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Research Article

Positive Response of *Hyoscyamus pusillus* Callus Cultures to Exogenous Melatonin on Biochemical Traits and Secondary Metabolites under Drought Conditions

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There is increased attention from specialists toward producing natural compounds from plant tissues through the combined use of elicitors. The present study investigated the effects of melatonin treatment in normal and drought stress conditions for sustainable production of biomass and secondary antioxidant metabolites in a *Hyoscyamus pusillus* four-month-old callus cultures. For this purpose, we used multiple concentrations of melatonin (0, 0.5, 1.0, 1.5, and 2.0 mg l^{-1}) to assess its ability to improve growth, physiological, and biochemical properties of *H. pusillus*. There was two drought stress levels (0 and 30 g l^{-1}) of polyethylene glycol (PEG). It was harvested in 28 days. The results showed a significant decrease with an increase in the concentration of PEG treatment in growth and physiological traits compared to the results of those samples when treated with melatonin. Results showed an increase in oxidative stress in tissue treated with PEG due to a significant increase in the content of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). The decreased oxidative stress was associated with an increased antioxidant enzyme activity superoxide dismutase (SOD), and catalase (CAT) in the samples was treated by melatonin, which resulted in increased membrane stability index (MSI) and enhanced growth traits under the PEG treatment compared to the control. By reducing the activity of phenylalanine ammonia-lyase (PAL) and peroxidase (POX), moisture tightening increased the production of phenolic compounds (PC) and flavonoid compounds (FC) in callus cultures, and high concentrations of melatonin were combined with them to improve their production. Therefore, it can be asserted that a moderate treatment with melatonin is more suitable under water stress conditions to produce secondary compounds from *H. pusillus*.

1. Introduction

Plants are essential to sustain human life. In addition to being used as food, they are used as the main source of pharmaceutical industries for most of the world's population. Wild plants are characterized by their content of phytochemicals known as secondary metabolites that are used in various industries [1]. The genus *Hyoscyamus* is an important plant genus that has a wide range of plant species, including the *pusillus*, a plant species with medicinal properties that belongs to the Solanaceae family [2]. The plant is classified among the endangered plants in the western desert of Iraq due to overgrazing and lack of rain, which leads to the loss of an important plant that can provide essential natural compounds for the pharmaceutical industry. The plant's rich content is characterized by inactive compounds such as alkaloids [3], phenolic acids, flavonoids, terpenoids, tannins, and steroids [4], from which a wide range of bioactivities can be derived such as antioxidant, antifungal, antibacterial, antibacterial [4], antispasmodic, anticholinergic, mydriatic, sedative, and analgesic derivatives [5]. Biotechnology, particularly plant tissue culture techniques, plays a distinct role in stimulating the metabolic pathways responsible for producing secondary metabolites.

It provides the necessary solutions to many of the problems facing the production of these compounds from their natural sources, such as the geographical distribution of the plant, the unfavorable environmental conditions, high economic cost, low productivity, difficult purification, the shortage of agricultural lands to grow medicinal plants due to the competition with other economic crops, and the lack of suitable land for cultivation [6–8].

Polyethylene glycol is a long-chain polymer compound, water-soluble and nonionic [9]. It has osmotic properties to enhance drought stress in the medium by increasing osmosis and growth of plant cells under the stress of aqueous deficit like dehydration under normal conditions without toxic effects [10, 11]. Several studies have confirmed that the induction of drought stress is one method used to increase the secondary compounds in calli cultures. While it negatively affects the accumulation of biomass, it can contribute to the production of secondary metabolites in calli cultures [12–15].

Melatonin (N-acetyl-5-methoxytryptamine) is a nontoxic chemical compound produced by various plant organs. It was classified as a newly discovered plant growth regulators [16, 17]. Several studies have reported the prominent role of melatonin in plant growth and development. It provides the necessary protection for the plant cell from the negative impact of various abiotic stresses [18–22] by stimulating gene expression [23] leading to the activation of the antioxidant enzymatic and nonenzymatic systems [24–29]. Melatonin is believed to have key role in increasing the mitochondrial efficiency of the electron transport chain. Thus, melatonin plays an important role in the stability of biofilms [30] and protecting them from the impact of oxidative damage [30–33]. Determining the efficacy of exogenous melatonin under water stress conditions is essential to study its ability to elicit defensive responses in calli cultures and their effects on the production secondary metabolites.

To our knowledge, till date, there is no research article available explaining the effects of the interaction between melatonin treatment and osmotic stress on the biomass accumulation of callus and the responses of plant defense systems as well as their implications for the production of plant antioxidants. Therefore, the present article aims to evaluate *H. pusillus* tissue cultures' response to exogenous addition of melatonin under normal and aqueous tension conditions using PEG in enhancing biomass and biochemical characteristics and reflecting these changes in the production of desired levels of phenolic metabolites and flavonoids in a *H. pusillus* calli cultures.

