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# Exogenous application of spermidine mitigates the adverse effects of drought stress in faba bean (*Vicia faba* L.)

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## ABSTRACT

In Tunisia, drought stress is a major environmental factor limiting crop production and causing relatively low and unstable faba bean yields. In the present study, we explored the putative role of spermidine (0.5, 1, 1.5 and 2 mM) in ameliorating the effects of drought stress induced by polyethylene glycol (PEG-6000, −0.58 MPa) in faba bean seedlings. Drought stress reduced photosynthetic performance, chlorophyll and relative water content in leaves of faba bean variety Badii. Moreover, drought increased proline, electrolyte leakage and malondialdehyde content by inducing reactive oxygen species (hydrogen peroxide) generation in leaves. However, applying spermidine increased the activities of catalase, superoxide dismutase, ascorbate peroxidase and guaiacol peroxidase. The results show that the application of spermidine especially at a rate of 1.5 mM effectively reduces oxidative damage and alleviates negative effects caused by drought stress. In addition, exogenous spermidine increased the expression of polyamine biosynthetic enzymes' genes (*VfADC*, *VfSAMDC* and *VfSPDS*), and reduced the expression of *VfSPMS* suggesting that exogenous spermidine can regulate polyamines' metabolic status under drought challenge, and consequently may enhance drought stress tolerance in faba bean. Real-time quantitative polymerase chain reaction analysis revealed that some drought responsive genes (*VfNAC*, *VfHSP*, *VfNCED*, *VfLEA*, *VfCAT*, *VfAPX*, *VfRD22*, *VfMYB*, *VfDHN*, *VfERF*, *VfSOD* and *VfWRKY*) from various metabolic pathways were differentially expressed under drought stress. Overall, these genes were more abundantly transcribed in the spermidine-treated plants compared to untreated suggesting an important role of spermidine in modulating faba bean drought stress response and tolerance.

**Keywords:** drought responsive genes, drought stress, faba bean, gene expression, oxidative damage, polyamine biosynthetic enzymes, spermidine, qRT-PCR.

## Introduction

In some Mediterranean countries including Tunisia, faba bean (*Vicia faba* L.) is incorporated into cereal crop rotation systems providing several benefits such as improved soil quality and fertility and reduced pest incidence in cereals. In Tunisia, faba bean covers more than 70% (54 907 ha) of the annual total area devoted to grain legume crops with 64 508 tonnes of yield (FAO 2018). However, the national average productivity is low (1.12 t/ha), 50% below the world average (2.26 t/ha) and characterised by wide fluctuation (FAO 2018). The variability of the yield is mainly due to its sensitivity to particular abiotic and biotic stresses (Kharrat and Ouchari 2011). Drought stress is the most prevalent environmental factor limiting grain legumes' growth and production in Tunisia. Faba bean is more drought-susceptible compared to other seed legumes including common bean, pea and chickpea (Amede and Schubert 2003). In this context, development of genotypes resistant to drought through breeding is a promising strategy for improving faba bean yield, seed quality and other agronomic

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traits to enhance crop management. Despite its importance, limited selection efforts to improve faba bean have occurred, and very restricted number of cultivars have been selected. Moreover, its physiological, biochemical and molecular adaptation to drought is still unknown. According to Karkanis *et al.* (2018) development of this pulse crop for enhanced drought resistance, among other things, requires the knowledge of physiological, biochemical, molecular and genetic mechanisms controlling responses to drought stress at different plant developmental stages. Plants have evolved various strategies at the morphological, physio-biochemical and molecular levels to react and adapt to water deficits and enhance drought tolerance (Fang and Xiong 2015).

Several studies reported the accumulation of polyamines (PAs) in many plant species under drought stress such as wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.), indicating the possible implication of PAs in plant drought tolerance (Alcázar *et al.* 2020). As an aliphatic amine, putrescine (Put), spermidine (Spd) and spermine (Spm) are the major PAs in plants involved in numerous physiological and biochemical processes associated with the regulation of plant growth and development like cell division and differentiation, seed germination, morphogenesis and senescence (Tavladoraki *et al.* 2012). Polyamines represent a group of naturally occurring compounds; have the advantage that they are non-toxic to plants when applied at effective concentrations and have been shown to play an essential role in drought stress tolerance in many plant species (Chen *et al.* 2019) but still not yet in *Vicia faba*.

In plants, arginine is decarboxylated by arginine decarboxylase (ADC) to agmatine, which is then hydrolysed by a combination of agmatine iminohydrolase (AIH) and *N*-carbamoylputrescine amidohydrolase (CPA) to form Put (Gupta *et al.* 2016). Then Spd and Spm are synthesised from Put by action of spermidine synthase (SPDS) and spermine synthase (SPMS), respectively. These reactions involve the addition of aminopropyl groups supplied from decarboxylated-S-adenosylmethionine (dcSAM), which is formed from methionine by S-adenosylmethionine (SAM) synthase and SAM decarboxylase (SAMDC). It has been widely demonstrated that exogenous application of Spd successfully enhanced the tolerance to drought stress in several plant species as white clover (Li *et al.* 2014), finger millet (Satish *et al.* 2018) and maize (Li *et al.* 2018*a*) demonstrating that Spd plays a crucial role in the regulation of the tolerance to drought in plants. Torabian *et al.* (2018) found that exogenously applied Spd improves leaf water status, photosynthesis, and membrane properties, as demonstrated by higher leaf relative water content (RWC), chlorophyll contents, stomatal conductance (gs), intercellular $CO_2$ concentration (Ci), transpiration rate (E), maximal quantum yield of photosystem II (PSII), net photosynthetic rate (Pn), proline and carotenoids content and lower malondialdehyde (MDA) level, thereby enhancing the drought tolerance of common bean and consequently increasing the grain yield and harvest index of bean plants under drought stress. Exogenous Spd alleviates oxidative damage by increased scavenging of the toxic reactive oxygen species (ROS) leading to reduced yield loss in rice (Liu *et al.* 2015*a*). Li *et al.* (2018*a*) reported that Spd alleviates the adverse effects of drought stress in maize by activating enzymatic and non-enzymatic antioxidant defence and increasing endogenous PAs content resulting in reduction of ROS production and oxidative stress. Recently, similar result was reported by Jiang *et al.* (2021) in rice under aluminium (Al) toxicity. Moreover, overexpression of *ADC*, *SPDS* and *SAMDC* genes in transgenic rice (Capell *et al.* 2004), *Arabidopsis* (Kasukabe *et al.* 2004) and tobacco (Wi *et al.* 2006), respectively, showed accumulation of higher levels of Spd and enhanced drought tolerance compared to controls.

