Impact of Putrescine on essential oil composition, free polyamines content and expression of related genes (DXR and TPS2) in Thymus daenensis under drought stress

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Abstract

Background Thymus daenensis

a medicinal plant native to Iran, produces essential oils rich in thymol and carvacrol, known for their strong antioxidant and antimicrobial properties. Exposure to drought conditions affects the production pathways of secondary metabolites, potentially contributing to improved stress tolerance in plants. Polyamines (PAs), particularly putrescine (Put), play a crucial role in mitigating drought stress by regulating stomatal behavior, improving osmotic adjustment, and modulating oxidative stress responses. This study aimed to investigate the effects of exogenous Put on *T. daenensis* under drought stress, focusing on gene expression related to terpenoid biosynthesis, secondary metabolite accumulation, and free PA content.

Results

Six-week-old *T. daenensis* plants were subjected to drought stress induced by 15% (w/v) polyethylene glycol (PEG) 6000, with or without foliar application of Put. Gene expression analysis (RT-qPCR), HPLC-based PA quantification, and GC-MS profiling of essential oil composition were performed. Put treatment significantly increased endogenous Put and spermidine content in drought-stressed plants, whereas no significant changes were observed in non-stressed plants. Furthermore, Put application upregulated *TPS2* but downregulated *DXR* expression in drought-stressed plants. Additionally, the relative contents of *γ*-terpinene and *p*-cymene—precursors of thymol and carvacrol—increased, while the contents of thymol and carvacrol decreased following Put treatment.

Conclusions

Exogenous application of putrescine modulates the expression of key genes in the terpenoid biosynthetic pathway and alters essential oil composition in *T. daenensis* under drought stress, potentially contributing to the plant's adaptive responses.

Background

T. daenensis is a species of thyme native to Iran (1). It's known for its medicinal properties and is widely used in traditional medicine (2). The essential oil of *T. daenensis* is rich in compounds like thymol, carvacrol, *p*-cymene, and *β*-caryophyllene, which have antioxidant, antibacterial, and antifungal properties (1). Thymol and carvacrol exhibit strong antioxidant properties, helping to neutralize reactive oxygen species (ROS) generated during stresses. These compounds reduce oxidative damage to cellular components by stabilizing cell membranes, protecting them from damage in relation to drought-induced dehydration (3). Thymol and carvacrol can influence stomatal behavior, promoting stomatal closure to reduce water loss and improve water-use efficiency (3). Drought stress can lead to increase in the production of some secondary metabolites. This enhanced production helps the plant cope with stress and defend against pathogens (4). Thymol and carvacrol are phenolic monoterpenes, and their

production is primarily driven by the methyl erythritol phosphate (MEP) pathway (5). The pathway begins with the generation of dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IPP), which are key building blocks for terpenes biosynthesis (5). DMADP and IPP are converted into 1-deoxy-D-xylulose-5-phosphate (DXP) through a series of enzymatic reactions. DXP is then transformed into 2C-methyl-D-erythritol-4-phosphate (MEP) via 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*DXR*) (5). MEP is further processed to produce isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMADP), which are used to synthesize monoterpenes (5). IPP and DMADP are converted into geranyl diphosphate (GPP), which is then cyclized and oxidized to form monoterpene compounds such as thymol and carvacrol by Gamma-terpinene synthase (*TPS2*) (6). The enzymes involved in this pathway, such as *DXR* and *TPS2*, play crucial roles in the monoterpene biosynthesis (7).

PAs are small aliphatic nitrogen-containing compounds characterized by the presence of two or more amino groups (8). These molecules are widely distributed across all living organisms, including both eukaryotic and prokaryotic cells (9). The most prevalent forms of PAs include the diamine Put, the triamine spermidine (Spd), and the tetraamine spermine (Spm) (10, 11). Put is considered the central product in the biosynthetic pathway of PAs, serving as the primary precursor for the synthesis of both Spd and Spm (12).

Several studies have indicated that the application of exogenous PAs can improve plant resistance to drought stress (13, 14). These compounds have been found to stimulate the production of secondary metabolites, including alkaloids, phenolic compounds, and terpenoids, which are essential for plant defense and overall health (15). Additionally, it has been shown that optimal foliar application of Put can activate key physiological processes, promoting the synthesis of osmotic regulators such as free amino acids, soluble sugars, and proline. This mechanism may help mitigate the negative impacts of drought on plant biomass, thus improving both the quality and quantity of specific bioactive compounds (13, 16).

