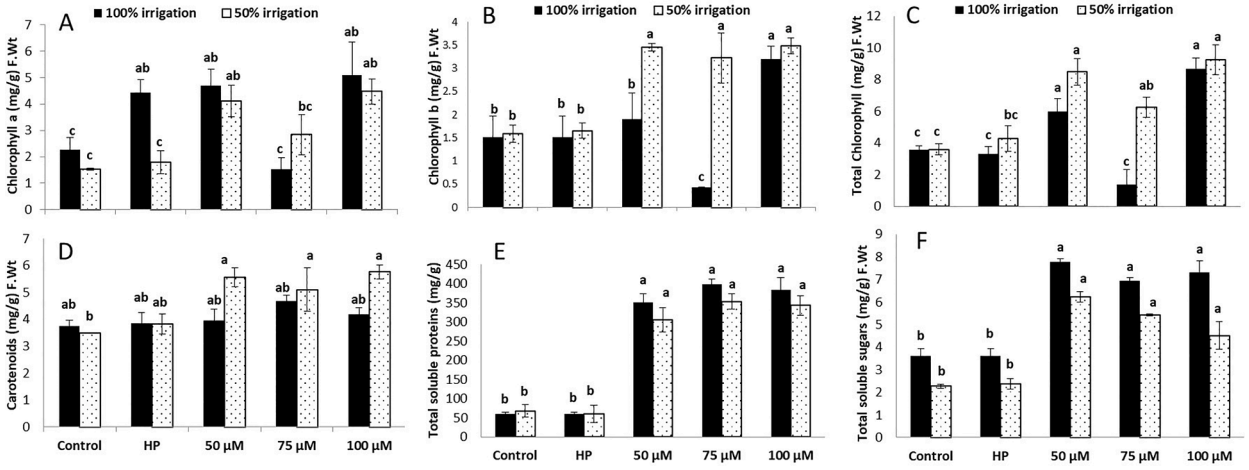

Fig. 1. Effects of water deficits on biochemical attributes including leaf chlorophyll contents (a, b and total), carotenoids, total soluble proteins, soluble sugars, of sunflower (*Helianthus annuus* L.) with hydropriming and primed with melatonin. Means with same letters, for each trait, do not differ significantly from each other at $P \leq 0.05$. Data shown are mean ± S.D. (n = 4 independent biological replicates).

(Table 2). Melatonin primed plants increased leaf area under 50 % irrigation only, while significant differences were observed in primed and non-primed plants for plant height leaf fresh and dry weight.

In yield parameters there was significant effect of priming including capitulum weight (P≤0.001), achenes/ capitulum (P≤0.001), achene weight/plant, and there was a marked decline with water stress. However, notable reduction in % oil yield and 50 achenes weight remained unaffected for both factors (irrigations and Melatonin). Treated plants (melatonin all levels) were found significant for number of achenes per capitulum, achene weight/plant in both water environments and capitulum weight in 100 % water supply (Table 2).

For leaf chlorophyll a content, unprimed and hydro-primed plants experienced reduction under reducing water supply while chlorophyll b (P≤0.001) remained unaffected in both regimes of water (100 and 50 %) (Fig. 1A). With regard to treatment and its levels (melatonin @ 50, 75 and 100 μM), plants grown under stress exhibited significant rise as compared to control. The plants grown after pretreatment of 75 μM melatonin showed reduced chlorophyll contents in 100 % irrigation (Fig. 1A–C). Leaf carotenoids contents did not show reduction for no-primed, water primed and melatonin primed plants under different irrigations and remained same (Fig. 1D)

Upon reducing water irrigation, non-significant effect was observed in non-primed and unprimed plants for soluble proteins, soluble sugars and reducing sugars (Fig. 1E-F; 2A). Considering melatonin pre-seed treatment, all levels (50, 75 and 100 μM respectively) varied for the effect upon soluble proteins and soluble sugars in both water regimes while reducing sugars were elevated under 100 % water only (Fig. 1F).