There are few reports focused on the drought tolerance of faba bean, especially about the role of Spd in drought tolerance. Therefore, in this study, we examine whether application of exogenous Spd could alleviate drought damages in Badii plants. In this regard, the study enlarges the better understanding of physico-biochemical adaptability to drought stress and will further expand insight on function of PAs, especially Spd in improving drought tolerance in faba bean. Therefore, after verifying the hypothesis that PAs are able to increase drought tolerance and ameliorate the harmful impact of osmotic stress in faba bean plants, the use of PAs seed priming against drought stress in faba bean might find future practical applications for faba bean crop protection against environmental stresses.

## Material and methods

### Plant material, growth conditions and treatments

The faba bean (*Vicia faba* L. var. *minor*) cultivar Badii (cultivated in sub-humid areas of Tunisia) was provided by the National Institute of Agronomic Research of Tunisia (INRAT). Seeds were germinated on perlite at 23 ± 2°C. Three weeks later, seedlings (corresponding to four fully expanded leaves) with uniform sizes were uprooted from the perlite and transplanted on plastic boxes (height 10 cm, width 17 cm, length 40 cm) filled with 5 L of Hoagland nutrient solution (Hoagland and Arnon 1950). Afterwards, seedlings were grown hydroponically with density of 15 seedlings per box for 1 week in the growth chamber under controlled conditions (temperature of 23 ± 2°C, relative humidity 55–65%, light 270 μmol of photons $m^{-2}$ $s^{-1}$ photosynthetic active radiation and a 14/10 h day/night photoperiod). The Hoagland solution was changed every 2 days. After acclimatisation period the seedlings' roots were exposed to Spd pre-treatment for 2 days by adding

0.5, 1, 1.5 and 2 mM of Spd to the nutrient solution in order to allow Spd absorption. Moreover, at the same hour all seedlings were sprayed three times per day before exposure to water deficit with equal volumes of 0.5, 1, 1.5 and 2 mM of Spd or water (non-Spd-treated control). The drought stress was induced by adding of 18% (−0.58 MPa) polyethylene glycol (PEG-6000) in the nutrient solution (Michel and Kaufmann 1973). All experiments were accomplished in a completely randomised design and six treatments (four biological replicates for each treatment) were set up for drought stress: (1) Control; (2) PEG treatment (18% PEG-6000); (3) Spd (0.5 mM) + PEG (18% PEG-6000); (4) Spd (1 mM) + PEG (18% PEG-6000), (5) Spd (1.5 mM) + PEG (18% PEG-6000) and (6) Spd (2 mM) + PEG (18% PEG-6000). The Spd concentrations were chosen on the basis of a preliminary test for the obvious effects on morphological and physiological changes (Hendawey *et al.* 2018). After 2 days of PEG treatment, roots and leaves were sampled for further physiological and biochemical analysis. For molecular analysis, tissue samples were immediately frozen in liquid nitrogen and stored at −80°C prior to analyses.

## Determination of leaf gas-exchange parameters

Leaf photosynthetic gas exchange rates were performed on the intact, fully expanded leaves using a Portable Photosynthesis System (LCpro+, Inc., UK). Measurements of net photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs), and intercellular $CO_2$ concentration (Ci) were done between 10:00 and 12:00 h and the photosynthetically active radiation in the leaf chamber was set at 980 μmol $m^{-2}$ $s^{-1}$ during the monitoring. Five randomly selected leaves (of similar age) were measured as replicates for each treatment.

## Determination of leaf relative water content (RWC) and chlorophyll content

RWC was calculated using the formula: RWC (%) = [(FW − DW)/(TW − DW)] × 100, where FW is fresh weight, DW is dry weight and TW is turgid weight. The collected leaves were immediately weighed for FW determination followed by immersion in deionised water for 24 h at 4°C and again weighed for TW. At the end, samples were dried in an oven at 70°C for 72 h and weighed for DW (Barrs and Weatherley 1962). Chlorophylls were extracted in 5 mL of 80% acetone in the dark for 48 h at 4°C. Total chlorophyll (Chlt), chlorophyll *a* (Chla) and chlorophyll *b* (Chlb) were determined according to the method described by Lichtenthaler and Wellburn (1983) by measuring the absorption of the extracts at 663 and 645 nm using a spectrophotometer (Spectro UV-Vis Dual Beam PC, UV-S-2007; LABOMED, INC.).

## Determination of proline, soluble sugars, malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$) content and electrolyte leakage (EL) level

Proline content was determined on the basis of its reaction with ninhydrin according to the method of Bates *et al.* (1973). Leaf samples (0.1 g) were extracted in 5 mL of 3% sulfosalicylic acid and filtered. An equal volume of glacial acetic acid and ninhydrin were added to the extract and heated in a water bath at 100°C for 1 h. The reaction was terminated on ice for 2 min followed by adding of 2 mL of toluene to the mixture. After the phase separation, the toluene layer was read at 520 nm, on a spectrophotometer. L-proline was used for the standard curve.

Soluble sugars were determined according to the method described by Dubois *et al.* (1956). Leaf samples (0.5 g) were mixed with deionised water for 12 h. After filtration, 0.5 mL of the extract was homogenised with 5 mL of 5% phenol and 7.5 mL of concentrated sulfuric acid and the mixture was kept for 1 h at room temperature. Total soluble sugars were determined at 490 nm by spectrophotometer using D-glucose as standard curve.

