Integrated analysis of antioxidant responses, phenolic profiles and Phytohormonal changes in Thymus daenensis treated with Paclobutrazol under in vitro conditions

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Research Article

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Integrated analysis of antioxidant responses, phenolic profiles and Phytohormonal changes in *Thymus daenensis* treated with Paclobutrazol under *in vitro* conditions

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Abstract

Background Paclobutrazol (PBZ), a triazole-type plant growth regulator, is widely used to modulate plant growth and stress tolerance by influencing phytohormonal signaling and oxidative metabolism. However, its effects on medicinal plants, particularly *Thymus daenensis*, under *in vitro* conditions remain largely unexplored. This study aimed to investigate the impact of different concentrations of PBZ (0, 2.5, 5, and 10 mg/L) on growth traits, antioxidant defense, phenolic metabolism, oxidative stress markers, and endogenous phytohormones in *T. daenensis* cultured *in vitro*.

Results PBZ treatments induced pronounced changes in growth performance. The fresh weight of shoots progressively decreased with increasing PBZ concentrations, reaching 46% below the control at 10 mg/L, whereas root fresh weight markedly increased by 66% and 330% at 2.5 and 10 mg L^{-1}, respectively. Shoot length remained unaffected at 2.5 mg/L but declined significantly at 5 and 10 mg/L, while root length increased at 2.5 mg L^{-1} but decreased at higher doses, with the strongest reduction at 10 mg/L. Consequently, the shoot-to-root fresh weight ratio rose at 2.5 and peaked at 5 mg/L, but declined at 10 mg/L, indicating a biomass allocation shift under elevated PBZ levels.

In parallel, PBZ significantly modulated enzymatic antioxidant activities, with the greatest enhancement at 5 mg/L, and stimulated phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) at moderate concentrations. Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents declined at 2.5 and 5 mg/L, suggesting reduced oxidative damage, while DPPH radical scavenging capacity and total flavonoid content peaked at 5 mg/L but decreased at 10 mg/L. Hormonal analysis revealed a concentration-dependent reduction in gibberellic acid (GA), indole-3-acetic acid (IAA), and brassinosteroids (BR), with the lowest values at 10 mg/L, whereas abscisic acid (ABA) progressively increased, reaching its maximum at the highest PBZ concentration.

Conclusion These findings demonstrate that PBZ exerts a dose-dependent modulation of growth, antioxidant metabolism, and hormonal balance in *T. daenensis* under in vitro conditions. Moderate concentrations (particularly 5 mg/L) enhance antioxidant capacity, phenolic metabolism, and biomass allocation toward roots, while higher doses suppress shoot growth and accentuate hormonal shifts (reduced GA, IAA, BR; elevated ABA). Overall, PBZ shows promise as an elicitor to improve physiological resilience and potentially the medicinal quality of *T. daenensis* during micropropagation.

Keywords *Thymus daenensis*, paclobutrazol, antioxidant defense system, oxidative stress, *in vitro* culture

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Background

Thymus daenensis Celak., a perennial aromatic herb endemic to the Zagros Mountains of Iran (1), is a highly valued species in traditional medicine due to its abundant production of secondary metabolites, particularly monoterpenes such as thymol and carvacrol (2). These bioactive constituents contribute significantly to the plant's antimicrobial, anti-inflammatory, antioxidant, and anticancer properties, making *T. daenensis* an important target for pharmacognostic research and commercial cultivation (3,4). However, the biosynthesis and accumulation of these metabolites are not fixed traits; rather, they are dynamically influenced by both genetic and environmental factors, including nutrient availability, light conditions, water stress, and the presence of plant growth regulators (5).

Paclobutrazol (PBZ) is a triazole-based plant growth regulator widely applied as a growth retardant and abiotic stress protectant (6). While the specific structural features conferring PBZ's biological activity are not fully elucidated, its growth-regulating effects are primarily attributed to the stereochemical configuration of its substituents and its interference with gibberellin biosynthesis (7). PBZ inhibits the cytochrome P450-dependent monooxygenases responsible for converting *ent*-kaurene to *ent*-kaurenoic acid, a crucial step in the gibberellin biosynthetic pathway (8). This disruption reduces cell elongation and division, and its growth-inhibitory effects can be reversed by exogenous gibberellin application, further validating its mode of action (9,10).

The suppression of gibberellin biosynthesis by PBZ alters the flux within the isoprenoid pathway, resulting in increased accumulation of precursors that are redirected towards the synthesis of other hormones, most notably abscisic acid (ABA) (11). ABA, synthesized through the same terpenoid pathway (12), is a key phytohormone involved in plant responses to drought and other abiotic stresses (13). PBZ application enhances endogenous ABA levels by both stimulating its biosynthesis and inhibiting its catabolism (14,15). Elevated ABA concentrations promote stomatal closure, reduce transpirational water loss, limit shoot elongation, and induce leaf anatomical adaptations that enhance water retention. These responses collectively improve water-use efficiency and drought tolerance in treated plants (16).

Beyond gibberline and ABA, PBZ significantly modulates the levels of other plant hormones. It has been shown to suppress indole-3-acetic acid (IAA) and ethylene levels, while increasing cytokinins such as zeatin and zeatin riboside (9,17). Enhanced cytokinin levels delay leaf

senescence, promote chlorophyll biosynthesis, and maintain photosynthetic efficiency for prolonged periods—known as the “stay-green” effect (6,18). PBZ-induced cytokinin accumulation has also been associated with enhanced chloroplast development and delayed onset of senescence in various plant species, including *Camelina sativa* and grapevine (16,17).

PBZ’s protective role under stress conditions also involves the modulation of antioxidant defense systems (20). PBZ has been widely reported to enhance the activity of key enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX), which collectively mitigate the harmful effects of reactive oxygen species (ROS) under stress conditions (21–23). These enzymatic responses lead to reduced lipid peroxidation, enhanced membrane stability, and greater oxidative stress tolerance (6,24).