PAs play a dual role in regulating oxidative stress in plants (17). On one hand, they enhance the activity of various antioxidant enzymes, thereby helping to mitigate oxidative stress induced by environmental factors (18). On the other hand, PAs can serve as a source of reactive oxygen species (ROS), as their catabolism generates potent oxidants such as hydrogen peroxide (H_2O_2) and acrolein, which may contribute to cellular damage under stress conditions. However, H_2O_2 also functions as a signaling molecule, initiating the stress signal transduction cascade and activating antioxidant defense mechanisms. Consequently, PAs act as key regulators of redox homeostasis, balancing both protective and potentially harmful effects in response to oxidative stress.

We hypothesized that the application of Put alleviates drought stress in *T. daenensis* by enhancing the biosynthesis and accumulation of some compounds present in the essential oil, regulating the expression of key biosynthetic genes (*DXR* and *TPS2*) and increasing PAs content. This effect is expected to occur through the modulation of stress-responsive metabolic and genetic pathways, leading to improved drought tolerance and enhanced secondary metabolite production in the plant.

In essence, our hypothesis suggests that Put plays a protective and stimulatory role in plants under drought conditions, likely involving the coordinated regulation of secondary metabolite biosynthesis, PA accumulation, and specific gene expression.

Materials and methods

Plant materials and treatments

To conduct this study, *T. daenensis* seeds were sourced from the Isfahan Agricultural and Natural Resources Research and Educational Center in Iran. The seeds were sown in individual plastic pots filled with perlite. The pots were kept in a phytotron chamber under light conditions of 16 hours of light and 8 hours of darkness, with a temperature of 25°C and light intensity of 1200–1400 lux (17–20 µmol photons/m²/s) for six weeks. During this time, each pot was irrigated uniformly with a half-strength Hoagland solution.

After this initial growth period, drought stress was induced using a polyethylene glycol (PEG) solution (15% W/V), applied with and without Put at a concentration of 0.2 mM as described by (13). 100 mL of the PEG-containing half-strength Hoagland solution was added to each pot, while 10 mL of Put solution was uniformly sprayed onto the plants using an atomizer on alternate days.

Each experimental treatment was performed in triplicate to ensure data reliability. At the end of the three-week treatment period, shoots were collected from each condition and a portion of the them were dried, while the remaining ones were stored − 80°C.

Total RNA extraction and cDNA synthesis

Total RNA was isolated utilizing the Riboex reagent according to the protocol provided by the manufacturer. The concentration and purity of the extracted RNA were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and verified by agarose gel electrophoresis. Samples exhibiting an A260/A280 ratio between 1.8 and 2.1 and an A260/A230 ratio above 1.7 were selected for downstream complementary DNA (cDNA) synthesis.

cDNA was synthesized from the purified RNA using reverse transcription kits obtained from Thermo Fisher (USA), adhering strictly to the provided instructions. Quantitative real-time PCR (RT-qPCR) was performed to evaluate the expression of *DXR* and *TPS2* genes, employing SYBR Green PCR Master Mix (2×) (Yekta Tajhiz Azma, Tehran, Iran). The amplification protocol involved an initial denaturation at 95°C for 5 minutes, followed by 40 cycles comprising 10 seconds at 95°C for denaturation, 10 seconds at 60°C for annealing, and 15 seconds at 72°C for extension. Reactions were run on an Applied Biosystems® StepOne™ instrument (Thermo Fisher, USA), and data analysis was conducted using the StepOne Software v2.3. Gene expression levels were normalized to *GAPDH* and analyzed using the 2^–

ΔΔCt method described by Livak and Schmittgen (19). All assays were carried out with two independent biological replicates and two technical replicates per sample.

Primers designing

For primer designing, the sequence of target genes was first examined on the NCBI website (gov.nih.nlm.ncbi.www). The sequence for the *Gamma terpinene synthase* (*TPS2*) gene was found for the *T. daenensis* species. However, no registered sequences for the *GAPDH* and *DXR* genes of this species were available. Therefore, gene sequences from a closely related species, *T. vulgaris*, were used. The accuracy of these sequences was confirmed by comparison with the gene bank, and primers for Real-Time PCR (RT-qPCR) were then designed based on these sequences (Table 1).