The content of MDA was assessed according to Dhindsa *et al.* (1981). Fresh leaf samples (1 g) were homogenised with extraction buffer containing 20% (w/v) trichloroacetic acid (TCA) and 0.5% (w/v) thiobarbituric acid (TBA) and the mixture was heated at 95°C for 15 min and then cooled in ice for 30 min followed by centrifugation at 11 300*g* for 15 min. The absorbance of the supernatant was determined at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

The content of $H_2O_2$ was determined as described by Velikova *et al.* (2000). Fresh leaf samples (0.5 g) were homogenised with 5 mL 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath. The homogenate was centrifuged at 11 300*g* for 15 min and 1 mL of the supernatant was homogenised with 1 mL of 10 mM potassium phosphate buffer and 2 mL of 1 M KI. The mixture was incubated at room temperature for 15 min and the absorbance of supernatant was recorded at 390 nm. $H_2O_2$ content was calculated using a standard curve.

EL was determined by using the following formula (Blum and Ebercon 1981): EL % = (E1/E2) × 100. Samples of fresh leaves (0.1 g) were immersed in the tube with 15 mL of deionised water for 24 h at room temperature and initial conductivity of the solution (E1) was evaluated by using a conductivity meter. Afterwards, samples were autoclaved for 30 min and cooled to 25°C and the conductivity (E2) was recorded.

## Determination of antioxidant enzyme activities

Leaf samples (0.5 g) were ground on ice with 2 mL of 50 mM cold potassium phosphate buffer (pH 7.8) containing 1 mM of EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). Each extract

was centrifuged at 4°C for 15 min at 11 300*g* and the supernatant was used to measure the activities of antioxidant enzymes.

Catalase (CAT) activity was measured by the method of Hasheminasab *et al.* (2012) as the decrease in $H_2O_2$ at 240 nm for 2 min by spectrophotometer. The reaction was carried out in a final volume of 2 mL of reaction mixture containing 50 μL enzyme extract, 15 mM $H_2O_2$ and 25 mM phosphate buffer (pH 7.8). Activity was calculated by using the extinction coefficient of $H_2O_2$ ($\varepsilon$ = 39.4 $mM^{-1}$ $cm^{-1}$).

Superoxide dismutase (SOD) activity was determined as described by Dhindsa *et al.* (1981) by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin. The 2 mL assay mixture contained 100 μL enzyme extract, 2 mM EDTA, 9.9 mM methionine, 55 μM NBT, 2 μM riboflavin in 50 mM phosphate buffer (pH 7.8). The mixture was illuminated with a fluorescent lamp during 15 min and the absorbance was recorded at 560 nm. One unit (U) of SOD was the amount that causes a 50% inhibition of NBT photoreduction.

Ascorbate peroxidase (APX) activity was assayed according to the method of Nakano and Asada (1981) by the decrease in absorbance of ascorbate at 290 nm for 2 min using extinction coefficient of ($\varepsilon$ = 2.8 $mM^{-1}$ $cm^{-1}$). Reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.5 mM $H_2O_2$ and 100 μL enzyme extract; $\varepsilon$ = 2.8 $mM^{-1}$ $cm^{-1}$.

Guaiacol peroxidase (GPX) activity was determined by monitoring the increase in absorbance at 470 nm during the oxidation of guaiacol to tetra-guaiacol (Urbanek *et al.* 1991) using the extinction coefficient ($\varepsilon$ = 25.5 $mM^{-1}$ $cm^{-1}$). The reaction mixture contained 50 μL of the enzyme extract, 50 mM phosphate buffer (pH 7.8), 0.1 μM EDTA, 10 mM guaiacol and 5 mM $H_2O_2$ in a total volume of 2 mL.

The total protein was determined using Bradford (1976) method and bovine serum albumin (BSA) as a standard.

### Total RNA extraction and qRT-PCR analyses

Total RNA was extracted from leaf and root tissues (0.5 g) following the protocol described by Chang *et al.* (1993). cDNA synthesis was carried out by using a revert Aid First Stand cDNA Synthesis Kit (Biomatik; Wilmington, Delaware, USA). Primer pairs (Table 1) were designed by Primer3 Input (version 0.4.0) software (Rozen and Skaletsky 2000) (http://frodo.wi.mit.edu/primer3/). Faba bean *VfEF1α* was used as an internal control for data normalisation. The PCR amplification (quantitative real-time polymerase chain reaction; qRT-PCR) was performed using the 7300 Real-Time PCR Detection System (Applied Biosystems, Foster City, USA) and the Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (Biomatik; Wilmington, Delaware, USA) with the following programme: 95°C denaturation for 10 min, then 40 cycles of denaturation at 95°C for 30 s, annealing/elongation at 60°C for 1 min. Each sample was amplified in triplicate. The abundance of transcribed genes was calculated according to the formula $2^{-\Delta\Delta Ct}$ described by Schmittgen and Livak (2008). The heatmaps were generated using gplots R package (http://www.r-project.org/) in order to compare the expression profiling of the transcriptome in different stress treatments.

### Statistical analysis

Three independent repetitions at least were performed for all measurements and data are shown as the mean ± standard deviation (s.d.). Analysis of variance (ANOVA) was performed using SPSS 17.0 statistical program (Statistical Package for the Social Sciences). The multiple comparisons for mean values between the treatments were performed by Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$).

## Results

### Photosynthetic gas-exchange parameters

Results of ANOVA showed that all gas-exchange parameters were notably affected by PEG and spermidine (Fig. 1). In general, PEG (18%) treatment caused a remarkable decrease of A, gs and E, but Ci showed a slight decline compared with the no stress treatment. Indeed, Ci decreased only by 5.5%, 6.8%, 4.4%, 4.6% and 2.7% in plants treated with PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd, PEG + 1.5 mM Spd and PEG + 2 mM Spd, respectively (Fig. 1*a*). A was greatly decreased in drought-stressed plants; however the highest level was shown in plants pretreated with exogenous Spd (1.5 mM). This decreased percentage was 52%, 42%, 41%, 37% and 49% in the PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd, PEG + 1.5 mM Spd and PEG + 2 mM Spd treatments respectively, in comparison with the controls (Fig. 1*b*). For gs, the values significantly declined in drought-stressed plants and showed similar trends to A (Fig. 1*c*). Indeed, the values declined in the PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd and PEG + 2 mM Spd treatments by 47%, 45%, 46% and 48%, respectively; while gs decreased only by 38% in the PEG + 1.5 mM Spd treatment compared with that in controls. PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd and PEG + 2 mM treatments resulted in a 62%, 51%, 56% and 55% decrease in E, respectively compared to control plants (Fig. 1*d*). However, in the PEG + 1.5 mM Spd group the E in Badii decreased only by 42%. Therefore, compared to Spd-treated plants, drought-stressed plants without exogenous application of Spd displayed significantly lower values of A, gs and E.