2. Materials and Methods

2.1. Seed Germination. H. pusillus seeds were provided from the desert environment museum (University of Anbar, Iraq). The seeds were surface sterilized by placing them in an Erlenmeyer flask with detergent liquid added and washed well under tap water for 30 minutes. They were inserted into a laminar flow and sterilized using 3% sodium hypochlorite solution for 15 minutes. They were then rinsed with sterile distilled water five times. The sterilized seeds were cultured in a preprepared MS medium with 30.0 g l^{-1} sucrose and 7.0 g l^{-1} agar, incubated at 25°C and a light/dark period of 8/16 hours to obtain the sterile seedlings.

2.2. Callus Induction. H. pusillus was callus induced by cultures of cotyledons from sterilized seedlings in MS medium supplemented with 3.0 mg l^{-1} 2,4-D, 0.5 mg l^{-1} Kin., 0.5 mg l^{-1} GA, 0.25 mg l^{-1} NAA and 30.0 g l^{-1} sucrose. The pH was adjusted to 5.7, after which the 7.0 g l^{-1} agar was added. The explants were incubated at 25°C for 28 days in the darkness.

2.3. Establishment and Treatment of Callus Cultures. H. pusillus callus cultures were initiated from several subcultures on the MS medium (pH 5.8) supplemented with 2.0 mg l^{-1} 2,4-D, 0.5 mg/l kin., 30 g l^{-1} sucrose, and 7.0 g l^{-1} agar as a callus cultures reproduction media, and the cultures were incubated at 25 ± 2°C in darkness. The formed callus cultures were treated with study agents that included polyethylene glycol at concentration (0 and 30) g l^{-1} and melatonin at concentrations 0, 0.5, 1.0, 1.5, and 2.0 mg l^{-1}. The callus was harvested after 28 days for the specified measurements.

2.3.1. Callus Morphology and Growth Traits. Callus color was determined by visual inspection. The fresh weight of the callus was determined by separating the callus samples from the culture media and removing the remaining media attached to it. The dry weight of the callus was determined based on oven-dried callus weight at 40°C for 48 h.

2.3.2. Callus Physiological Traits. The total chlorophyll content was determined based on the method described by Lichtenthaler and Buschmann [34]. Briefly, 0.25 g was taken from a homogeneous sample of soft callus crushed by liquid nitrogen. Then, 10 ml of acetone was added and centrifuged at 5000 rpm for 5 min. The absorbance of the supernatant was determined at wavelengths 660 and 642.5 nm. The membrane stability index was determined in a sample of 200 mg fresh weight of callus according to the addition of 10 ml of distilled water. It was divided into two groups. The first group was placed in a water bath at 40°C for 30 minutes. Then, the electrical conductivity was recorded after being cooled to 25°C. In the same way, the second group was heated to 100°C for 15 minutes. Then, the electrical conductivity was measured, followed by measuring the membrane stability index [35]. The relative water content of callus cultures was calculated by soaking fresh callus in water distilled and then weighed to obtain the turgid weight (TW). Then, the calculation was done based on the previously described formula by Karimi et al. [36].

2.3.3. Content H_2O_2. The spectrophotometric method according to Velikova et al. [37] was used to determine the content of H_2O_2 from callus samples. Briefly, 250 mg of fresh callus was added in 2.5 ml of 0.1% tri-chloroacetic acid

(TCA) and centrifuged at 12,000 rpm for 15 min. Then, 500 μl of the supernatant solution was added to 500 μl of 10 mM K-phosphate buffer (pH 7.0) and 1000 μl of KI (1.0 M). The absorbance of the supernatant was determined at 390 nm. The concentration of H_2O_2 was determined using a H_2O_2 standard curve.

2.3.4. Lipid Peroxidation Assay. The amount of malondialdehyde (MDA) production in the assay was measured based on the report provided by De Vos et al. [38]. In short, 300 mg of fresh callus was homogenized in 10 ml of 0.25% TBA dissolved in 10% TCA. The extract was heated at 95°C for 30 minutes and then immediately cooled on ice. After that, we used the centrifugation at 3,000 rpm for 10 min. The concentration of MDA was measured by deducting the absorbance at 600 and 532 nm using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.3.5. Determination of Enzyme Activity. Antioxidant enzymes analysis was carried out according to the provided method of Gapinska et al. [39]. The enzyme was extracted from 500 mg of fresh callus which was symmetrized in potassium phosphate buffer (50 mM, pH 7) including 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). Then, the centrifugation of 10,000 rpm at 4°C for 15 min was applied to measure the enzymatic activity.