Plants grown (non-primed/hydro-primed) under limited irrigation showed considerably (P≤0.001) higher level of proline in contrast to normally irrigated plots. In discussion to treatment, none of the melatonin level was statistically significant for this attribute (Fig. 2B)

For leaf percent oil contents, statistically (P≤0.001) similar results were noted in both water and treatment conditions (Fig. 2C). With regard to catalase activity, lowering the number of irrigations caused reduction in the activity of this attribute in control and water primed plants (Fig. 2D). Melatonin triggered the activity via 50 and 100 μM seed priming in 50 % irrigations (Fig. 2D). Irrespective of water conditions, 75 μM plants remained indifferent.

At flowering stage, leaf phenolic contents displayed non-significant changes under altered number of irrigations for non-primed and hydro-primed plants (Fig. 2E). Seeds primed with melatonin (50, 75 and 100μM), successfully enhanced phenolic accumulation with the lowering of irrigations percent, whereas these applied levels were noted non-significant in 100 % water medium (Fig. 2E)

Flavonoids accumulations were observed non-significant for both (irrigation and melatonin) conditions (Fig. 2F. Single exception was recorded for leaf flavonoids (P≤0.001) where plants grown in normal water supply after 100 μM seed priming (Fig. 2F) exhibited marked increase.

Statistical analysis of data for anthocyanin revealed (P≤0.001) non-significant value for irrigation factor in control and water- primed plants. In case of melatonin, only 50 μM treatment enhanced this attribute under 50 % irrigation conditions (Fig. 3A).

With reference to metabolizable energy and nutritional value, all levels of melatonin (50, 75 and 100 μM) showed significant (P≤0.001) outcome for both attributes (metabolizable energy and nutritional value) as compared to control and hydro-primed plants (Fig. 3B-C).

Based upon findings of above-mentioned experiment plants pre-treated with melatonin 100 μM and untreated as control were selected for metabolomic profiling. Water reduction induced marked decline in soluble phenolics (26.6 %) (Fig. 4A and C). With respect to melatonin seed treatment, in both water irrigations there was significant increase in phenolic value. Each phenolic variably responded towards both factors (stress and priming) as depicted in pie diagram with variable pattern of percentage in each treatment. Water limitation indicated % increase in quercitin, gallic acid, vanillic acid, chlorogenic acid, syringic, p-coumaric, m-coumaric, cinnamic acid and sinapic acid (34.80, 100, 62.2577, 100, 100, 5.59, 6.29, 81.15 & 100 respectively). Whereas, caffeic acid (121.08), benzoic acid (146.51) and ferulic acid (0.04) decreased in untreated stressed plants (Fig. 4A and C). Melatonin primed (100 μM) plants exhibited rise of caffeic acid, syringic acid, p-coumaric, m-coumaric and ferulic acid increased (100,100, 100, 9.79 and 100 % respectively) under 100 % water irrigations. Whereas, quercetin (4.7), gallic acid (76.18), vanillic acid (34.03), benzoic acid (334.52), cinnamic (0.61), sinapic acid (22.45) exhibited % decline (Fig. 4B) in above mentioned plants. Under 50 % irrigations melatonin primed plants showed % rise in caffeic acid (24.25), p-coumaric acid (21.71), ferulic acid (56.12) and cinnamic acid (100). The phenolics like quercetin (12.95), vanillic acid (116.96), benzoic acid (125.77) and m-coumaric (1.86) showed % decrease (Fig. 4D). Gallic acid, chlorogenic acid, syringic acid, sinapic acid did not appear in profile.