### RWC and electrolyte leakage (EL) level, total chlorophyll (Chlt), proline soluble sugars, $H_2O_2$ and MDA content

RWC was significantly reduced under PEG treatment by 25% compared to controls (Fig. 2*a*). Similarly, a considerable

**Table 1.** Sequences of primers used for real-time quantitative polymerase chain reaction (qRT-PCR).

| Gene | Accession number | Gene description | Sequence (5′–3′) | Product size (bp) | Tm (°C) |
|---|---|---|---|---|---|
| *VfWRKY* | GARZ01000588 | WRKY transcription factor | F: 5′-CCGCTGTTTGCAGTTATTGA-3′<br>R: 5′-TCATTCATTTCGGTCCACAA-3′ | 126 | 58 |
| *VfNAC* | JR969093 | NAC family transcription factor | F: 5′-ATGCTGCATCGTTCTCAGTG-3′<br>R: 5′-TGATTGGGTTCTGTGTCGAA-3′ | 214 | 56 |
| *VfMYB* | GASA01006303 | Myeloblastosis oncogene | F: 5′-TCCGTTCGACCAGGTAACTT-3′<br>R: 5′-ATCCTGGTCTCAAACGTGGT-3′ | 117 | 60 |
| *VfHSP* | FL504801 | Heat-shock protein | F: 5′-TCTCAAGCTGGTGGGTCTTT-3′<br>R: 5′-AAATCCTTCAATCGGCGCTC-3′ | 131 | 60 |
| *VfNCED* | FL505829 | Nine-*cis*-epoxycarotenoid dioxygenase | F: 5′-ACAATGTCAGCAGATCCCGT-3′<br>R: 5′-GCAATGGTTGTCTGCCTGTT-3′ | 115 | 59 |
| *VfDHN* | GASA01012523 | Dehydrin | F: 5′-CAGATGAAACAAACTACTCAAAC-3′<br>R: 5′-AAGCTTCCTGGTACTGGAGGA-3′ | 129 | 55 |
| *VfERF* | EU543659 | Ethylene response factor | F: 5′-TGCTGCTTTTCATTTTCGTG-3′<br>R: 5′-AGGCGCTGTAAGAGGCATAG-3′ | 106 | 59 |
| *VfLEA* | GARZ01002376 | Late embryogenesis abundant protein | F: 5′-TGACCAGAAGCCAGTGTGAG-3′<br>R: 5′-CGGGAGTACCAACGGATATG-3′ | 139 | 56 |
| *VfRD22* | JR966180 | Responsive to dehydration | F: 5′-AGAGTTTCCCTTGTCGGTGA-3′<br>R: 5′-TGCCCCCAATGAGAAGTATC-3′ | 80 | 56 |
| *VfAPX* | FL507355 | Ascorbate peroxidase | F: 5′-CATTGAAAAGGCCAAGAGGA3′<br>R: 5′-TGCTTAATGGTTCCGAAAGG-3′ | 141 | 59 |
| *VfCAT* | JQ043348 | Catalase | F: 5′-TGCATTTTGTCCTGCCATTA-3′<br>R: 5′-TCCAAGTCTGTGCCTCTGTG-3′ | 106 | 57 |
| *VfSOD* | JQ043347 | Superoxide dismutase | F: 5′-CAGGGCTTCATGGTTTTCAT-3′<br>R: 5′-GACGGGTTTCATCTTCAGGA-3′ | 120 | 58 |
| *VfSPMS* | CSVX01007906 | Spermine synthase | F: 5′-ACCGACATTCACACCAGACA-3′<br>R: 5′-TGGCATCCCAAATCTCTTTC-3′ | 87 | 56 |
| *VfSPDS* | CSVX01024702 | Spermidine synthase | F: 5′-GGTGAACTGTCGCCAGGTAT-3′<br>R: 5′-CAATAGGATTCACCGGATGC-3′ | 143 | 57 |
| *VfSAMDC* | CSVX01022230 | S-adenosylmethionine decarboxylase | F: 5′-GGAGAAGGTGGTGCTGTTGT-3′<br>R: 5′-CCTCGCTCTCATCCTCACTC-3′ | 103 | 58 |
| *VfADC* | CSVX01000024 | Arginine decarboxylase | F: 5′-CACAATGGCCCTACCACTCT-3′<br>R: 5′-TGCTGTTGTTCATGCTGTGA-3′ | 88 | 57 |
| *VfELF1A* | AJ222579 | Elongation factor 1A | F: 5′-GACAACATGATTGAGAGGTCCACC-3′<br>R: 5′-GGCTCCTTCTCAATCTCCTTACC-3′ | 542 | 58 |

reduction in RWC was observed under drought stress in plants treated by Spd at 0.5 mM, 1 mM and 2 mM. This decreased percentage was 30%, 43% and 40%, respectively. However, exogenous application of Spd (1.5 mM) showed improved RWC in drought-stressed plants compared to other Spd concentrations. Indeed, the treatment of Spd at 1.5 mM maintained the RWC compared to non-stressed plants.

It is well shown from data in Fig. (2*b*) that the Chlt in Badii leaves was considerably decreased (13%) in drought-stressed compared to non-stressed plants. Moreover, in faba bean treated by exogenous application of Spd at 0.5 mM, 1 mM and 2 mM, PEG treatment caused a significant decline in Chlt by 17%, 23% and 15%, respectively. However, Chlt content was maintained in Spd-pretreted plants by 1.5 mM compared to controls and showed higher Chlt content than untreated plants under water deficit.

Data presented in Fig. (2*c*) clearly indicate that drought stress significantly increased (20%) electrolyte leakage (EL) level in Badii leaves compared to the well-watered treatment. Similar results were observed by application of different concentrations of Spd. However, PEG + 0.5 mM Spd, PEG + 1 mM Spd, PEG + 1.5 mM and PEG + 2 mM Spd-treated plants displayed significant lower EL level (only 8%, 4%, 13% and 10%, respectively) in comparison to controls. In general, PEG treatment caused a considerable increase of EL level in leaves of Badii, but exogenous application of Spd, especially at 1 mM effectively reduced the increased trend of EL.