Table 1
Specifications of primers used for RT-qPCR (Real time PCR)

Gene	Sequences	Amplicone size (bp)	Accession no.
GAPDH	Forward:5′GTGCTTCCAGCTTTGAACG-3′ Riverse: 5′GTTCTCTGACTCCTCCTTGATG-3′	147	>MF373628.1
DXR	Forward: 5′-GTGCTAGCTCAGTTAGGA TGG-3′ Riverse: 5′-TTAGATCGACGTTGCAGAGG	124	>KY621335.1
TPS2	Forward: 5′-CTTACAAGGCGAGGA AGG ACA-3′ Riverse: 5′-CACAAATGGGAGTTTCTCGG-3′	149	>MH686193.1

Measurement of PA content using HPLC

PAs were extracted following the method of Sharma & Rajam (1995). 0.2 g of samples was homogenized with 1 mL of 2% perchloric acid (PCA) and centrifuged at 4°C for 20 minutes. Then, 150 µL of the supernatant was mixed with 200 µL of saturated sodium carbonate and 500 µL of dansyl chloride solution (5 mg/mL) for dansylation. The mixture was incubated at 60°C in the dark for 1 hour. Afterward, 200 µL of proline solution (0.1 g/mL) was added, and the solution was kept under the same conditions for an additional 30 minutes. The dansylated PAs were extracted by vortexing with 500 µL of toluene, and the upper phase containing the PAs was collected for subsequent analysis. PAs quantification was performed using an ODS18-C5 column. The mobile phase consisted of acetonitrile and acidified water (deionized water with 0.01% acetic acid) applied using a standard gradient system. The column used was an ODS18-C3 type with a length of 250 mm and a diameter of 4.6 mm. PAs absorption was detected using a UV detector at a wavelength of 254 nm. The quantification of PAs was performed based on the chromatogram, using their respective standard curves (21).

GC.MS

20 mg portion of powdered plant dry tissue was placed in 20 mL vials. Static headspace analysis was performed using an A7697 Headspace Sampler (Agilent Technologies). The headspace oven temperature was maintained at 80°C for 30 minutes to allow volatile compounds to evaporate and reach equilibrium between the headspace and the sample in the vial. Subsequently, a specialized syringe was used to extract 1 mL of the headspace, which was then injected into a gas chromatography-mass spectrometry (GC-MS) system for analysis.

An Agilent 7890 gas chromatograph, coupled with an HP-5MS column (30 m length, 0.25 mm internal diameter, and 250 µm film thickness), and an ELCD 5320 detector, was employed for the analysis. The temperature program for the oven was as follows: an initial ramp from 60°C to 210°C at a rate of 3°C/min, followed by a temperature increase to 240°C at 20°C/min, and a subsequent hold at 240°C for 8.5 minutes. The total run time was 60 minutes, with the electron ionization energy set to 70 eV. Helium was used as the carrier gas with a flow rate of 1 mL/min. Data analysis was conducted using ChemStation software.

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) based on a completely randomized design (CRD) with three replications. Duncan's test was applied to determine statistically significant differences between treatment means at a significance level of $p \leq 0.05$.

Results and discussion

Relative Content of Essential Oil Compounds and Expression of DXR and TPS2 Genes:

The impact of drought stress on essential oil content in plants varies depending on the plant species, the intensity and duration of stress, and the growth stage (22). Some studies have shown that drought stress can enhance essential oil content by stimulating the biosynthesis of terpenoids, phenylpropanoids, and other aromatic compounds (22, 23). Conversely, other studies have indicated that drought stress can reduce essential oil content by limiting biomass production and the availability of precursors (24). Thus, the optimal irrigation level for maximizing essential oil content depends on the plant species and environmental conditions (23).

A study on *T. daenensis* demonstrated that Put increased the content of thymol and other secondary metabolites such as carvacrol, *α* and *γ*-terpinene, *p*-cymene, and *β*-caryophyllene (26). Moreover, it increased the content of terpenoids, especially mono- and sesquiterpenes, in tea plants under saline stress (24). Another study found that Put reduced terpenoid content in thyme under drought stress but increased the phenolic compound content (25). These findings suggest that Put regulates the biosynthesis of various secondary metabolites in plants, depending on environmental conditions and genetic factors.