The SOD activity level was described by Beauchamp and Fridovich [40]. The assay mixture consisted of 100 μl of the enzyme extract, 50 mM K-phosphate buffer (pH 7.8), 2 μM riboflavin, 13 mM methionine, 0.1 mM EDTA, and 75 μl nitroblue-tetrazolium (NBT). The reactions mixture was carried out at 25°C under a light bank consisting of eight fluorescent lamps with intensity of around 15 W for 10 min. After the completion of the reaction, the activity of SOD enzyme was determined at an absorbance level of 560 nm, based on a level of photoreduction by 50% of NBT.

The CAT activity was measured based on the decrease in H_2O_2 absorption at 240 nm for 30 s using 3.8 mM^{-1} cm^{-1}as an extinction coefficient [41]. The reaction mixture consisted of 15 mM of H_2O_2, and 50 mM K-phosphate buffer at pH 7.0 and 25°C with 50 μl of enzyme extract.

PAL activity was determined as described by Lin and Wu [42]. Briefly, 100 μl of the enzymatic extract was added to 1000 μl of borate buffer at pH 8.8. The callus cultures were ground with a pestle and mortar on a makeshift pretzel (0.15 g ml^{-1}) for 2 minutes on ice. Then, centrifugation of 1000 rpm for 20 min under 4°C was carried out. The PAL activity was determined based on its transformation to cinnamic acid.

POX activity was estimated based on the report of Abeles and Biles [43]. In short, the components of the reaction mixture included 2 ml of acetate buffer (0.2 M, pH 4.8), and 200 μl of benzidine (40 mM). 200 μl of H_2O_2 was added to the reaction, and purified anionic POX was added at 20 μl. The absorbance was measured at 530 nm.

2.3.6. Nonenzymatic Antioxidants. The phenolic content in callus tissue was determined based on modified protocol of Velikova et al. [37]. Briefly, 100 mg of dried callus was added in 20 ml of the methanol 80% and sonicated at 25°C for 20 min. Then, we centrifuged for 20 min at 15,000 rpm. The supernatant was dried by a vacuum evaporator, resolved in 1 ml methanol, and used for the determination of phenolic compounds. The absorbance of the mixed solution was measured at 725 nm using gallic acid in the standard curve [44]. Total flavonoids were estimated as catechin equivalents. The absorbance was measured at 510 nm. The results were expressed as mg catechin g^{-1} DW [45].

2.4. Experimental Design and Statistical Analysis. The coefficients were distributed using the complete randomized design system (CRD). The data of three replicates were subjected to one-way analysis of variance (ANOVA), and Duncan's multiple range test (DMRT) was used to determine the significant differences for the several samples at $P \leq 0.05$ by the statistical software package (GenState 24th Edition).

3. Results and Discussion

3.1. Morphological Changes and Biomass Accumulation. The effect of PEG and melatonin on morphological changes and biomass fresh weight (FW) and dry weight (DW) of calli cultures was tested in a *H. pusillus* calli cultures.

Greenish white color was obtained for calli cultures nontreated with PEG and melatonin. A color change from greenish white to greenish brown was observed with an increased concentration of melatonin, whereas the melatonin-free PEG 4% treated callus cultures acquired a dark brown color. This color changed to light brown with increasing melatonin concentration (Table 1 and Figure 1). The main reason for the dark brown coloration of calli tissue is the accumulation of phenolic compounds due to drought stress [12, 13, 46], whereas melatonin treatment contributed to relieving stress by changing the dark color to light brown or brownish white. This may be due to its ability to protect the cell from the negative effects of abiotic stress [47].

The highest mean values significantly increased in FW, and DW (7.115 and 0.515 g) was observed in 4% PEG with treating calli cultures at a concentration of 1.0 or 1.5 mg l^{-1}, respectively, under drought-free conditions. The biomass of the callus culture in the combination (4% PEG + 0 melatonin) was significantly decreased by 2.522 and 0.180 g in FW and DW, respectively. Conversely, melatonin-enhanced biomass accumulation under drought stress by increasing the FW and DW (Table 1). Our observations were supported by investigations revealing the role of melatonin processing in biomass enhancement [27]. It was observed that the inclusion of PEG in the culture medium reduced the growth of tissue cells, thus causing their deterioration [12, 48, 49]. Melatonin can enhance plant tolerance to abiotic stress and stimulate cell growth [50, 51]. This may be due to the positive role

Table 1: The morphological traits, fresh weight (FW), and dry weight (DW) in *H. pusillus* calli cultures treated with melatonin concentrations and PEG levels after 28 days.