In addition to enzymatic antioxidants, PBZ also elevates the levels of non-enzymatic antioxidants such as ascorbic acid, reduced glutathione (GSH), and α-tocopherol (25–28). These compounds are vital for scavenging free radicals and preserving cellular redox homeostasis, especially in chloroplasts and cytosol under abiotic stress (29,30).

Collectively, these multifaceted physiological and biochemical responses underscore PBZ’s potential as a powerful agronomic tool. Its ability to modulate phytohormonal profiles, delay senescence, and activate antioxidative defenses makes it particularly valuable for enhancing plant performance and productivity under both optimal and stress-prone conditions.

Therefore, this study aimed to evaluate the effects of different PBZ concentrations on growth traits, antioxidant defense responses (enzymatic and non-enzymatic), oxidative stress markers (H_2O_2 and MDA), phenolic metabolism (including PAL, TAL, total phenolics, and total flavonoids), and phytohormone profiles in *T. daenensis* under in vitro conditions. Our findings are expected to provide new insights into the regulatory mechanisms linking growth modulation, antioxidant defenses, and phytohormonal balance with secondary metabolite accumulation in response to PBZ, thereby contributing to a broader understanding of stress adaptation and metabolic regulation in medicinal plants.

Material and methods

Plant Material and *in vitro* Culture Conditions

Seeds of *T. daenensis* were obtained from Isfahan Agricultural and Natural Resources Research and Educational Center, Isfahan, Iran. To eliminate surface contaminants, seeds were sterilized by immersion in 5% (v/v) sodium hypochlorite solution for 10 minutes, followed by five thorough rinses with sterile distilled water under aseptic conditions. The sterilized seeds were inoculated onto Murashige and Skoog (MS) basal medium supplemented with 30 g/L sucrose and solidified with 2 g/L Phytagel. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for

20 minutes. Cultures were maintained in a growth chamber at 25 ± 2 °C with a 16/8 h (light/dark) photoperiod and a light intensity of approximately 40 µmol m^{-2} s^{-1}.

After 21 days of culture, uniform shoots were excised and transferred to fresh MS medium containing different concentrations of PBZ (0, 25, 50, and 100 mg/L) to evaluate its effects on *in vitro* growth and biochemical responses. Following 14 days of treatment, whole plantlets were harvested, immediately frozen in liquid nitrogen, and stored at –80 °C for subsequent physiological, biochemical, and molecular analyses.

Plant Growth Parameters and Photosynthetic Pigments

Fresh weights of root and shoot samples were recorded immediately after harvest. For dry weight determination, samples were dried in a shaded, well-ventilated area until their weight remained constant over three consecutive days, indicating complete drying. Total chlorophyll (a and b) and carotenoid contents in fresh shoot tissue were quantified following the method described by Arnon (1949) (31). Approximately 0.1 g of fresh tissue was homogenized in 80% acetone using a mortar and pestle. The extract's absorbance was measured at 480, 510, 645, and 663 nm using a spectrophotometer. Photosynthetic pigment concentrations were calculated and expressed as milligrams per gram of fresh weight (mg/ g FW).

Anthocyanins

Total anthocyanin content was quantified following the method described by Wagner (1979) (32) using acidified methanol (methanol:HCl, 99:1 v/v) as the extraction solvent. Approximately 0.1 g of fresh shoot tissue was homogenized in 10 mL of acidified methanol and incubated in the dark at 25 ± 1 °C for 24 hours. The extract was then centrifuged at 4000 rpm for 10 minutes at room temperature, and the absorbance of the supernatant was measured at 550 nm. Anthocyanin concentration was calculated using an extinction coefficient of 33,000 L/ mol/ cm.

DPPH Radical Scavenging Activity

The free radical scavenging activity was assessed using the method described by Abe et al. (1998) (33). Briefly, 0.1 g of fresh shoot tissue was homogenized in 1 mL of 96% ethanol, and the mixture was centrifuged at 3500 rpm for 5 minutes to remove insoluble debris. Then, 20 µL of the clear supernatant was mixed with 800 µL of 0.5 mM DPPH solution prepared in ethanol. The reaction mixtures were incubated in the dark at room temperature for 30 minutes, after which the absorbance was measured at 517 nm using a spectrophotometer. The percentage of DPPH radical inhibition was calculated using the formula:

Phenylalanine Ammonia-Lyase (PAL) and Tyrosine Ammonia-Lyase (TAL) Activity

Phenylalanine ammonia-lyase (PAL) activity was determined following the protocol described by Berner et al. (2006). Briefly, 20 µL of enzyme extract was incubated with 500 µL of boric acid buffer (pH 8.0). The reaction mixture was incubated for 30 minutes, and the absorbance was measured at 290 nm. Enzyme activity was quantified using a standard curve generated with varying concentrations of E-cinnamic acid (34).

For tyrosine ammonia-lyase (TAL) activity, the assay mixture consisted of 20 µL of enzyme extract, 500 µL of boric acid buffer (pH 8.5), and 30 mM tyrosine as substrate. After 30 minutes of incubation, absorbance was recorded at 310 nm. TAL activity was calculated based on a standard curve prepared using p-coumaric acid (34).

Stress Markers Assays

Hydrogen peroxide (H_2O_2) content was quantified following the method described by Velikova et al. (35). Briefly, 0.2 g of fresh shoot tissue was homogenized in 2.5 mL of ice-cold 0.1% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 10,000 rpm for 15 minutes at 4 °C. To 0.5 mL of the resulting supernatant, 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of freshly prepared 1 M potassium iodide (KI) were added. The reaction mixture was incubated in the dark, and absorbance was measured at 390 nm using a UV-visible spectrophotometer. The H_2O_2 concentration was calculated using a standard curve generated with known concentrations of hydrogen peroxide.

Malondialdehyde (MDA) content, as an indicator of lipid peroxidation, was determined according to the thiobarbituric acid (TBA) reaction described by Heath and Packer (36). In this assay, 0.5 g of shoot tissue was homogenized in 0.1% TCA and centrifuged at 13,000 rpm for 10 minutes at 4 °C. An equal volume (1 mL) of the supernatant was mixed with 1 mL of 0.5% (w/v) TBA prepared in 20% TCA. The mixture was incubated in a water bath at 95 °C for 30 minutes, then rapidly cooled on ice to terminate the reaction. After centrifugation at 13,000 rpm for 15 minutes, the absorbance of the supernatant was measured at 532 and 600 nm. The MDA concentration was calculated by subtracting nonspecific absorbance at 600 nm from the absorbance at 532 nm.