Fatty acid composition of sunflower seeds

Sunflower seeds samples were rich in Palmitic acid (8.01 %), Stearic acid (4.11 %), Oleic acid (36.5 %) and linoleic acid (50.76 %). Stress and priming not only altered individual percentage of each fatty acid but also changed overall unsaturation to saturation ratio (U/S). Under

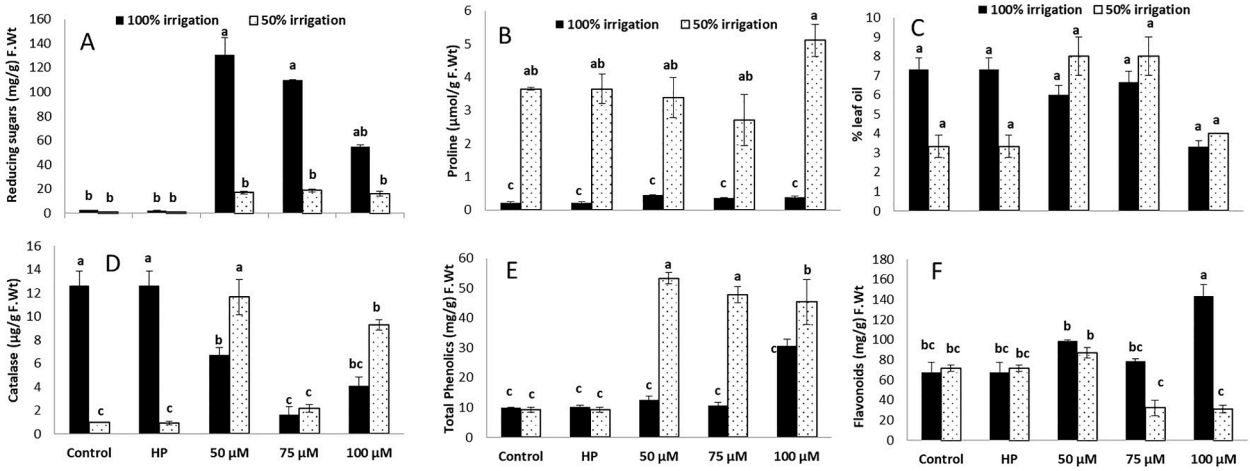

Fig. 2. Effects of water stress on biochemical attributes including reducing sugars, proline, % leaf oil, catalase, leaf total phenolics, flavonoids, of sunflower (*Helianthus annuus* L.) plants with hydropriming and primed with melatonin (50, 75 and 100 μM). Means with same letters, for each trait, do not differ significantly from each other at P ≤ 0.05. Data shown are means ± S.D. (n = 4 independent biological replicates).

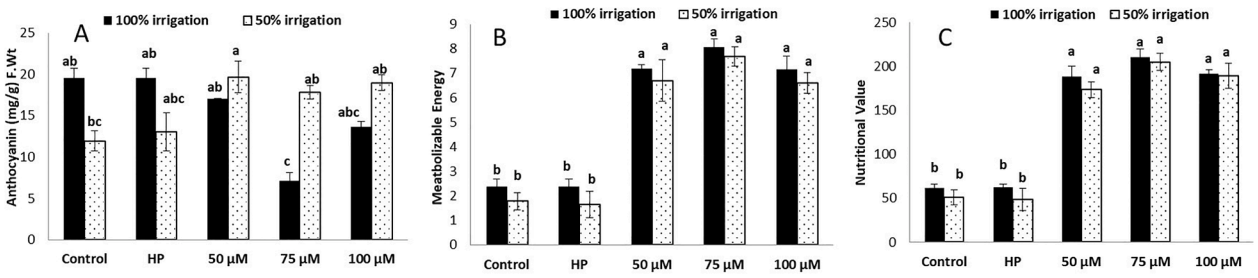

Fig. 3. Effects of water stress on biochemical attributes including anthocyanin, metabolizable energy and nutritional value of sunflower (*Helianthus annuus* L.) plants with hydropriming and primed with melatonin (50, 75 and 100 µM). Means with same letters, for each trait, do not differ significantly from each other at $P \leq 0.05$. Data shown are means ± S.D. (n = 4 independent biological replicates).