Treatments	Callus color	FW (g)	DW (g)
PEG 0% + melatonin 0 mg l^{-1}	Greenish white	5.457 ± 0.121^{e}	0.377 ± 0.025^{d}
PEG 0% + melatonin 0.5 mg l^{-1}	Green	6.051 ± 0.080^{f}	0.431 ± 0.006^{e}
PEG 0% + melatonin 1.0 mg l^{-1}	Green	7.115 ± 0.343^{i}	0.502 ± 0.012^{fi}
PEG 0% + melatonin 1.5 mg l^{-1}	Greenish brown	6.698 ± 0.218^{g}	0.515 ± 0.007^{i}
PEG 0% + melatonin 2.0 mg l^{-1}	Brown	6.707 ± 0.275^{gh}	0.492 ± 0.009^{f}
PEG 4% + melatonin 0 mg l^{-1}	Dark brown	2.522 ± 0.181^{a}	0.180 ± 0.013^{a}
PEG 4% + melatonin 0.5 mg l^{-1}	Reddish brown	3.575 ± 0.189^{ab}	0.259 ± 0.017^{b}
PEG 4% + melatonin 1.0 mg l^{-1}	Brown	3.758 ± 0.116^{b}	0.266 ± 0.006^{b}
PEG 4% + melatonin 1.5 mg l^{-1}	Light brown	4.044 ± 0.140^{c}	0.309 ± 0.010^{c}
PEG 4% + melatonin 2.0 mg l^{-1}	Brownish white	4.460 ± 0.306^{d}	0.312 ± 0.023^{c}

Data show the mean values ± standard errors of triplicates. Different alphabets indicate significant statistical differences between the mean values based on Duncan's test at $P \leq \%5$.

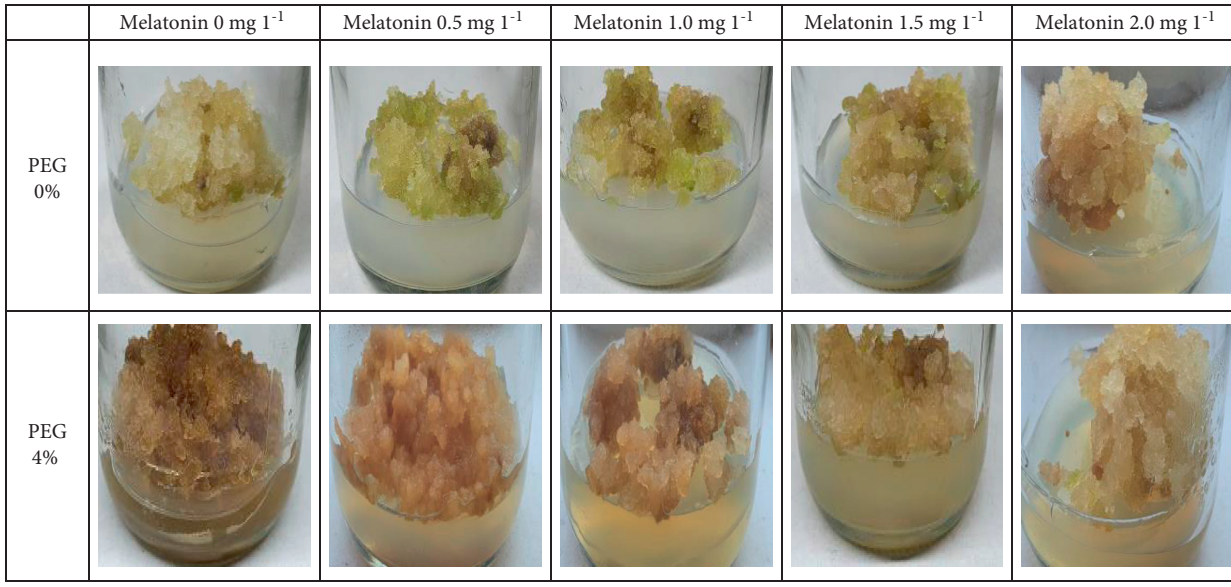


Figure 1: Morphological changes and biomass traits in melatonin treated *H. pusillus* callus cultures on medium with or without PEG.

that melatonin plays in inducing the biosynthesis of indole-acetic acid in plant cells [52], contributing to the promotion of biomass accumulation [27].

3.2. Physiological Traits. Physiological changes in melatonin treated *H. pusillus* calli cultures in media free or containing of drought stress by PEG are shown in Table 2.

The total chlorophyll content was significantly increased in callus cultures grown in PEG-free media. The melatonin concentration was reached at 2.85 mg g^{-1} FW in 1.0 mg l^{-1}, compared to the control treatment, which was 2.47 mg g^{-1} FW. Furthermore, melatonin treatment caused an improvement in the total chlorophyll content of the developing callus under drought conditions, the concentration of 2.0 mg l^{-1}, a significant increase of 2.15 mg g^{-1} FW compared to the control treatment, which showed the lowest total chlorophyll content at 0.99 mg g^{-1} FW.