The PEG treatment significantly increased the $H_2O_2$ content by 76% compared to control plants (Fig. 2*d*). Interestingly, application of Spd exhibited reduction in $H_2O_2$ production relative to PEG-stressed plants. Indeed, no significant change of $H_2O_2$ content in leaves of Badii

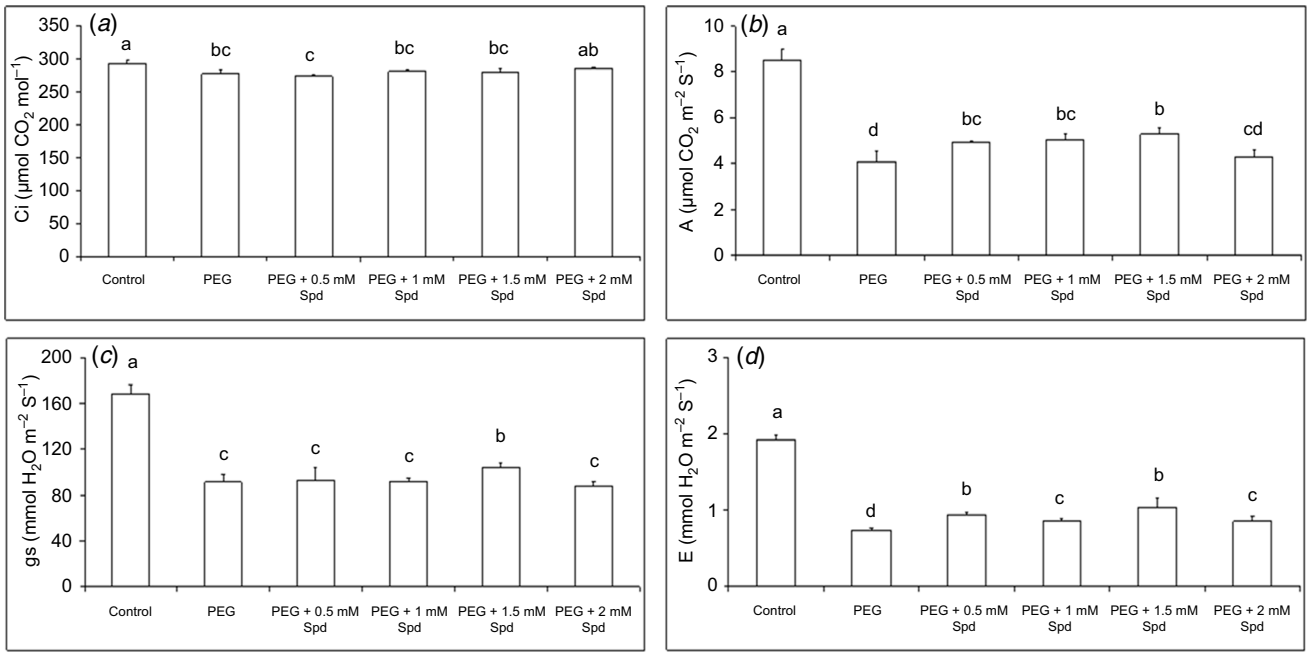


**Fig. 1.** Effects of PEG (18%) and Spd treatments (0.5 mM, 1 mM, 1.5 mM and 2 mM) on intercellular $CO_2$ concentration (*a*), net photosynthetic rate (*b*), stomatal conductance (*c*) and transpiration rate (*d*). Different letters indicate significant differences (Tukey's HSD, $P < 0.05$).

treated with 0.5 mM and 1 mM Spd was observed after comparison to the controls. In the other hand, PEG + 1.5 mM Spd and PEG + 2 mM Spd-treatment exhibited 31% and 23%, respectively, lower $H_2O_2$ content than controls (Fig. 2*d*), suggesting that exogenous Spd-pretreatment was effective in decreasing $H_2O_2$ content during drought stress in *Vicia faba*.

Proline content of Badii leaves was markedly increased by 265%, 445%, 368%, 443% and 446% in PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd, PEG + 1.5 mM Spd and PEG + 2 mM Spd treatment respectively compared to controls (Fig. 2*e*). It is clear from data that under drought challenge, all Spd treatments accumulated more proline under drought stress relative to non-Spd treated plants. The best treatment in this concern was Spd at 0.5 mM, 1.5 mM and 2 mM.

Similar to the proline parameter, drought stress had strongly affected the accumulation of soluble sugars in leaves in different treatments (Fig. 2*f*). Indeed, drought-stressed faba bean significantly increased soluble sugars content by 21% compared to the control treatment, whereas PEG treatments induced significant increase of soluble sugars by 55%, 92%, 95% and 42% in Spd-pretreated plants at 0.5 mM, 1 mM, 1.5 mM and 2 mM, respectively. Indeed, the highest soluble sugars content was recorded when Spd was applied.

Drought stress caused a significant increase in the MDA content in the leaves of Badii by 52% compared to the control treatment (Fig. 2*g*). The MDA accumulation was slightly increased (7%) under drought and exogenous application of 1 mM Spd. Interestingly, exogenous application of 1.5 mM Spd resulted in a reduction (15%) in MDA content to the level lower than that of the controls, suggesting that Spd could reduced lipid peroxidation in drought-stressed faba bean. In the other hand, the highest MDA content was recorded in drought-stressed plants when 0.5 mM and 2 mM Spd was applied.

## Antioxidant enzyme activities (CAT, SOD, APX and GPX)

CAT activity was markedly induced by PEG (18%) and increased by 27 % compared with the control and CAT activity increased under drought pressure by a wide range in Spd-treated plants (Fig. 3*a*). Exogenous Spd could significantly increase the activity of CAT by 46%, 40% and 54% in PEG + 0.5 mM Spd, PEG + 1.5 mM Spd and PEG + 2 mM Spd, respectively. By contrast, its activity remarkably decreased by 35% in PEG + 1 mM Spd treatment. The highest CAT activity was observed in stressed plants treated with Spd at 2 mM.