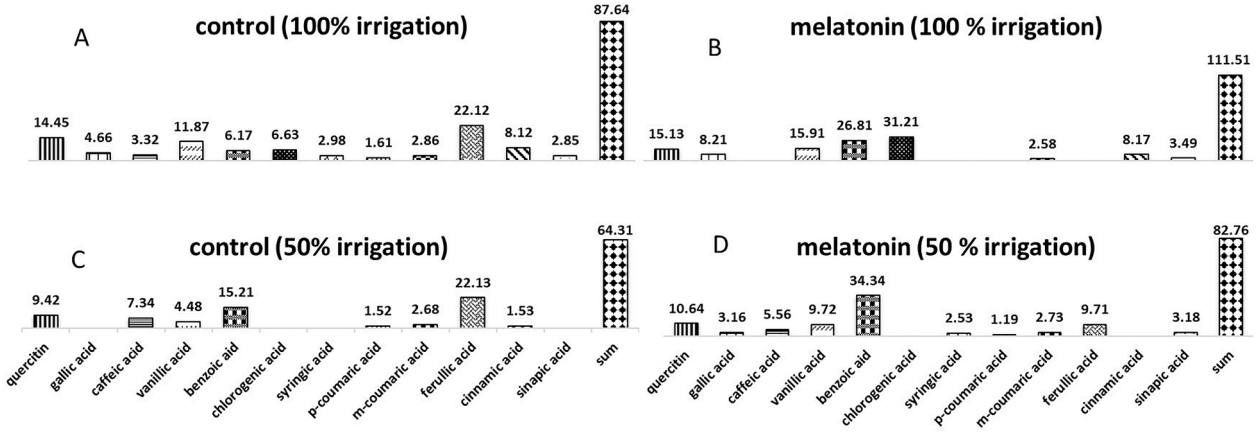

Fig. 4. Fatty acid composition of oil yield extracted from sunflower rose in control (100 %) and water deficit (50 % irrigation) conditions after melatonin (100 µM) priming. S indicates saturated fatty acid U indicates unsaturation.