The membrane stability index was observed to increase with increasing melatonin concentration significantly. The concentration of melatonin at 2.0 mg l^{-1} showed the highest mean values of 86.48 compared with the concentration of 0.5 mg l^{-1} by 73.61. However, the results showed the same concentration of melatonin treated in media containing drought condition by 66.77, compared to the control treatment, which showed the lowest mean values for this trait (45.48).

The RWC significantly increased in callus cultures grown in media free from drought conditions and treated with a high melatonin concentration by 6.12%, compared to 0.5 mg l^{-1} of melatonin concentrations. Melatonin treatment caused an increase in the RWC of the enhancing callus under drought conditions. The concentration of 2.0 mg l^{-1} achieved a significant increase of 66.77% compared to the control, which showed the lowest percentage of 57.41%.

The callus cultures grown under abiotic stress conditions are negatively affected in Chl.T, MSI, and RWC, leading to increased oxidative stress. This caused damage to some cellular components [27, 53–55].

Table 2: Total chlorophyll (Chl T), membrane stability index (MSI), and relative water content (RWC) in *H. pusillus* calli cultures treated with melatonin and PEG levels after 28 days.

Treatments	Chl T (mg g−1 FW)	MSI	RWC (%)
PEG 0% + melatonin 0 mg l^{-1}	2.47 ± 0.121^{ef}	75.49 ± 2.030^{fg}	82.88 ± 0.697^{f}
PEG 0% + melatonin 0.5 mg l^{-1}	2.62 ± 0.025^{gh}	73.61 ± 1.581^{f}	82.06 ± 1.320^{f}
PEG 0% + melatonin 1.0 mg l^{-1}	2.85 ± 0.076^{i}	80.23 ± 2.096^{h}	83.93 ± 2.165^{f}
PEG 0% + melatonin 1.5 mg l^{-1}	2.72 ± 0.123^{h}	85.21 ± 1.616^{i}	87.03 ± 1.243^{g}
PEG 0% + melatonin 2.0 mg l^{-1}	2.52 ± 0.090^{fg}	86.48 ± 2.761^{i}	87.08 ± 0.656^{g}
PEG 4% + melatonin 0 mg l^{-1}	0.99 ± 0.084^{a}	45.48 ± 2.336^{a}	57.41 ± 1.234^{a}
PEG 4% + melatonin 0.5 mg l^{-1}	1.43 ± 0.139^{b}	54.19 ± 2.237^{b}	66.14 ± 1.279^{b}
PEG 4% + melatonin 1.0 mg l^{-1}	1.95 ± 0.100^{c}	57.22 ± 1.257^{c}	67.73 ± 2.041^{c}
PEG 4% + melatonin 1.5 mg l^{-1}	2.14 ± 0.031^{d}	63.11 ± 1.926^{d}	72.07 ± 1.206^{d}
PEG 4% + melatonin 2.0 mg l^{-1}	2.15 ± 0.174^{d}	66.77 ± 1.424^{e}	75.28 ± 1.252^{e}

Data show the mean values ± standard errors of triplicates. Different alphabets indicate significant statistical differences between the mean values based on Duncan's test at $P \leq \%5$.