Generally, the mechanism of PAs effects under drought stress is not fully understood but may involve regulation of enzyme gene expression and their activity, transcription factors, and signaling pathways (26). In this study, the thymol content increased by 4.4% in drought-stressed plants compared to controls (Table 2). Similar results were previously observed in garden thyme (26) and oregano (27). Research on *T. daenensis* subsp. *daenensis* showed that drought stress (20% field capacity) increased thymol content by 17.5% (29). Additionally, drought stress (50% field capacity) increased thymol content by 13.6% in the first growth season and 9.8% in the second growth season, compared to controls (30). In addition to drought stress at 40% field capacity, thymol content increased by 10.6% in the first year and 7.8% in the second year in *T. daenensis* (30). These results suggest that drought stress enhances thymol biosynthesis as a defensive mechanism against oxidative stress in *T. daenensis*. However, the optimal level and duration of drought stress depend on plant genotype, climate, and soil conditions (31). The ratio of thymol to other compounds in the essential oil affects the quality of cosmetic, food, and pharmaceutical products derived from this plant (32), Therefore, it can be suggested that the quality of *T. daenensis* essential oil may improve under drought stress.

Table 2
The effect of drought stress and Put on the relative content of compounds in the essential oil of *T. daenensis*.

substance	KI	RT (min)	Relative content of substance (%)				Average
			Control	Put	PEG	Put + PEG	(%)
Thymol	1290	18.5	$41.53^{c} \pm 0.89$	$45.49^{a} \pm 0.57$	$43.37^{b} \pm 0.59$	$36.27^{d} \pm 0.54$	41.66
Carvacrol	1298	18.7	$1.6^{a} \pm 0.04$	$1.36^{b} \pm 0.01$	$1.50^{a} \pm 0.04$	$1.25^{b} \pm 0.04$	1.42
γ-Terpinene	1050	9.2	$9.34^{d} \pm 0.06$	$17.71^{a} \pm 0.11$	$11.67^{c} \pm 0.29$	$15.00^{b} \pm 0.20$	13.43
p-cymene	1011	8.0	$15.60^{a} \pm 0.29$	$15.18^{a} \pm 0.05$	$8.87^{b} \pm 0.17$	$15.87^{a} \pm 0.30$	13.88
α-Thujene	905	5.5	$1.42^{c} \pm 0.19$	$2.50^{c} \pm 0.29$	$5.07^{b} \pm 0.40$	$5.30^{a} \pm 0.28$	3.57
Camphene	943	5.9	$0.57^{a} \pm 0.76$	nd	$0.45^{a} \pm 0.58$	$0.66^{a} \pm 0.78$	0.44
β-Pinene	970	6.9	$0.66^{a} \pm 0.07$	nd	$0.69^{a} \pm 0.08$	$0.69^{a} \pm 0.08$	0.51
β -Myrcene	980	6.9	$0.50^{c} \pm 0.05$	$1.82^{b} \pm 0.08$	$3.49^{a} \pm 0.15$	nd	1.45
α -Terpinen	1010	7.8	$2.50^{a} \pm 0.08$	$1.44^{c} \pm 0.04$	$2.14^{b} \pm 0.03$	nd	1.52
β -Phellandrene	1030	8.4	0.90	nd	nd	nd	0.22
α – Phellandrene	1008	7.6	nd	nd	0.73	0.91	0.41
Endo-Borneol	1165	13.3	$1.00^{a} \pm 0.28$	$1.35^{a} \pm 0.08$	$1.48^{a} \pm 0.10$	$1.14^{a} \pm 0.02$	1.24
Thymol methyl ether	1235	16.1	$0.25^{c} \pm 0.05$	$0.37^{bc} \pm 0.04$	$0.61^{a} \pm 0.06$	$0.46^{ab} \pm 0.05$	0.42
Carvacrol methyl ether	1244	16.5	$3.63^{b} \pm 0.28$	$4.46^{a} \pm 0.11$	$4.88^{a} \pm 0.17$	$4.35^{a} \pm 0.11$	4.33
Thymoquinone	1249	16.7	$1.95^{c} \pm 0.11$	$3.57^{a} \pm 0.06$	$2.94^{b} \pm 0.17$	$0.33^{d} \pm 0.05$	2.19
Caryophyllene	1414	23.7	$4.50^{b} \pm 0.08$	$6.72^{a} \pm 0.06$	$5.73^{ab} \pm 0.07$	$5.20^{b} \pm 0.9$	5.53