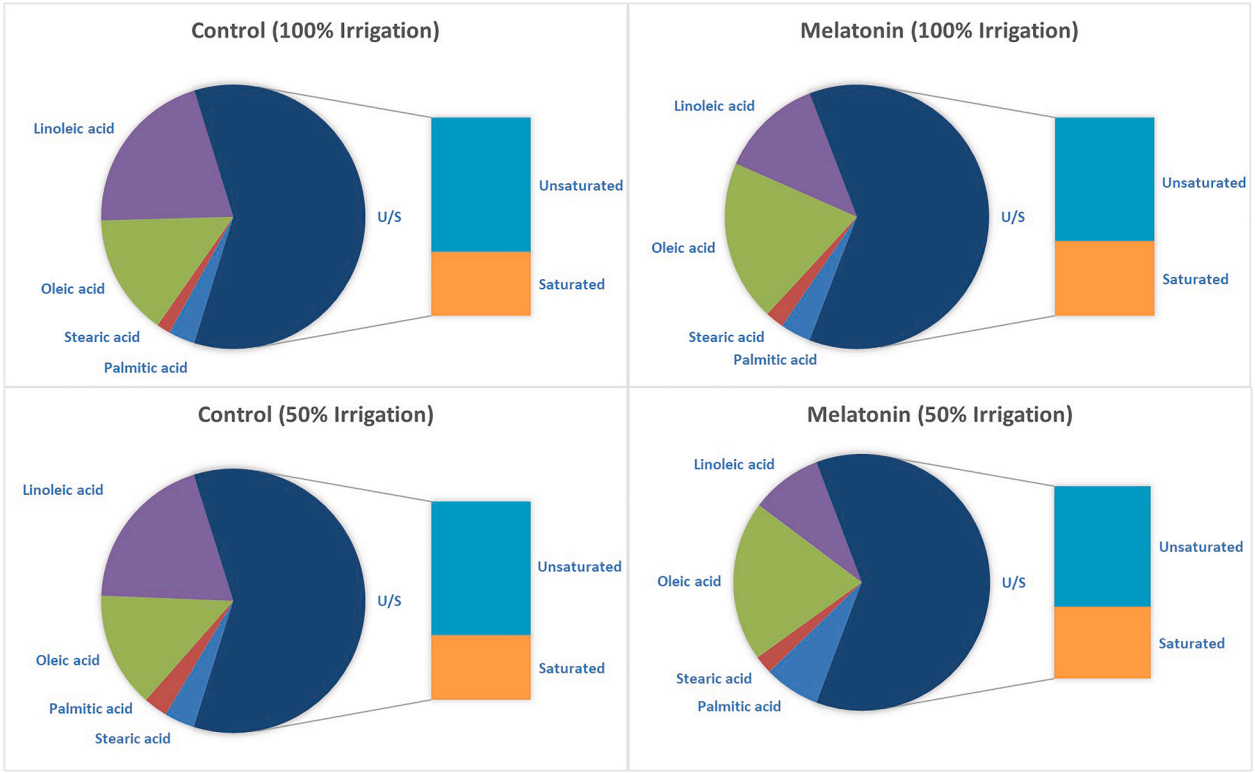

Fig. 5. Effect of water stress (100 and 50 % irrigation) and melatonin priming (100 µM) upon phenolic profile of sunflower (*Helianthus annuus* L).

normal irrigation, unprimed plants manifested 46.96 U/S, in water stress this ratio was reduced up to 43.63. In case of melatonin (100 μM) priming there was small effect under % irrigation but in 50 % irrigations, there was marked improvement of unsaturation to saturation ratio that was 58.12 (Fig. 5).

Discussion

Plants need strategic and effective solutions to cope with various environmental stresses in their growth environment (Gupta et al., 2020; Sani et al., 2023; Suzuki et al., 2014; Yong et al., 2010). Melatonin, an amine hormone is the focus of recent research due to its significant importance in plant physiology with special emphasis to stress tolerance (Tiwari et al., 2021; Gatasheh et al., 2023). Previously endogenous (Liu et al., 2021) and exogenous melatonin has been reported to alleviate water stress in different plants (Sharma et al., 2020; Rajora et al., 2022); where the effectiveness of melatonin was found dose and species specific (Park et al., 2021). In the current study three levels (50, 75 100 μM respectively) of melatonin were applied and found to be effective for sunflower plants.

Water scarcity caused significant reduction of growth in untreated sunflower plants as indicated by decreased plant height, number of leaves, yield attributes and % oil contents. Similar reduction was recorded in other species previously (Bakery et al., 2012; Ahmed & Sadak 2016; Sharma et al., 2020; Bidabadi et al., 2020; Saleem et al., 2023). Priming of sunflower seeds with melatonin lowered these negative effects on later growth and oil yield; and improve the physiological stress tolerance potential during water deficit. Melatonin was reported to improve root growth and development; the mechanism is possibly through some auxin dependent and some independent signal transduction mechanisms (Banerjee et al., 2021).

Plant growth is directly associated with photosynthesis, which must be secured to retain biomass production under water shortage (Abd Elhamidet al., 2016; Tiwari et al., 2021). Stressful conditions may reduce stomatal opening, reducing CO_2 diffusion in mesophyll cells that alters photosynthetic rate. In addition, water reduction may lower chlorophyll contents. In each plant it is totally dependent on crop and genetic makeup. Hence, there is parallelism between chlorophyll pigments and rate of photosynthesis (Ezzo et al., 2018; Li et al., 2021). Currently, water limitation reduced photosynthetic pigments that were mitigated by melatonin application. Melatonin primed plants exhibited higher level of chlorophyll contents when compared with control plants under both water regimes. Interestingly, best was displayed at its highest level (100μM) in terms of stress alleviation underlying its key adaptability against water deficit conditions. It is well justified due to documented role of melatonin in biosynthesis of chlorophyll components, defense of photosynthetic pigments, related biomass production, oil yield (Bidabadi et al 2020 and Sadak et al 2020) and decreasing stomatal inhibition and elevating photosynthetic rate (Ahmad et al., 2021)