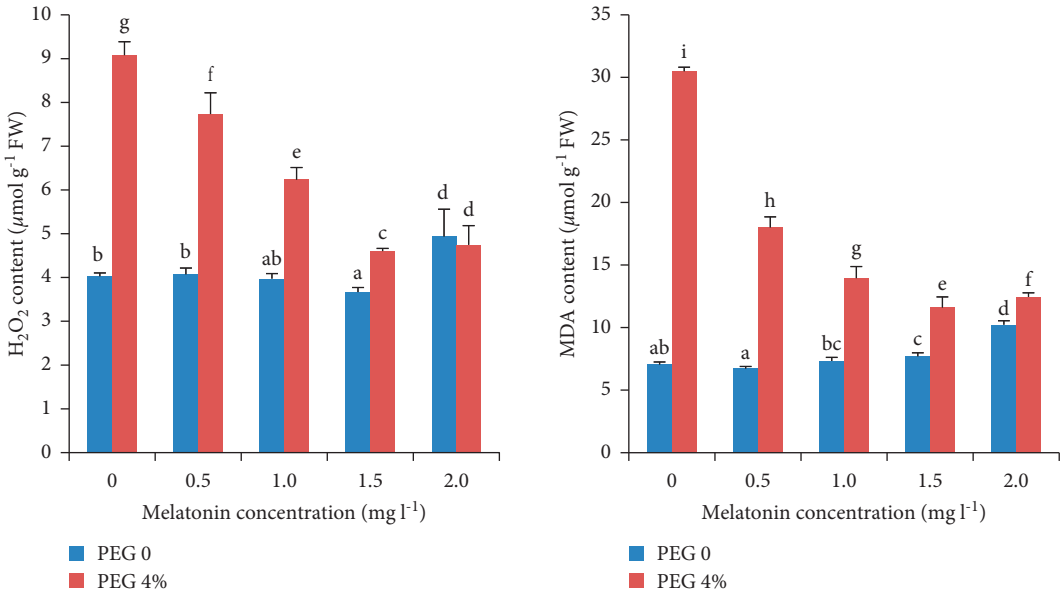


Figure 2: H_2O_2 and MDA content in *H. pusillus* calli cultures treated with melatonin concentrations and PEG levels after 28 days. Vertical bars indicate the standard errors of triplicates. Different alphabets indicate significant statistical differences between the mean values based on Duncan's test at $P \leq \%5$.

Melatonin treatment improved the harmful effects of dehydration stress on all measured physiological characteristics. Our data are consistent with previous literature indicating a role for melatonin in the improvement of total chlorophyll content in *Lupinus termis* L. and *Solanum lycopersicum* L. under water stress conditions [28, 56]. Melatonin treatment contributes to protecting chlorophyll pigments from degradation by the harmful effects of drought by stimulating gene expression responsible for regulating some genes such as Chl-PRX, Chlase and PPH. that protect the degradation of plant cell pigments [57]. It stimulates the cell's defence system against the adverse effects of ROS, which leads to the maintenance of the various cellular components [58].

3.3. H_2O_2 and MDA Content. H_2O_2 and MDA assays showed significant differences in melatonin-treated callus cultures in PEG-free or PEG-containing media as shown in Figure 2.

H_2O_2 content was reduced by higher melatonin concentrations. This role is apparent in media containing PEG as melatonin concentration of 1.5 mg l^{-1} showed decreased by 97.23% compared to the comparison treatment. In comparison, most of the melatonin concentrations were not significantly affected for the mean values of PEG-free media.

The MDA content increased at PEG levels, and was enhanced further by the melatonin treatment.

Melatonin contributed to a significant increase in PEG-free media with a concentration of 2.0 mg l^{-1} that reached 4.94 μmol g^{-1} FW. Conversely, a significant decrease was obtained by treating media containing PEG with melatonin, with a decrease of 2.25-fold compared to the control treatment.

In our study, PEG treatment induced an increase in oxidative stress by increasing the level of free radicals, resulting in damage to cell membranes. The negative impact of drought stress on H_2O_2 and MDA accumulation has been reported [36, 49].