Antioxidant Enzyme Assays

For enzymatic antioxidant analyses, 0.5 g of fresh shoot tissue was homogenized in ice-cold 1 M Tris–HCl buffer (pH 6.8) at 4 °C. The homogenate was centrifuged at 13,000 rpm for 20 minutes at 4 °C, and the resulting supernatant was collected and stored at −70 °C until further use for enzyme activity assays. Total soluble protein content was quantified using the Bradford method (37), with bovine serum albumin (BSA) employed as the standard protein.

SOD (EC 1.15.1.1) activity was assessed following the method described by Giannopolitis and Ries (38). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 75 μM riboflavin, and 100 μL of enzyme extract. After exposing the mixture to fluorescent light for 18 minutes, absorbance was recorded at 560 nm to estimate SOD activity based on NBT photoreduction inhibition.

CAT (EC 1.11.1.6) activity was determined according to the method of Chaoui et al. (39). The assay mixture included 250 μL of 100 mM potassium phosphate buffer (pH 7.0), 250 μL of freshly prepared 70 mM hydrogen peroxide (H_2O_2), 500 μL distilled water, and 10 μL of enzyme extract. The decomposition of H_2O_2 was monitored spectrophotometrically at 240 nm by measuring the decrease in absorbance.

POD (EC 1.11.1.7) activity was evaluated as described by Abeles and Biles (40). The reaction mixture consisted of 50 μL enzyme extract, 100 μL of 40 mM benzidine solution, 200 μL of 3% hydrogen peroxide, and 2 mL of 0.2 M acetate buffer (pH 4.8). The change in absorbance was measured at 530 nm.

PPO (EC 1.14.18.1) activity was assayed following the protocol by Raymond et al. (41), using a reaction mixture containing 2.5 mL of 200 mM potassium phosphate buffer (pH 6.8), 200 μL of 20 mM pyrogallol, and 20 μL of enzyme extract. The formation of oxidized products was monitored at 430 nm.

Phytohormone Analysis

For phytohormone extraction, fresh shoot tissues were homogenized in 3 mL of absolute methanol on ice under dark conditions. The homogenates were sonicated for 1 hour to enhance extraction efficiency, followed by overnight concentration via evaporation. The concentrated samples were then filtered through a sterile 0.22 μm syringe filter. Aliquots of 20 μL filtrate were injected into a high-performance liquid chromatography (HPLC) system for analysis.

Phytohormones were separated using a gradient elution at a flow rate of 1 mL/min with solvents consisting of methanol (solvent B) and acidic water (0.001% acetic acid in deionized water, solvent A). The gradient program was as follows: 0–0.6 min, 60% B; 0.6–10 min, 45% B; 10–20 min, 20% B; 20–25 min, 60% B. IAA, ABA, GA, BS and MJ were detected at 220 nm and quantified by comparing their retention times and peak areas with authentic standards (42).

Results

Growth parameters

The fresh weight of shoots decreased progressively with increasing concentrations of PBZ, reaching the lowest value at 10 mg/L, which was approximately 46% lower than the control. In

contrast, the fresh weight of roots increased significantly at 2.5 and 10 mg/L by 66% and 330%, respectively, compared to the untreated plants (Table1).

Table1 Effects of PBZ concentrations on growth parameters of *T. daenensis* under *in vitro* conditions.

PBZ (mg/ L)	Shoot FW (gr)	Root FW (gr)	Shoot length (cm)	Root length (cm)	Shoot / Root FW
0	0.50± 0.04[a]	0.069± 0.001[b]	5.8± 0.6[a]	2.1± 0.1[b]	0.5± 0.04[c]
2.5	0.47± 0.03[b]	0.115± 0.02[d]	5.8± 0.4[a]	1.1± 0.1[c]	4.1± 0.6[b]
5	0.46± 0.03[b]	0.054± 0.005[c]	3.0± 0.2[c]	0.9± 0.08[a]	8.4± 0.7[a]
10	0.27± 0.01[c]	0.300± 0.01[a]	4.7± 0.5[b]	1.0± 0.2[c]	0.9± 0.8[c]

Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

Shoot length remained unchanged at 2.5 mg/L but showed a significant reduction at higher concentrations (5 and 10 mg/L). Root length significantly increased at 2.5 mg/L PBZ but declined at 5 and 10 mg/L, with the greatest reduction observed at 10 mg/L (Table1).

The shoot-to-root fresh weight ratio increased at 2.5 and 5 mg/L, reaching a maximum at 5 mg/L. However, this ratio declined at 10 mg/L, indicating a shift in biomass allocation under higher PBZ levels (Table1).

Photosynthetic pigments

The contents of chlorophyll a, chlorophyll b, and carotenoids significantly increased at all tested concentrations of PBZ (2.5, 5, and 10 mg/L) compared to the control plants. The maximum levels of all pigments were observed at 5 mg/L, indicating an optimal response at this concentration. However, a slight decline was noted at 10 mg/L, though pigment levels remained significantly higher than the control (Table2).

Table2 Effects of PBZ concentrations on photosynthetic pigments of *T. daenensis* under *in vitro* conditions.

PBZ (mg/L)	chl a (mg/g FW)	Chl b (mg/g FW)	Total chl (mg/g FW)	chl a/b (mg/g FW)	carotenoids (mg/g FW)
0	0.80± 0.10^{c}	0.90± 0.08^{b}	1.71± 0.96^{d}	1.12± 0.25^{b}	0.29± 0.03^{d}
2.5	1.04± 0.12^{b}	0.98± 0.08^{a}	2.83± 0.61^{b}	1.70± 0.61^{a}	0.72± 0.02^{b}
5	2.35± 0.17^{a}	2.00± 0.15^{a}	4.36± 0.44^{a}	0.85± 0.04^{c}	0.90± 0.01^{a}
10	1.03± 0.10^{b}	0.96± 0.09^{b}	2.00± 0.14^{c}	0.93± 0.12^{c}	0.43± 0.04^{c}

Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

Phytohormones content

A concentration-dependent decline was observed in the levels of GA, IAA, and BR with increasing PBZ application, with the lowest values recorded at 10 mg/L. Conversely, ABA content showed a consistent upward trend, reaching its maximum at the highest PBZ concentration (Table3).