substance	KI	RT (min)	Relative content of substance (%)				Average
			Control	Put	PEG	Put + PEG	(%)
Aromandendrene	1414	24.5	0.14 ^{a}±0.01	0.17 ^{a}±0.01	0.18 ^{a}±0.01	0.19 ^{a}±0.02	0.17
Humulene	1450	25.0	0.08 ^{b}±0.02	0.15 ^{a}±0.02	0.10 ^{b}±0.02	0.09 ^{b}±0.03	0.10
β-Bisabolene	1500	27.3	0.32 ^{b}±0.02	0.57 ^{a}±0.04	0.44 ^{ab}±0.08	0.43 ^{ab}±0.07	0.44
(Z)- *α*-Bisabolene	1538	28.6	1.07 ^{a}±0.10	1.34 ^{a}±0.19	1.11 ^{a}±0.06	1.39 ^{a}±0.10	1.22
α-Pinene	930	5.9	nd	0.25 ^{b}±0.07	0.93 ^{a}±0.17	0.73 ^{a}±0.10	0.47
Eucalyptol	1023	8.3	nd	1.39 ^{a}±0.16	1.57 ^{a}±0.08	1.63 ^{a}±0.11	1.14
(+)-2-Carene	995	7.8	nd	nd	nd	2.76	0.69
Unknown	-	-	8.25 ^{a}±1.0	2.53 ^{bc}±0.17	1.91 ^{c}±0.23	5.22 ^{b}±0.12	3.72

The data are the mean of three replicates ± SE. Different letters show statistically significant differences at $p \leq 0.05$ level.

Table 3
The effect of drought stress and Put on the relative expression of the *DXR* and *TPS2* gene in *T. daenensis*.

Treatment	*DXR* expression (fold change)	*TPS2* expression (fold change)
Control	1 ^{C}	1 ^{C}
Put (0.2 mM)	1.51 ± 0.16 ^{C}	23.31 ± 3.69 ^{a}
PEG (15%)	13.34 ± 2.61 ^{a}	1.52 ± 0.23 ^{C}
Put + PEG	5.66 ± 1.20 ^{b}	4.17 ± 0.76 ^{b}

The data are the mean of three replicates ± SE. Different letters show statistically significant differences at $p \leq 0.05$ level.

As shown in Table 2, Put treatment increased thymol content by 9.5%. However, in plants under drought stress, Put caused a 16.37% reduction in thymol content (Table 2). One possible explanation for this reduction is that Put regulates the biosynthesis of various secondary metabolites based on genetic factors and environmental conditions (33). Consequently, Put may reduce thymol content while increasing the levels of other secondary metabolites that could play a more protective role under stress conditions (34).

Based on our results, drought stress also reduced carvacrol content by 6.25% (Table 2). Previous studies reported a decline in carvacrol content in *T. daenensis* (35), garden thyme (26, 30), and Himalayan thyme (22) under drought stress. In contrast, it is found that carvacrol content increased under mild drought stress but decreased under severe stress in *Satureja hortensis* (36). Under non-stress and stress conditions, Put reduced the carvacrol content by 15% and 16.6%, respectively (Table 2). Previous studies reported a positive effect of Put on carvacrol content; for instance, Put increased carvacrol content by 2.10% in oregano. A potential explanation for the reduction in carvacrol content with Put treatment is the conversion of carvacrol to thymoquinone, as Put increased thymoquinone (TQ) content by 83% in non-stressed plants.

The content of *p*-cymene, a precursor for thymol and carvacrol, showed a significant decrease (43.14%) under drought stress (Table 2). Similar results were observed in *T. daenensis* (33) and *Satureja hortensis* (36). This negative effect could be due to the inhibitory role of drought stress on the monoterpene biosynthesis pathway and reduced activity of related enzymes (37). Also, our results indicated that Put reduced *p*-cymene content by 2.6% under non-stress conditions while increased it's content by 56% under drought stress (Table 2).

The expression of *TPS2*, the gene encoding the enzyme involved in *γ*-terpinene biosynthesis, increased 1.5-fold under drought stress (Table 3). Studies have shown that *γ*-terpinene content increases under mild to moderate drought stress in thyme species (36, 27), while it decreases under severe stress, as observed in garden thyme (39) and *T. daenensis* (33). The discrepancy in results may be related to the plant's adaptive mechanisms, such as changes in proline content, potassium levels, and antioxidant activity, in response to drought (35, 33). Put treatment increased *γ*-terpinene content by 89.6%, (Table 2) with a 23.3-fold increase in *TPS2* gene expression compared to controls (Table 3). Under drought stress, Put enhanced *γ*-terpinene content by 28.5-fold and *TPS2* gene expression by 2.7-fold (Table 2).