Notable differences of SOD activity between different treatments were also detected under drought application. PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd, PEG + 1.5 mM Spd and PEG + 2 mM Spd treatment showed almost 24%, 27%, 55%, 12% and 59% higher SOD activity than non-stressed and Spd-treated plants (Fig. 3*b*). Similar to CAT

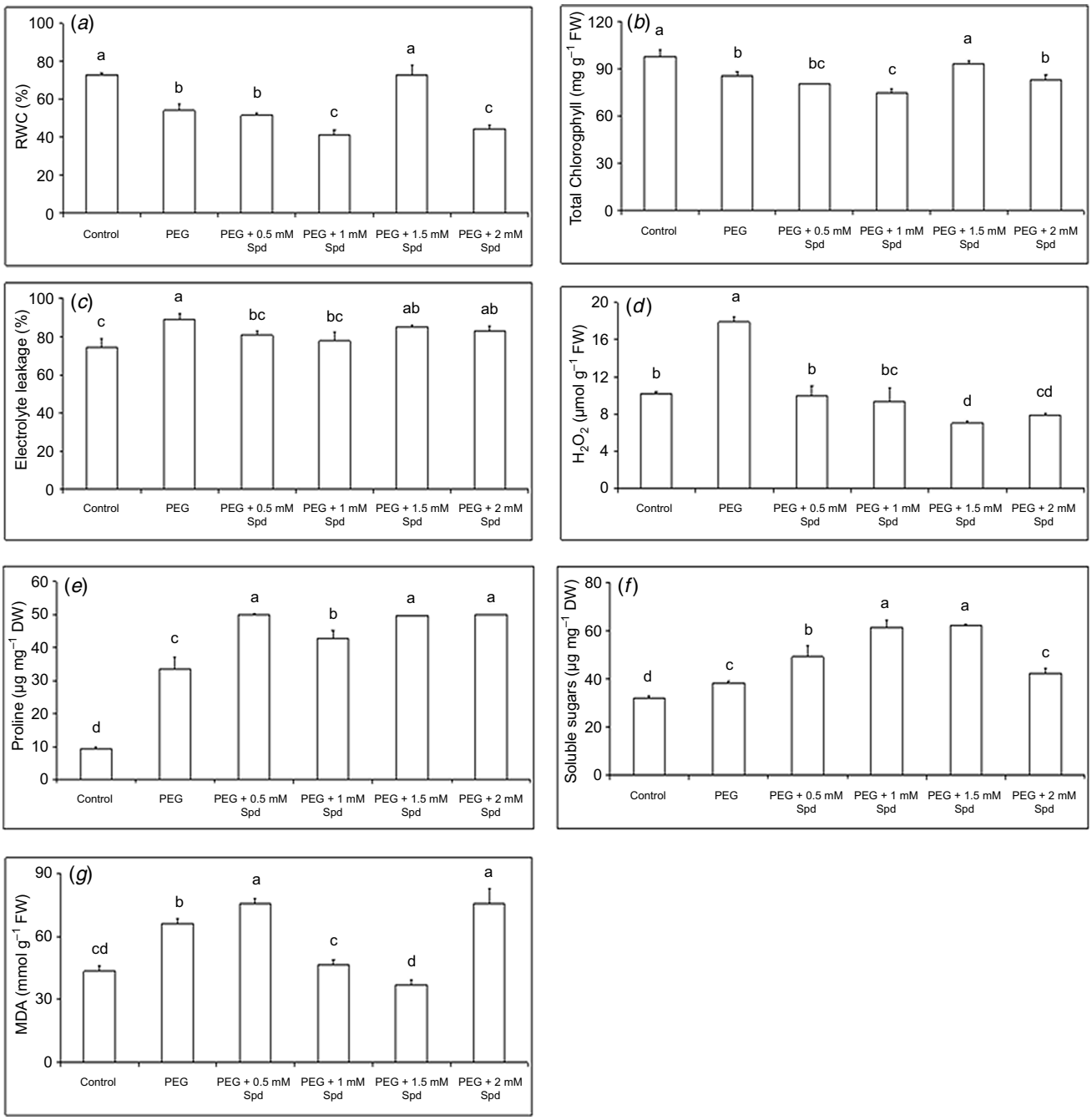


**Fig. 2.** Effects of PEG (18%) and Spd treatments (0.5 mM, 1 mM, 1.5 mM and 2 mM) on relative water content (*a*), total chlorophyll (*b*), electrolyte leakage level (*c*), hydrogen peroxide (*d*), proline (e), soluble sugars (*f*) and malondialdehyde (*g*) content. Different letters denote significant differences (Tukey's HSD, $P < 0.05$).

activity, the highest SOD activity was displayed in drought-stressed plants and treated with Spd at 2 mM.

APX activity significantly differs between treatments under drought stress (Fig. 3*c*). The effect of Spd at 1 mM was the most obvious, and the activity of APX under drought stress was higher by 99% compared to the control. However, APX activity in PEG + 0.5 mM Spd was relatively unchanged, but distinctly increased by 63%, 74% and 34% in PEG, PEG + 1.5 mM Spd and PEG + 2 mM Spd treatment, respectively.

Drought-stressed plants exhibited significantly higher GPX activity than non-stressed (Fig. 3*d*). GPX activity in PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd, PEG + 1.5 mM Spd and PEG + 2 mM Spd treatment was about 43%, 17%, 60%, 55% and 83% higher than that in control treatment. Similar to CAT and SOD activity, the highest GPX activity