Table3 Effects of PBZ concentrations on phytohormones content of *T. daenensis* under *in vitro* conditions.

PBZ (mg/L)	GA (ng/g FW)	ABA (ng/g FW)	IAA (ng/g FW)	BR (ng/g FW)
0	281.09± 42.11^{a}	31.15± 2.45^{d}	5375.28± 37054^{a}	356.59± 77.99^{a}
2.5	112.14± 15.01^{b}	75.20± 6.25^{c}	4440.21± 430.00^{b}	253.66± 16.95^{b}
5	45.32± 3.25^{c}	85.87± 7.25 ^{b}	2019.87± 115.0^{c}	34.45± 25.82^{c}
10	13.73± 1.03^{d}	151.88± 13.87^{a}	386.37± 32.59^{d}	5.42± 15.71^{d}

Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

Anthocyanines content

Anthocyanins content was significantly influenced by PBZ application. The maximum accumulation was observed at 5 mg/L, showing an approximately 50% increase compared to the control. This indicates a stimulatory effect of moderate PBZ concentration on anthocyanins

biosynthesis. In contrast, anthocyanins content markedly declined at 10 mg/L, reaching the lowest value among all treatments, suggesting that higher concentrations may suppress pigment accumulation (Fig1).

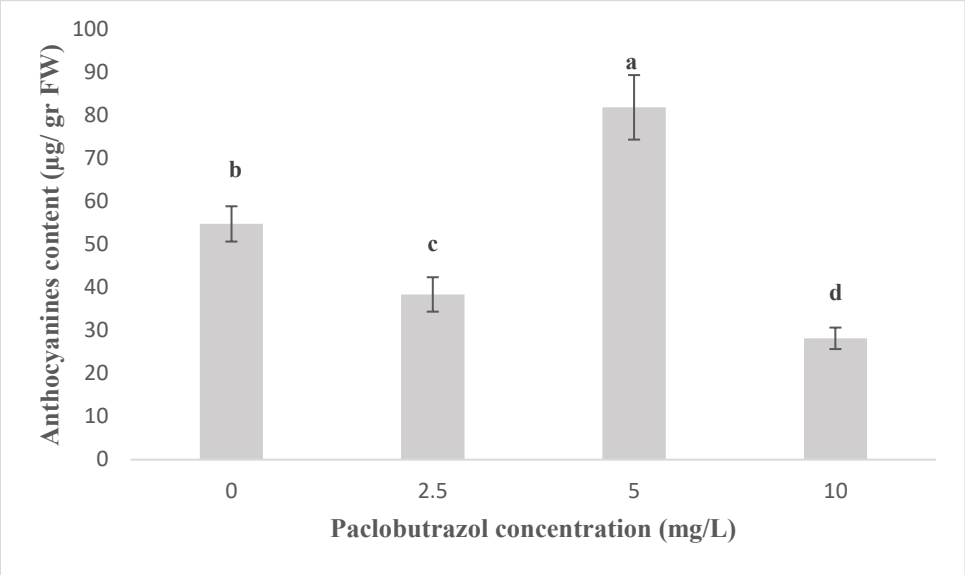

Fig1 Effects of PBZ concentrations on anthocyanines content of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

Total Phenoles content

Total phenoles content exhibited a remarkable increase (up to 27%) in response to 5 mg/ L PBZ. However, a sharp reduction (by 15%) was observed at 10 mg/ L, whereas the 2.5 mg/ L treatment did not result in any significant change (Fig2).

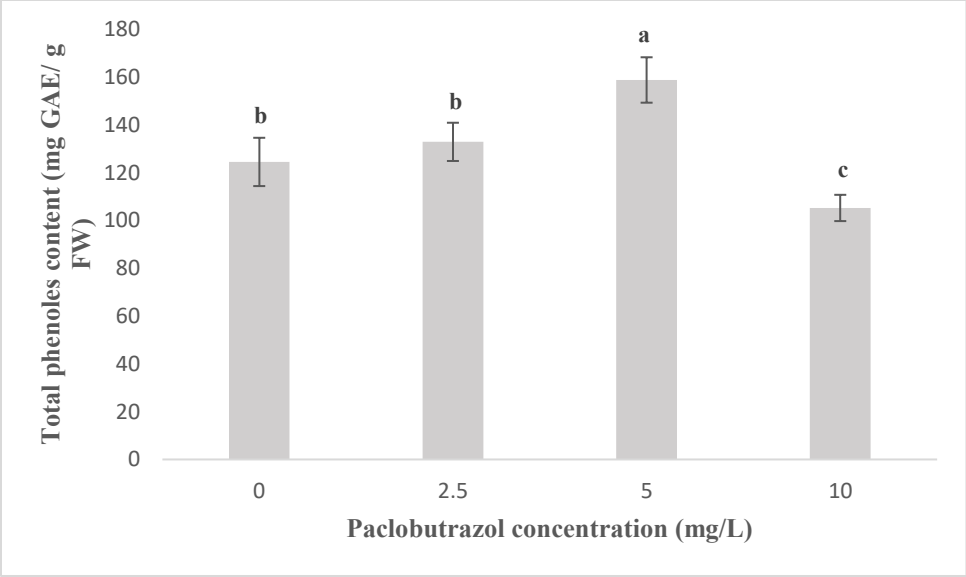

Fig2 Effects of PBZ concentrations on phenols content content of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

Flavonoids content

Total flavonoid content increased at 2.5 and 5 mg L^{-1} PBZ, reaching its maximum at 5 mg/L, but declined at 10 mg/L (Fig3).