TQ, an important compound in the essential oil of *T. daenensis*, is derived from thymol and carvacrol. Drought stress increased TQ content by 50.7% (Table 2). Mild drought stress (40% field capacity) increased TQ content, while severe drought stress (20% field capacity) decreased it (27). TQ has been suggested to enhance the antioxidant and antimicrobial activities of thyme essential oil. Studies indicate that TQ mitigates drought stress by modulating growth regulators like abscisic acid, salicylic acid, and jasmonic acid (38, 39).TQ also boosts the activity of enzymes like superoxide dismutase, catalase, and ascorbate peroxidase (42), as well as non-enzymatic antioxidants such as ascorbic acid, glutathione,

and phenolic compounds. Additionally, TQ improves relative water content (RWC), water use efficiency (WUE), and proline accumulation (42).

Free PAs Content

During various stress conditions, the accumulation of PAs contributes to plant survival and stress tolerance. The ability of PAs to mitigate stress effects is attributed to their antioxidant properties (43) and their role in maintaining cellular turgor pressure (32). PAs in plants are synthesized through the enzymatic activities of ornithine decarboxylase and arginine decarboxylase. Under stress conditions, the biosynthesis of these compounds is increased through the activity of arginine decarboxylase (44).

Numerous studies have been reported that PAs have a modulating function in plants exposed to drought stress (41). Osmotic stress induced by mannitol has been shown to increase the levels of Put, Spd, and Spd in wheat plants (43). Research findings indicate that under drought stress, the activity of ornithine decarboxylase and arginine decarboxylase, as well as the content of PAs, is higher in drought-tolerant species compared to drought-sensitive ones (44). This underscores the role of PAs in enhancing plant resistance to stress.

Drought stress decreased the content of endogenous Put by 26.3% and had no effect on Spd content. A study on barley (*Hordeum vulgare* L.) seedlings reported that 21-day drought stress resulted in an 80% reduction in endogenous Put levels. However, the same stress conditions led to increases in Spd (50%) level.

Put treatment increased the free Put content (Fig. 1.a) by 55% and free Spd content (Fig. 1.b) by 23.1% in plants under drought stress. The application of exogenous putrescine under drought conditions was found to mitigate some of the adverse effects of drought stress (47). Similarly, researchers observed that drought stress led to a decrease in free Spd levels at all developmental stages in both traditional and semi-dwarf triticale (×*Triticosecale Wittm.*) cultivars. Additionally, a reduction in free Put was noted in the traditional cultivar under drought conditions (48). In another study, the effects of osmotic stress induced by PEG on polyamine content in soybean (*Glycine max* L.) leaves were examined. The findings indicated that drought-tolerant soybean cultivars exhibited higher increases in free Spd and Spm levels, while the drought-sensitive cultivars showed a higher increase in free putrescine under stress conditions. This suggests a complex relationship between polyamine levels and drought tolerance in soybean (49).

Exogenous application of Put significantly influences the endogenous levels of PAs, including Put itself and Spd, in plants. The effects observed can vary depending on the plant species, developmental stage, and environmental conditions (50). These changes are mediated through the regulation of genes associated with PA metabolism, highlighting the intricate balance plants maintain in PA homeostasis. Applying Put externally often leads to an increase in the plant's internal Put concentration. For instance, in *Picea abies* somatic embryos, treatment with exogenous Put at concentrations of 10, 100, or 500 µM resulted in a significant, concentration-dependent rise in endogenous Put levels. This suggests that externally supplied Put can be absorbed and utilized by plant tissues, thereby elevating internal Put (50).

Conversely, research on *Vitis vinifera* (grapevine) seedlings under drought stress demonstrated that exogenous Put application not only increased endogenous Put content but also influenced Spd levels. Specifically, certain treatments led to a significant rise in Spd content, suggesting that exogenous Put can serve as a precursor for the biosynthesis of higher PAs like Spd, especially under stress conditions (51).

Conclusion

This study highlights the significant role of Put in mitigating drought stress in *T. daenensis* by modulating gene expression, PAs accumulation, and secondary metabolite composition. The findings highlight that exogenous Put application significantly increases endogenous Put and Spd levels under drought conditions, suggesting its involvement in osmotic adjustment and oxidative stress regulation.