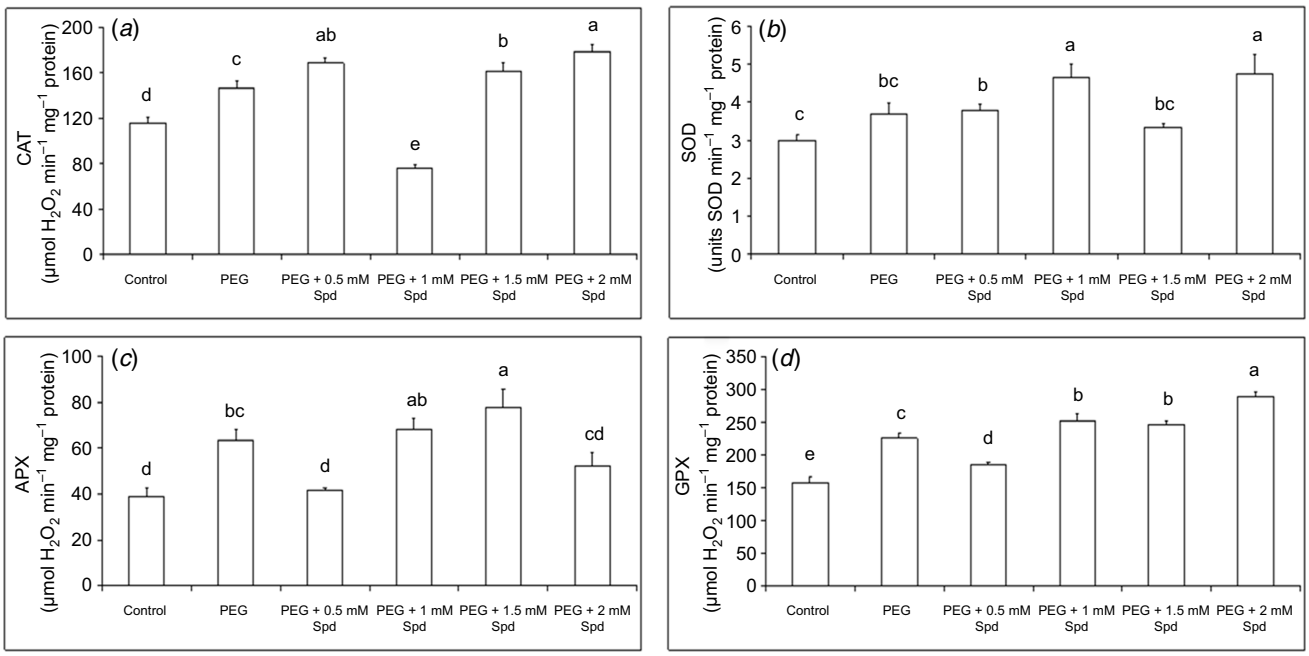


**Fig. 3.** Effects of PEG (18%) and Spd treatments (0.5 mM, 1 mM, 1.5 mM and 2 mM) on the activities of catalase (*a*), superoxide dismutase (*b*), ascorbate peroxidase (*c*) and guaiacol peroxidase (*d*) in leaves of Badii. Different letters denote significant differences (Tukey's HSD, $P < 0.05$).

was observed in drought-stressed plants treated with Spd at 2 mM.

## Transcriptional profiles of genes involved in polyamines (PAs) synthesis

The expression pattern of some representative genes encoding enzymes involved in PAs synthesis, including *VfADC*, *VfSAMDC*, *VfSPDS* and *VfSPMS,* was measured in leaves of Badii by quantitative real-time PCR (qRT-PCR) in order to determine the effects of exogenous Spd application on PAs synthesis under drought stress (Fig. 4). PEG (18%) treatment significantly increased the expression level of *VfADC*, *VfSAMDC*, *VfSPDS* and *VfSPMS* in leaf tissues by 85%, 42%, 345% and 25%, respectively, compared to the control condition. Moreover, the transcription of four genes was significantly upregulated in drought-stressed plants compared to controls due to Spd application, except for *VfSPMS* when 2 mM Spd was applied. Spd treatment at 1.5 mM resulted in the greatest *VfADC* expression level (increased by 194%) in drought-stressed plants (Fig. 4*a*). Under drought challenge the level of *VfSAMDC* transcripts was greater in Spd-treated plants than in untreated (Fig.4*b*). In contrast to *VfSAMDC*, expression of *VfSPDS* in Spd-treated plants was markedly lower compared with the PEG-treated ones (Fig. 4*c*). *VfSPMS* transcripts were abundantly induced in the Spd-treated samples by 0.5 mM (89%), 1 mM (134%) and 1.5 mM (69%), followed by a marked decline (30%) when 2 mM Spd was applied (Fig. 4*d*).

## Expression of drought stress-related genes

The expression pattern of 12 drought stress-related genes (*VfNAC*, *VfHSP*, *VfNCED*, *VfLEA*, *VfCAT*, *VfAPX*, *VfRD22*, *VfMYB*, *VfDHN*, *VfERF*, *VfSOD* and *VfWRKY*) was accomplished by quantitative real-time PCR (qRT-PCR) in leaves (Fig. 5) and roots (Fig. 6) of Badii subjected to Spd and PEG treatments. Some of the selected genes, in addition to their implication in drought tolerance are also involved in pathogen defence, oxidative, osmotic and salt stress responses (Table 1). The objective of this study was to determine whether alleviation of drought stress in faba bean by exogenous application of Spd was associated with the differential transcript accumulation of genes involved in stress tolerance.

As shown in Fig. 5, all tested genes were differentially expressed in leaves of Badii in treated and untreated-Spd plants under drought stress conditions. In leaves, the expression pattern of studied genes could be divided into four groups (Fig. 5). The first group included five members (*VfAPX*, *VfNCED*, *VfLEA*, *VfRD22* and *VfSOD*, 43%) which displayed a significant increase transcript expression in PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd, PEG + 1.5 mM and PEG + 2 mM Spd-treated plants compared to control plants. However, these genes exhibited significantly higher expression levels under drought stress in treated-Spd plants than that in untreated-Spd plants. These observations also revealed a high expression level of *VfNCED*, *VfRD22* and *VfSOD* in PEG + 0.5 mM Spd-treated plants, while stronger