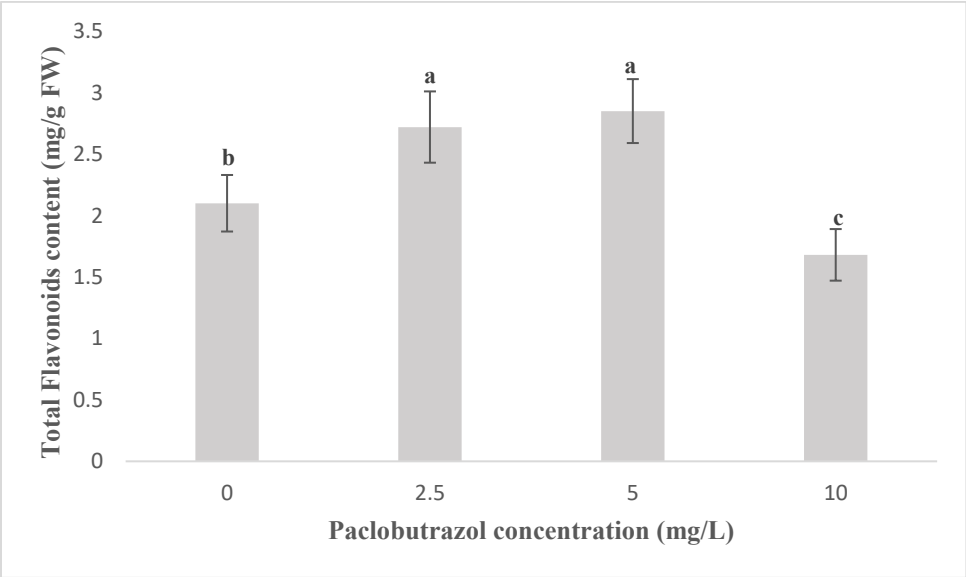

Fig3 Effects of PBZ concentrations on flavonoids content content of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

DPPH radical scavenging activity

DPPH radical scavenging activity significantly increased in response to 2.5 and 5 mg/L PBZ treatments, with enhancements of 29% and 64.25%, respectively. However, a notable reduction of 19.8% was observed at the highest concentration (10 mg/L), indicating a potential inhibitory effect at elevated doses (Fig4).

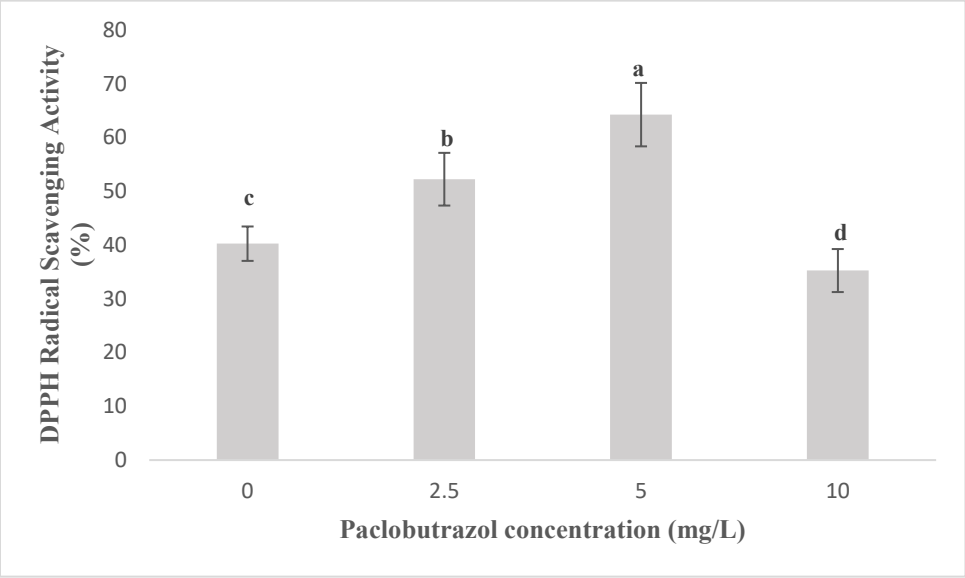

Fig4 Effects of PBZ concentrations DPPH radical scavenging activity of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

PAL and TAL activity

PAL enzyme activity exhibited a considerable increase under 2.5 and 5 mg/L PBZ treatments, with respective enhancements of 55.5% and 63.8% compared to the control. In contrast, a 30.5% decrease was recorded at the highest concentration (10 mg/L), suggesting a dose-dependent biphasic response (Fig5).

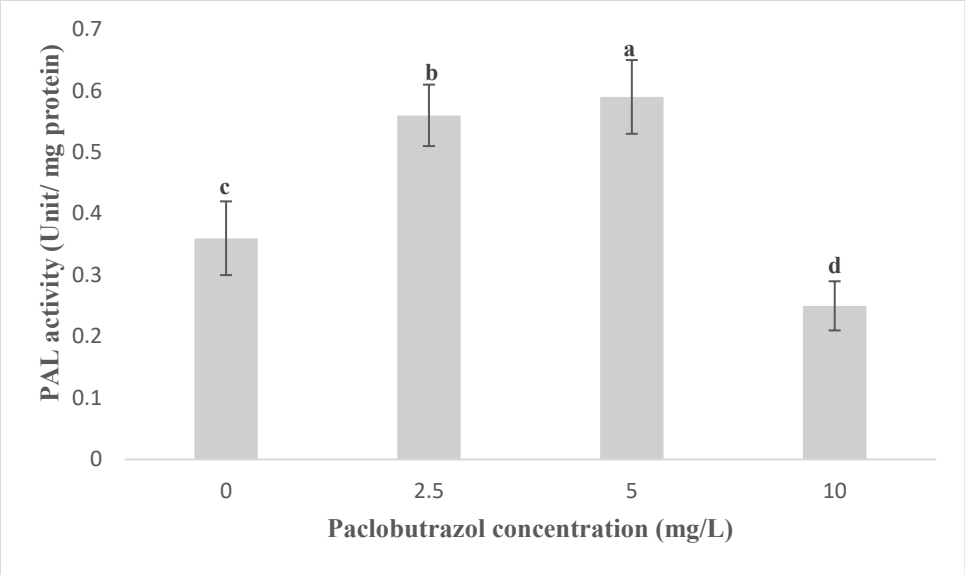

Fig5 Effects of PBZ concentrations on PAL activity of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

TAL enzyme activity increased in all PBZ treatments compared to the control. The highest activity was observed at 2.5 mg/L, while a declining trend was evident at 5 and 10 mg/L concentrations, indicating a possible dose-dependent modulation (Fig6).