To adjust the stress induced osmotic pressure and cell balance, plants produce metabolites like proteins and sugars (Elewa et al., 2017a; Afzal et al., 2021). Many studies have reported that the effect of various forms of stress on plant proteins and sugars. In the current work, unprimed sunflower plants showed small decline of proteins and sugars in stress that might be the reason behind known potential of moderate tolerance of sunflower against abiotic stresses (Elewa et al., 2017b; Chi et al., 2019). Moreover, in melatonin primed plants, there was marked elevation of both metabolites.These findings indicate positive role of melatonin against water stress by executaion of stable metabolic response. Chen et al. (2020) had positively correlated melatonin treatment with physiological cell balance in stressed plants preventing protein degradation and improved biosynthesis. A rapid rise was observed in the proline value in stressed plants which are in accordance with the finding of earlier studies on different crop species (Sadak et al 2020; Ghosh et al., 2022). Deposition of metabolites called osmo-protectants such as proline may be a plants protective strategy to alleviate abiotic stresses (Ghosh et al., 2022). It is directly related to plants improved performance under environmental stresses as we observed in case of sunflower in this study. Melatonin priming lead to increase this attribute non-significantly only via 100 uM plants in 50 % irrigations where in 100 % irrigations, response was small. Previous studies have also documented enhanced levels of proline under suitable levels of melatonin application for abiotic stresses (Ahmad et al., 2021). In contrast to this, Zamani et al (2020) reported that melatonin application lowered the proline attribute in fenugreek (Trigonella foenum-gracum L.) under drought stress. Considering the relieving impact of melatonin on oxidative injury caused by abiotic stress, proline degradation may be activated during stress relief (Zamani et al., 2020). Furthermore, proline degradation is needed to maintain growth and development under different environmental stresses.

As defensive mechanism plants produce enzymatic and non-enzymatic antioxidants to cope with excessive ROS accumulation and oxidative damage. Numerous studies have explained that melatonin enhances the activity of enzymatic antioxidants under stressful conditions (Altaf et al., 2021). Our findings confirmed previous evaluations. Though, it was level specific as 50 and 100 μM melatonin priming performed excellently for regulation of catalase enzyme activity in 50 % irrigations. In the present work, catalase activity was decreased in untreated plants by lowering a number of irrigations. Recent findings endorse our results reporting crosstalk between melatonin and other growth-promoting regulators with a negative effect on reactive oxygen species (Li et al., 2023)

Higher levels of ROS in plants exposed to water deficit is commonly related with alterations in the net carbon assimilations that might have significant effect upon signaling pathways of secondary metabolites especially leaf polyphenols (Tiwari et al., 2021; Mittler et al., 2022). In present samples, water stress reduced caffeic acid, ferulic acid, cinnamic acid and quercetin with increase of gallic acid, benzoic acid, chlorogenic acid and vanillic acid. Whereas, gallic acid, benzoic acid and chlorogenic acids with known pharmaceutic value enhanced in melatonin treated stressed plants. Chlorogenic acid involved in regulation of enzymatic and non-enzymatic activities related to AsA-GSH cycle, maintain membrane integrity participated in regulation of saturated to unsaturated fatty acid (Arikan et al. 2022).