At the molecular level, Put treatment upregulated *TPS2* while downregulating *DXR* expression in drought-stressed plants, indicating its influence on the terpenoid biosynthetic pathway. These genetic modifications were accompanied by a decrease in thymol and carvacrol content, alongside an increase in their precursors, *γ*-terpinene and *p*-cymene. This shift in essential oil composition suggests that Put may regulate metabolic fluxes within the plant's secondary metabolism in response to drought stress.

Overall, these results underscore the potential of PAs, particularly Put, as biostimulants for improving drought tolerance and metabolic regulation in medicinal plants. Further investigations are needed to assess the long-term physiological effects and potential agronomic applications of Put in *T. daenensis* cultivation under field conditions.

Abbreviations

DMAPP
Dimethylallyl pyrophosphate
DXP
1-deoxy-D-xylulose 5-phosphate
DXR
1-deoxy-D-xylulose 5-phosphate reductoisomerase
GAPDH
Glyceraldehyde-3-phosphate dehydrogenase
GPP
Geranyl pyrophosphate
H_2O_2
Hydrogen peroxide
IPP
Isopentenyl pyrophosphate
MEP

Methylerythritol phosphate
PA
Polyamine
PEG
polyethylene glycol
Put
Putrescine
ROS
Reactive oxygen species
RT-qPCR
Quantitative real-time PCR
Spd
Spermidine
Spm
Spermine
T. daenensis
Thymus daenensis
TPS2
Terpene synthase 2

Declarations

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Author Contributions

Ensiyeh Shahroudi designed and performed the experiments, collected and analyzed the data, and wrote the manuscript. Dr. Fatemeh Zarinkamar supervised the physiological experiments conducted in her laboratory. Dr. Bahram M. Soltani supervised the molecular experiments conducted in his laboratory.

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Ethics approval and consent to participate

This study did not involve any human participants or animals. *Thymus daenensis* seeds were sourced from the Isfahan Agricultural and Natural Resources Research and Educational Center (Iran), and the plants were cultivated and used in accordance with institutional, national, and international guidelines. Therefore, no ethical approval or consent to participate was required.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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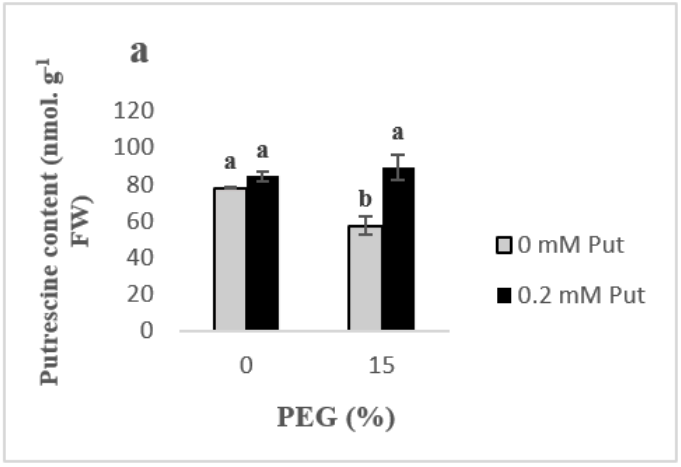
Figures



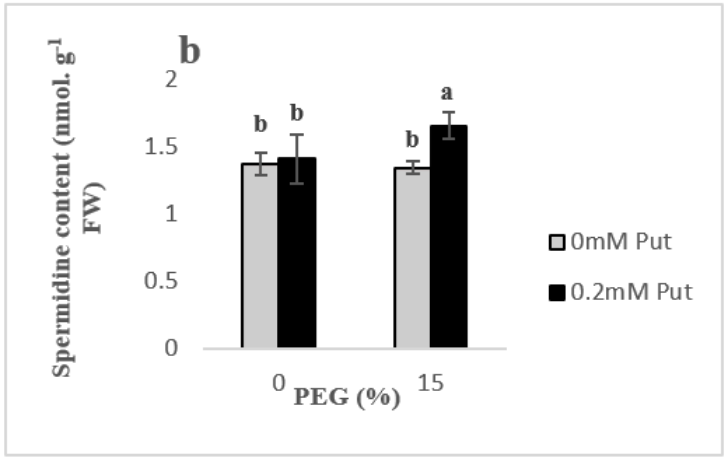


Figure 1

The effect of drought stress and Put on the content of free Put (a) and free Spd (b) in *T. daenensis*. The data are the mean of three replicates ± SE. Different letters show statistically significant differences at $p \leq 0.05$ level.