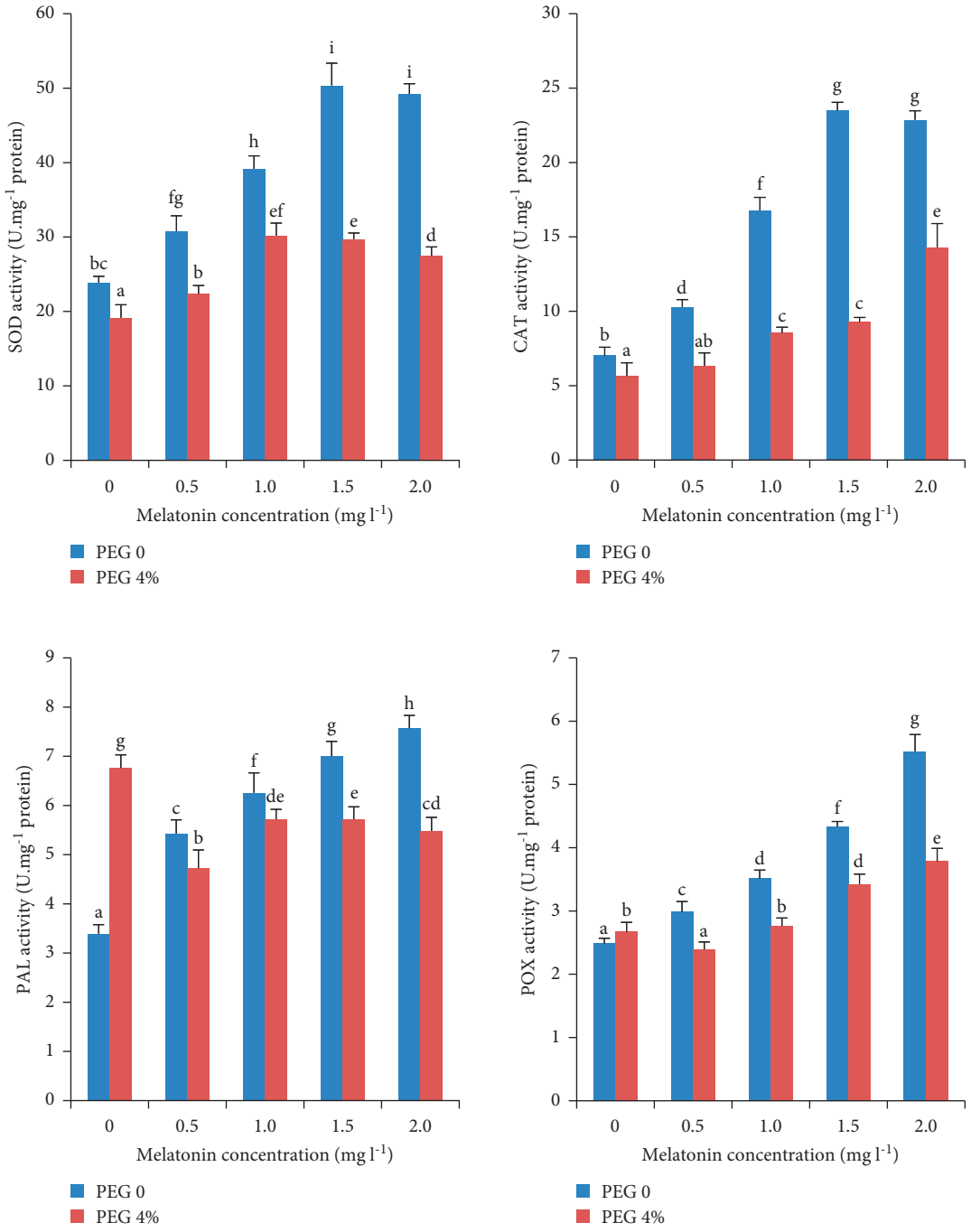


Figure 3: SOD, CAT, PAL and POX activity in *H. pusillus* calli cultures treated with melatonin concentrations and PEG levels after 28 days. Vertical bars indicate the standard errors of triplicates. Different alphabets indicate significant statistical differences between the mean values based on Duncan's test at $P \leq \%5$.

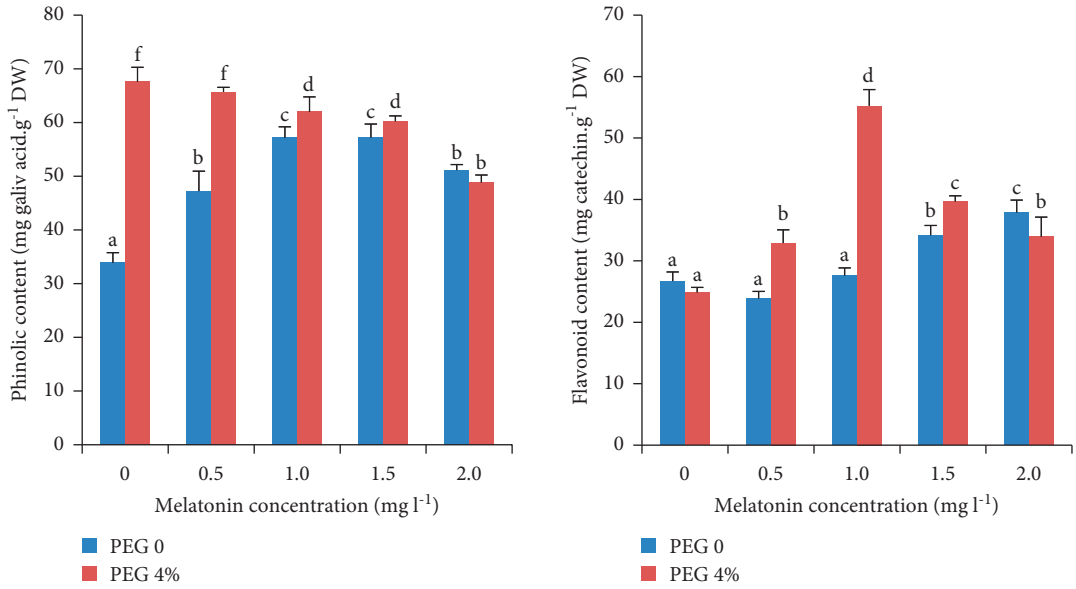


FIGURE 4: PC and FC activity in *H. pusillus* calli cultures treated with melatonin concentrations and PEG levels after 28 days. Vertical bars indicate the standard errors of triplicates. Different alphabets indicate significant statistical differences between the mean values based on Duncan's test at $P \leq \%5$.