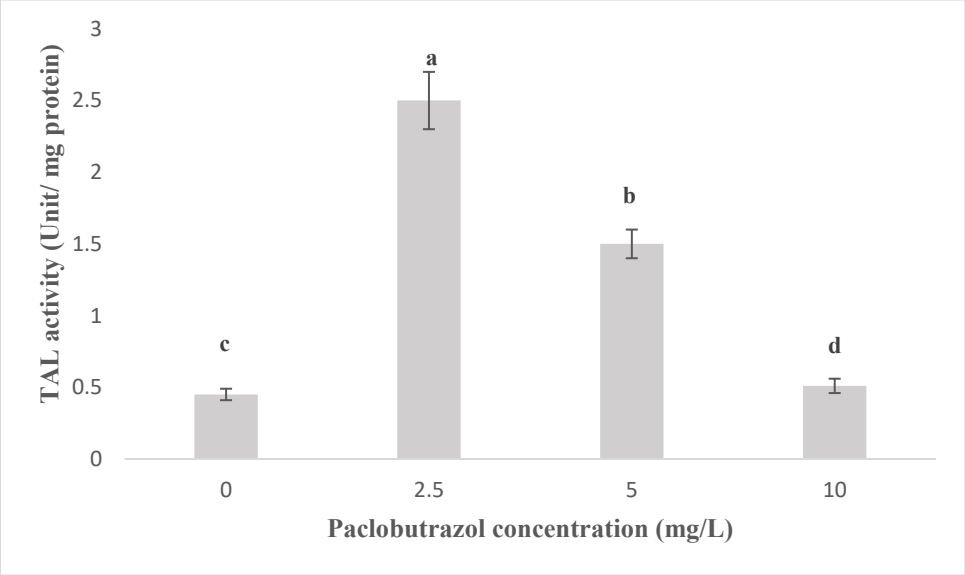

Fig6 Effects of PBZ concentrations on TAL activity of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

Stress markers

H_2O_2 content showed a reduction of 11% and 15% under 2.5 and 5 mg/L PBZ treatments, respectively. However, a 19.8% increase was recorded at 10 mg/L (Fig7). MDA levels remained statistically unchanged at 2.5 and 5 mg/L, while a substantial elevation (62.5%) was observed at 10 mg/L compared to the control, indicating enhanced lipid peroxidation at higher PBZ concentration (Fig8).

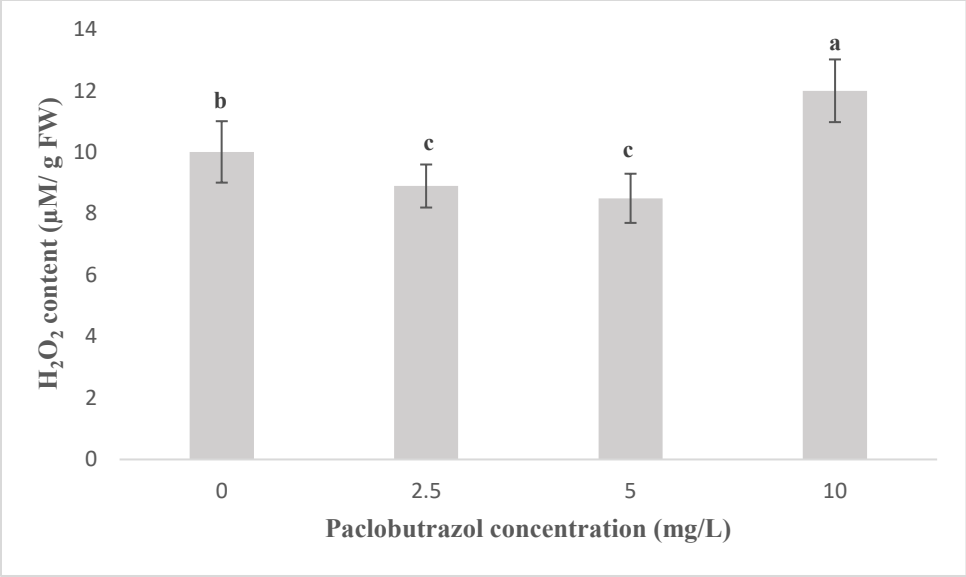

Fig7 Effects of PBZ concentrations on H_2O_2 content of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

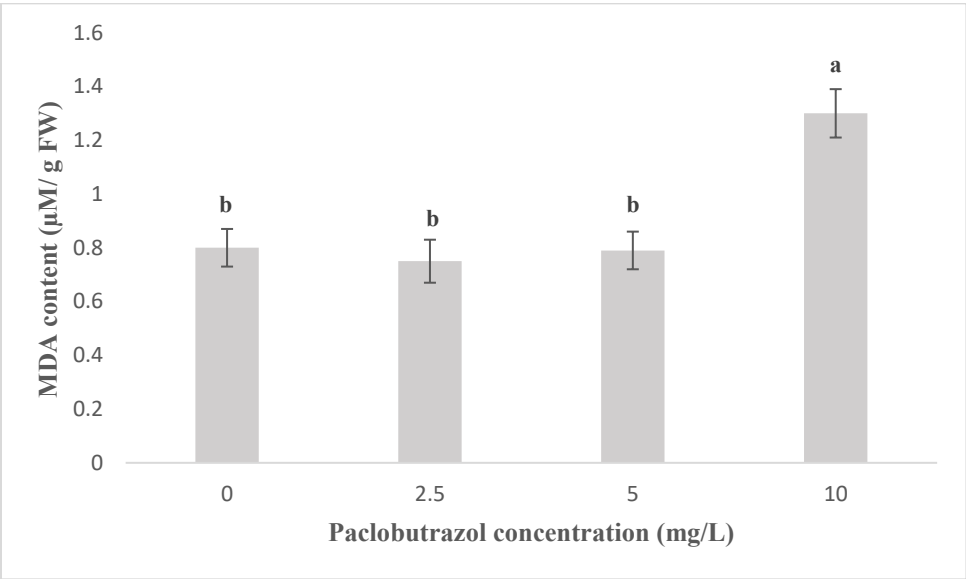

Fig8 Effects of PBZ concentrations on MDA content of *T. daenensis* under *in vitro* conditions. Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