Phenolics, flavonoids and anthocyanins (subfamily of flavonoids) are the products of PePP and known ROS scavengers. These plausible missing link can provide useful information about direct involvement of melatonin with PePP. At present, there is limited literature about positive effect of melatonin treatment upon products of PePP. In this study, it is noteworthy that not only stressed sunflower plants differed from control for their phenolic profiles, but melatonin treated plants also differed from the untreated ones. These observations highlighted possible biochemical linkages and more research is needed in future along these lines. An overview of PePP and associated pathways including endogenous melatonin biosynthesis (Fig. 6) showed that tryptophan is the precursor of melatonin, auxin and PePP. In exogenously treated plants there is expected rise in endogenous melatonin level (Wang et al., 2022). This increase in one of the products of tryptophan may results in its more utilization in other two pathways; that is in line to our findings as better growth might be associated with higher auxin level; and enhanced phenolics and flavonoids might be indicative for improved PePP. Supportive evidence is the knowledge of feedback inhibition; less utilization of tryptophan for melatonin production may cause feedback inhibition upon its immediate precursor chorismate (Eberhard et al., 1996). Hence, chorismate might be responsible for the overproduction of the other possible products (tyrosine and phenylalanin) that are direct precursors of PePP. Moreover, the possible linkage between melatonin treatment and PePP could indicate the role of melatonin in activation of its key enzyme PAL (phenylalanine ammonia lyase) (Sharma et al. 2020; Wang et al. 2020; Liu et al. 2021).

Yin et al. (2022) and Banothu and Uma (2022) revealed expression of genes associated with chalcone synthase, isoflavone reductase and