The H_2O_2 and MDA content treated with melatonin showed better protection of cellular membranes from oxidative stress. This is a guide for Melatonin role. In resistance of cellular membranes to oxidative stress damage, which was supported by Sharma and Zheng [58].

3.4. Antioxidant Activity Enzymes. SOD and ACT activity significantly increased in melatonin-treated *H. pusillus* calli cultures in media (with or without PEG) as shown in Figure 3.

Melatonin concentrations showed a significantly higher SOD activity in PEG-free media than their PEG-containing counterparts. The current study results revealed that the concentration of melatonin 1.5 mg l^{-1} achieved 50.63 U mg^{-1} protein and was 2.11-fold higher than the comparison treatment. At the same time, melatonin improved this trait in PEG-treated callus cultures, especially with high concentrations of it.

As for CAT activity, the increased concentrations of melatonin played a prominent role in enhancing this trait. For example, the media of free drought condition with a concentration of 1.5 mg l^{-1} showed a mean value of CAT activity of 23.49 U mg^{-1} protein, 4-fold more, than that of the control treatment. However, the concentration of 2 mg l^{-1} was optimal in improving this trait under the drought condition, increasing 4- fold compared to the control.

PAL and POX activity significantly increased with melatonin concentrations when treated in *H. pusillus* calli cultures with or without PEG level as shown in Figure 3.

In the activity of PAL, there was a significant effect of PEG levels and melatonin concentration. The increased melatonin concentrations in the media of drought condition showed a similar increase in PAL activity. The highest mean values of 7.57 U.mg^{-1} protein were obtained at a concentration of 2 mg l^{-1}. Conversely, media containing PEG showed a significant decrease in this trait, increasing melatonin concentrations. The highest mean values was 6.76 U.mg^{-1} protein in the control.

The activity of POX was significantly increased in callus cultures grown in media free from drought stress and treated with a high concentration of melatonin by 2.22-fold, compared to the control. The combination of melatonin with PEG caused an enhancement of POX for callus cultures. A melatonin concentration of 2.0 mg l^{-1} increased this trait by 3.79 U.mg^{-1} protein compared to the comparison treatment, which showed the lowest mean value 2.67 U.mg^{-1} protein.

It was observed that melatonin effectively contributed to reducing stress induced by PEG by increasing the activity of the antioxidant enzyme system such as SOD, CAT, PAL, and POX that scavenge ROS [59]. Exogenous melatonin improves cellular growth. Furthermore, it stimulates genes expression and encoding antioxidant enzymes, which leads to resistance to abiotic stress [23].

3.5. Phenolic and Flavonoid Content. Phenolic compounds (PC) and total flavonoid (TP) significantly increased in melatonin treated *H. pusillus* calli cultures in media (with or without PEG) as shown in Figure 4. Melatonin concentrations showed a significant improvement in PC production in PEG-containing media compared to their non-containing counterparts. The current study results revealed that the control of melatonin achieved 67.6 and was 2.00-fold more

than the comparison treatment of PEG. At the same time, melatonin improved this trait in non-treated PEG callus cultures, especially with 0.5, 1.0, and 1.5 mg l^{-1} concentrations of it.

As for TF content, the increased concentrations of melatonin played a prominent role in enhancing this compounds.

For example, the media of drought condition with a concentration of 1.0 mg l^{-1} from melatonin showed a mean value of TF production of 55.23, 2-fold more, than that of the same concentration nontreated PEG. However, the concentration of 0.5 mg l^{-1} from melatonin was the lowest production of TF in cultures free of drought conditions.

PC and TP are nonenzymatic antioxidants. They are a class of secondary receptors known for their important biological effects. The enzyme PAL plays a major role in the synthesis of phenols. Therefore, we explain the increase in its production by the impact of the study treatments in increasing the activity of PAL enzyme [13].

4. Conclusions

In this study, the role of different melatonin concentrations in influencing *H. pusillus* calli cultures with or without drought stress by PEG is reported for the first time. The drought stress by PEG negatively affected all measured morphological, biomass, physiological, and biochemical traits. Melatonin enhanced PAL, POX, SOD, and CAT activity, which was positively reflected in the regulation of ROS level and the improvement of calli tolerance to PEG. Thus, morphological, FW, and DW traits are improved through melatonin in maintaining MSI, RWC, and Chl.T. These results will be helpful to compensate for the decline of the plant in the wild through the production of secondary compounds *in vitro*. Moreover, the present study provided knowledge that can be applied to drought tolerance by this plant. Thus, the reduction of wild-type constant attrition using plant tissue culture approaches. However, this study needs to define the role of the nonenzymatic oxidative system and understand the biological mechanisms that can cause diuresis in the production of secondary metabolites.

Data Availability

The data used to support the findings of this study will be available on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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