Enzymatic antioxidants activity

CAT enzyme activity did not change significantly at 2.5 mg/L PBZ but showed a marked increase at 5 and 10 mg/L, with the highest activity observed at 10 mg/L. POD activity was also enhanced by PBZ treatment, reaching its maximum level at 5 and 10 mg/L. SOD activity increased across all treatments, peaking at 5 mg/L; however, a slight decline was noted at 10 mg/L compared to lower concentrations. PPO activity exhibited a consistent increase under all PBZ concentrations, with the highest value recorded at 5 mg/L (Table4).

Table4 Effects of PBZ concentrations on antioxidant enzymes activity of *T. daenensis* under *in vitro* conditions.

PBZ (mg/L)	CAT (U/mg protein)	POD (U/mg protein)	SOD (U/mg protein)	PPO (U/mg protein)
0	0.008± 0.001^{c}	0.006± 0.001^{c}	0.02± 0.96^{d}	0.10± 0.25^{d}
2.5	0.009± 0.002^{c}	0.020± 0.004^{b}	0.05± 0.61^{b}	0.45± 0.61^{c}
5	0.045± 0.007^{b}	0.07± 0.006^{a}	0.07± 0.44^{a}	0.85± 0.04^{a}
10	0.081± 0.008^{a}	0.07± 0.005^{a}	0.04± 0.14^{c}	0.62± 0.12^{b}

Means ±SE of three replicates are given. The same letter denotes lack of a statistically significant difference ($P \leq 0.05$) and determines using DMRT.

The data represent the means of three replicates ± SE.
Different letters indicate statistically significant differences among treatments based on Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

Discussion

PBZ, a triazole-derived growth regulator, has been widely documented for its ability to modulate plant growth and development through interference with gibberellin biosynthesis and modulation of hormonal crosstalk, particularly involving ABA, cytokinins, and auxins (6,43). Beyond its role in growth retardation, PBZ has emerged as a potent elicitor of stress-responsive metabolic pathways, including those associated with antioxidant defense and secondary metabolite production (27). The present study provides compelling evidence that PBZ, when applied under *in vitro* conditions, elicits a concentration-dependent modulation of the antioxidative system and phenylpropanoid metabolism in *T. daenensis*, an endemic medicinal species valued for its bioactive constituents.

A key finding of this study is the biphasic response of antioxidant and phenolic metabolism to increasing PBZ concentrations. Low to moderate doses (2.5 and 5 mg/L) were effective in enhancing both enzymatic and non-enzymatic components of the antioxidant machinery, while the highest concentration (10 mg/L) generally exerted suppressive or even deleterious effects. Such biphasic or hormonal responses are not uncommon in plant systems exposed to exogenous elicitors, where in a certain threshold of exposure triggers beneficial stress signaling, but beyond which metabolic homeostasis may be disrupted (44).

The enhancement of DPPH radical scavenging capacity (Fig4) at 2.5 and 5 mg/L PBZ aligns with the concurrent increase in total flavonoids (Fig3) content, suggesting an upregulation of secondary metabolite biosynthesis with antioxidant potential. These compounds, particularly flavonoids, not only function as direct ROS scavengers but also play crucial roles in modulating cellular redox status and signaling under abiotic stress (45). The subsequent reduction of both parameters at 10 mg/L may reflect feedback inhibition of flavonoid biosynthesis pathways or oxidative damage impairing metabolic flux through the phenylpropanoid pathway.

The enzymatic antioxidant system responded differentially to PBZ concentrations (Table4). SOD activity, which represents the primary line of defense against superoxide radicals, increased significantly under all treatments but peaked at 5 mg/L, suggesting optimal induction of ROS-detoxifying mechanisms at this concentration. A slight decline at 10 mg/L may be attributed to either substrate limitation or oxidative inactivation of the enzyme under elevated ROS levels. Similarly, CAT and POD—key enzymes involved in the decomposition of hydrogen peroxide—showed progressive activation, particularly at 10 mg/L, indicating a potential compensatory response to excess H_2O_2 accumulation (Table4). These patterns are consistent with prior studies reporting PBZ-induced enhancement of antioxidative enzymes in diverse plant species under both *in vitro* and *in vivo* conditions (46–49)

Notably, PPO activity also exhibited a consistent upward trend, peaking at 5 mg/L (Table4). PPO catalyzes the oxidation of phenolics to quinones (50), which can contribute to strengthening of cell walls and enhanced resistance to oxidative or pathogen-induced damage. Its upregulation under PBZ treatments likely reflects an activation of defense-related phenolic metabolism, possibly linked to systemic acquired resistance-like responses (51).

In parallel, PBZ modulated the activity of PAL and TAL, the two key gateway enzymes of the phenylpropanoid pathway (Fig5 and 6). PAL activity was significantly enhanced at 2.5 and 5 mg/L (Fig5), supporting the observed increase in flavonoids content and antioxidant capacity. However, its activity was notably suppressed at 10 mg/L, possibly due to feedback regulation or cellular damage affecting metabolic integrity. TAL activity, on the other hand, increased across all treatments but exhibited a declining trend beyond 2.5 mg/L (Fig6). The decoupling of TAL and PAL activities at higher PBZ concentrations may suggest differential regulation of phenylpropanoid branch pathways under stress-like conditions.