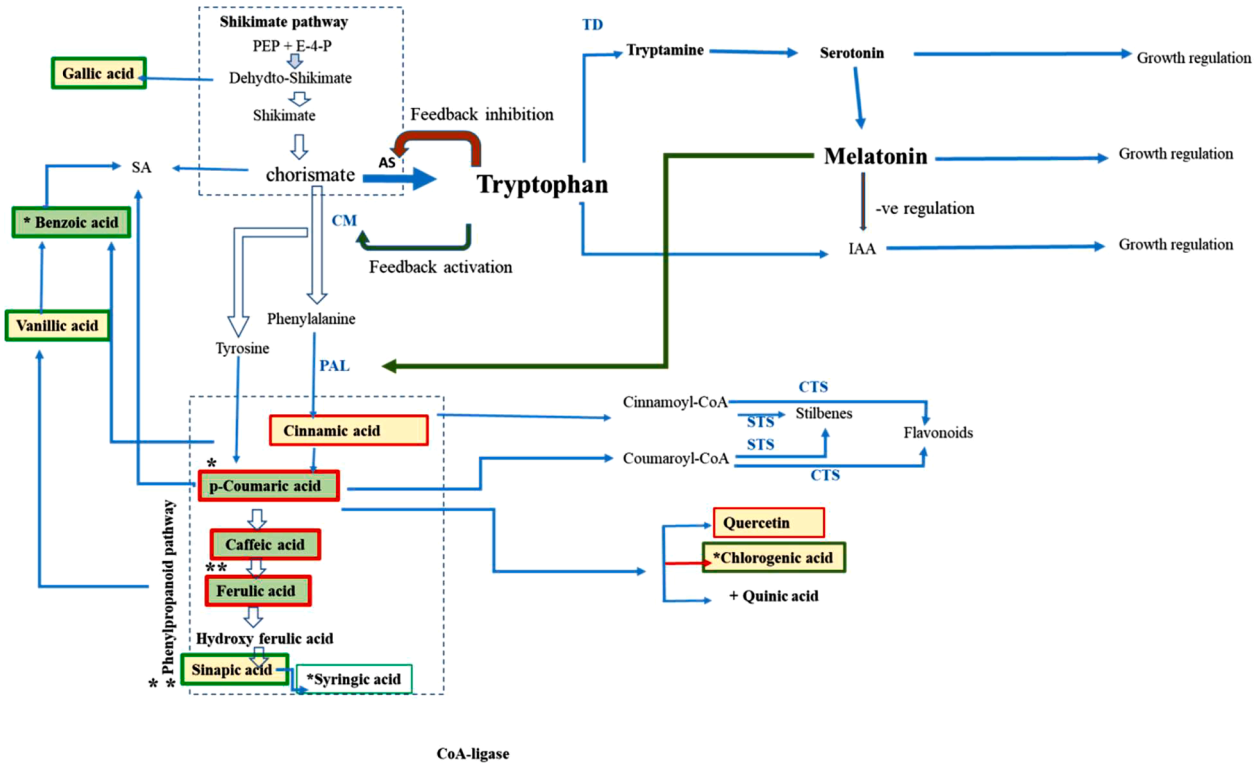

Fig. 6. The proposed metabolic scheme to illustrate a plausible link between Melatonin and PePP based upon literature (Sharma et al., 2020, Wang et al., 2020 and Mansouri et al., 2021; Yin et al., 2022) and current findings. Red arrows show all possible entries of Tyrosine (forward or backward) into PePP. Phenolics highlighted in green are significant while those in brown color indicating reduction in response to melatonin priming.

flavanone 3-hydroxylase in melatonin treated plants. These are considered as the enzymes of anthocyanin and flavonoid biosynthesis. In our study better accumulation of these secondary metabolites in melatonin pretreated plants is in support of their findings and indicating maintained secondary metabolism in sunflower (Fig. 2). However, better results were found in melatonin treated stressed plants underlying its significance against water deficiency. Here the combine effect of drought and melatonin was found superior to the individual effects of both factors. Better response of melatonin treatment in drought stressed plants may be justified by the findings of Hodaei et al., 2018that explored up regulation of genes encoding some enzymes of PePP, flavonoid and anthocyanin pathways under water stress that might be the reason behind negative impact of drought in sunflower even it is moderately drought tolerant. As regards melatonin seed treatment, in both water irrigations there was significant increase in phenolic value in line to previous literature (Yin et al., 2022).

For the oil food industry, WHO, (1985) and FAO (United Nations Food and Agriculture Organization) have a recommendation with the intake of saturated fatty acids up to 10 % of total calories. This recommended range may results in prevention of chronic diseases with a healthy use of nutrition (Darnton-Hill et al., 2004). In the current work, sunflower unprimed plants produced 7 U/S proving healthy use of sunflower edible oil. Upon lowering the number of irrigation it reduced its level to increasing saturated fatty acids. Water shortage reduces nutritive value in terms of U/N

Therefore, any treatment with enhancement of unsaturated fatty acids including oleic acid and linoleic acid would be candidate strategy for future sunflower production. Water reduction reduced oleic acid and linoleic acid in present study. Melatonin seed treatment (100uM) improved oleic acid and total unsaturated to saturated fatty acid ratio of sunflower achene oil. Oleic acid and linoleic acid are associated with plant stress tolerance too which are reported higher in melatonin treated plant under water deficit (Zamani et al., 2020). Our findings support previous literature about effect of melatonin upon U/S (unsaturated to saturated ratio) (Kong et al., 2020). Melatonin treatment can increase the ratio of unsaturated to saturated fatty acids by reducing enzymatic activities of phospholipase D (PLD) and lipoxygenase (LOX) at transcriptional level (Kong et al., 2020).

Conclusion

Our results confirmed that melatonin priming helped sunflower plants in mitigating the negative effects of water shortage. Our approach has further revealed that melatonin priming improve resilience, growth performance and yield of sunflower plants under either stressed or unstressed growth environment. Moving forward, future investigations are required to examine the genetic mechanism and metabolic pathways involved in understanding the recovery processes from water shortage conditions under melatonin treatment.

CRediT authorship contribution statement

Saqib Mahmood: Supervision, Conceptualization. **Beenish Afzal:** Writing – original draft, Methodology. **Rohina Bashir:** Writing – review & editing, Investigation. **Muhammad Bilal Shakoor:** Validation, Resources. **Zaib Un Nisa:** Validation, Investigation. **Muhammad Rizwan:** Investigation, Funding acquisition. **Muhammad Awais:** Writing – review & editing, Investigation. **Muhammad Azeem:** Visualization, Project administration. **Abdul Wahid:** Visualization, Formal analysis. **Jean Wan Hong Yong:** Project administration, Funding acquisition.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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