Biochemical indicators of oxidative damage, including H_2O_2 and MDA levels, further reinforced the interpretation of a dose-dependent stress response. At lower concentrations of PBZ, H_2O_2 content was reduced (Fig7) and MDA remained unchanged (Fig8), indicating that ROS production was efficiently managed by the upregulated antioxidant system. However, at 10 mg/L, both H_2O_2 and MDA levels increased markedly (Fig7 and 8), reflecting a state of oxidative stress and lipid peroxidation. These findings are in line with the oxidative burst and membrane destabilization frequently reported at high doses of PBZ or other triazoles (49).

In addition to modulating redox homeostasis and phenylpropanoid metabolism, PBZ treatment induced marked alterations in the endogenous phytohormonal profile of *T. daenensis* (Table3). A clear concentration-dependent decline was observed in the levels of growth-promoting hormones, including GA, IAA, and BRs, with all reaching their minimum concentrations at 10 mg/L PBZ (Table3). This pattern is in line with PBZ's established mechanism of action, which involves inhibition of ent-kaurene oxidase in the gibberellin biosynthetic pathway (52), as well as interference with auxin transport and BR signaling (53). The suppression of these hormones is thought to contribute to growth retardation and metabolic reallocation toward defense pathways, a shift that is commonly observed in plants undergoing abiotic stress or elicitor exposure.

In contrast, ABA levels increased progressively with rising PBZ concentrations, peaking at the highest treatment level (Table3). ABA is a central regulator of abiotic stress responses and is well known to orchestrate stomatal closure, antioxidant gene expression, and osmoprotectant accumulation under drought, salinity, and oxidative stress conditions (54). The elevated ABA levels observed here may reflect an adaptive hormonal reprogramming that enhances stress tolerance by priming both antioxidant systems and stress-related metabolic networks. The reciprocal regulation of ABA versus GA, IAA, and BRs suggests that PBZ exerts a tightly coordinated shift in hormonal homeostasis, favoring stress acclimation over growth under in vitro conditions.

These findings underscore the multifaceted role of PBZ in orchestrating a stress-adaptive hormonal milieu in *T. daenensis*, which likely interacts synergistically with the observed biochemical and physiological changes. Further integration of hormonal signaling with transcriptomic and metabolic profiling would be valuable in elucidating the upstream regulatory networks governing PBZ-induced stress resilience in medicinal plants.

Taken together, the data underscore the dualistic nature of PBZ as both a growth regulator and stress modulator, with beneficial effects at optimal concentrations and adverse impacts at supra-optimal levels. The findings presented here highlight a tightly regulated interaction between PBZ and redox metabolism in *T. daenensis*, with implications for its use in micropropagation systems and metabolic enhancement strategies. The stimulation of both enzymatic and phenolic defense systems suggests that PBZ could serve as a useful tool for priming antioxidant capacity and improving the phytochemical quality of medicinal plants under controlled environments.

Future work incorporating hormonal profiling, transcriptomic analysis of antioxidant- and phenylpropanoid-related genes, and metabolomic characterization of phenolic derivatives will be essential to further unravel the mechanistic basis of PBZ-mediated responses in *T. daenensis* and related species.

Conclusion

This study demonstrates that PBZ exerts concentration-dependent effects on the antioxidant system, phenylpropanoid metabolism, and hormonal homeostasis of *Thymus daenensis* under in vitro conditions. Moderate concentrations of PBZ (2.5 and 5 mg/L) effectively enhanced enzymatic (SOD, CAT, POD, PPO) and non-enzymatic (flavonoids, DPPH) antioxidant responses, alongside elevated activities of key phenolic biosynthetic enzymes (PAL and TAL). These changes collectively contributed to reduced oxidative damage, as reflected by decreased H_2O_2 and MDA levels. In contrast, exposure to a high PBZ concentration (10 mg/L) resulted in the accumulation of oxidative stress markers and downregulation of several defense-related parameters, indicating a threshold beyond which PBZ exerts phytotoxic effects.

In parallel, PBZ treatment induced significant hormonal reprogramming. Growth-promoting hormones including GA, IAA, and BRs were progressively reduced with increasing PBZ concentrations, while ABA levels increased, peaking at 10 mg/L. This reciprocal hormonal shift likely contributed to the observed enhancement in stress tolerance mechanisms under moderate PBZ levels, and to growth suppression and stress exaggeration at supra-optimal concentrations.

These findings underscore the potential application of PBZ as a biochemical modulator to enhance antioxidant capacity, secondary metabolite biosynthesis, and stress resilience in medicinal plants, provided its concentration is carefully optimized. Further investigations integrating hormone profiling, gene expression analysis, and comprehensive metabolomics are warranted to elucidate the underlying molecular mechanisms and to validate these effects *in vivo* and field conditions.

Abbreviations

ABA, Abscisic acid; APX, Ascorbate peroxidase; BR, Brassinosteroids; CAT, Catalase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GA, Gibberellic acid; H_2O_2, Hydrogen peroxide; IAA, Indole-3-acetic acid; KI, Potassium iodide; MDA, Malondialdehyde; MJ, Methyl jasmonate; PAL, Phenylalanine ammonia-lyase; PBZ, Paclobutrazol; POD, Peroxidase; PPO, Polyphenol oxidase; ROS, Reactive oxygen species; SOD, Superoxide dismutase; TAL, Tyrosine ammonia-lyase; TBA, Thiobarbituric acid; TCA, Trichloroacetic acid.

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Author Contributions

Ensiyeh Shahroudi was responsible for designing and conducting the experiments, collecting and analyzing the data, and preparing the manuscript. Dr. Fatemeh Zarinkamar supervised the physiological experiments carried out in her laboratory.

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Declarations

Ethics approval and consent to participate

This study did not involve any experiments on human subjects or animals. *Thymus daenensis* seeds were procured from the Isfahan Agricultural and Natural Resources Research and Educational Center, Isfahan, Iran. All plant cultivation and experimental procedures were conducted in strict compliance with institutional, national, and international guidelines and regulations. Consequently, formal ethical approval and consent to participate were not applicable or required for this study.

Clinical trial number:

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Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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