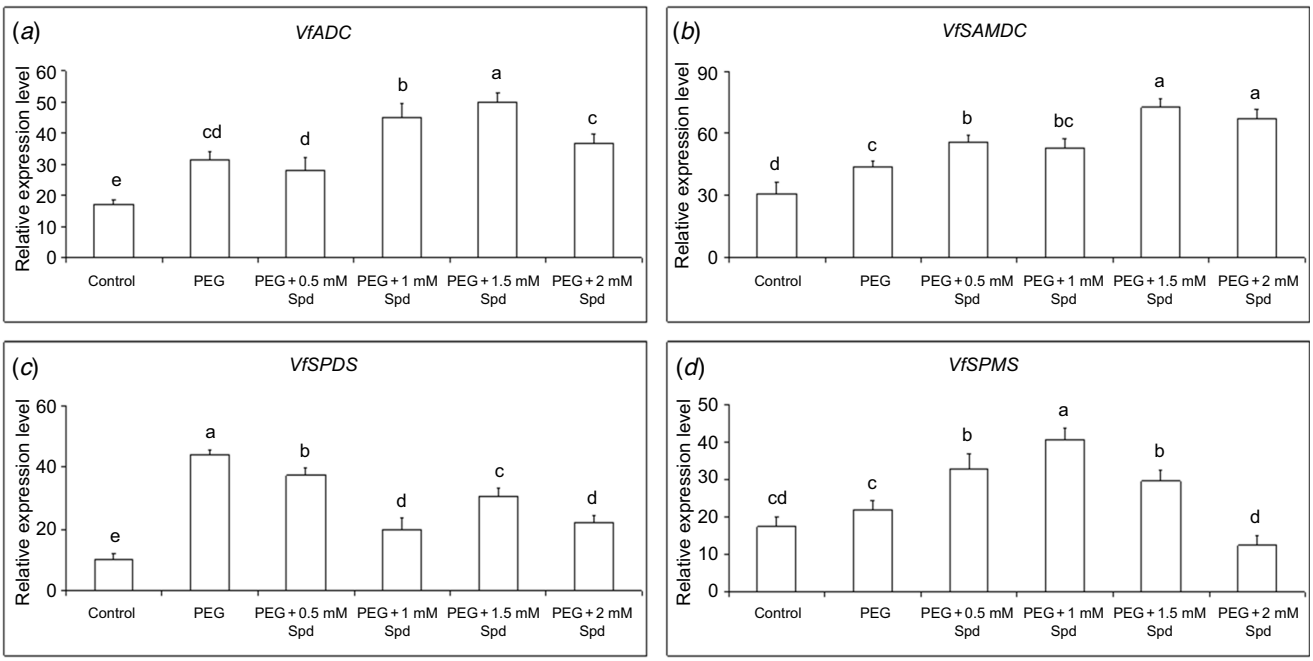


**Fig. 4.** Effects of PEG (18%) and Spd treatments (0.5 mM, 1 mM, 1.5 mM and 2 mM) on *VfADC* (*a*), *VfSAMDC* (*b*), *VfSPDS* (*c*) and *VfSPMS* (*d*) gene relative expression ratio in Badii under water stress. Different letters denote significant differences (Tukey's HSD, $P < 0.05$).

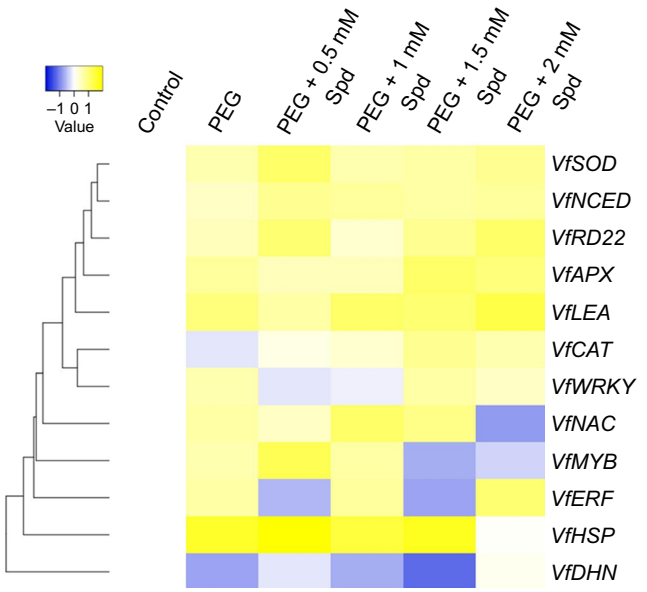


**Fig. 5.** Heat map representation of the effects of PEG (18%) and Spd treatments (0.5 mM, 1 mM, 1.5 mM and 2 mM) on gene expression in the leaves of Badii. Yellow and blue indicate higher and lower expression values, respectively. Intensity of the colours is proportional to the absolute value of log2 of the fold difference in expression.

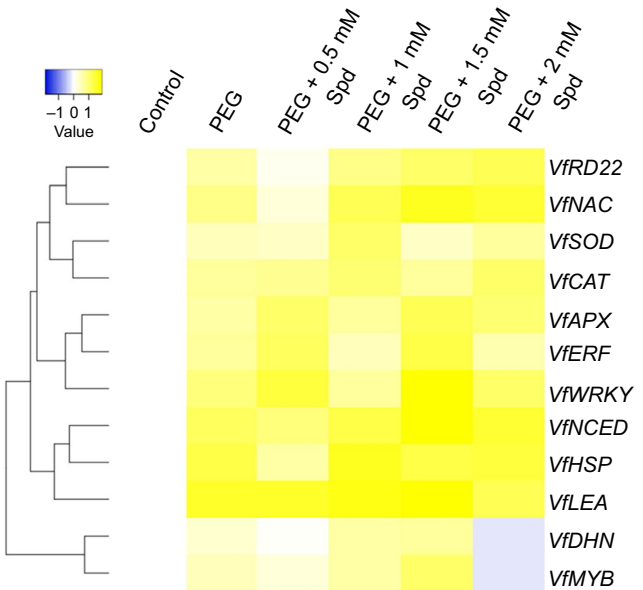


**Fig. 6.** Heat map representation of the effects of PEG (18%) and Spd treatments (0.5 mM, 1 mM, 1.5 mM and 2 mM) on gene expression in the roots of Badii. Yellow and blue indicate higher and lower expression values, respectively. Intensity of the colours is proportional to the absolute value of log2 of the fold difference in expression.

induction of *VfAPX* and *VfLEA* was recorded in PEG + 1.5 mM and PEG + 2 mM Spd-treated plants, respectively. The two members (*VfWRKY* and *VfCAT*, 16%) of group 2 were mainly upregulated in PEG + 1.5 mM and PEG + 2 mM Spd-treated plants compared to controls. However, under PEG + 0.5 mM Spd and PEG + 1 mM Spd treatment, *VfCAT* was weakly upregulated and weakly downregulated in PEG treatment. In contrast, under PEG + 0.5 mM Spd and

PEG + 1 mM Spd treatment *VfWRKY* was weakly downregulated and weakly upregulated in PEG treatment. The members (*VfNAC*, *VfMYB* and *VfERF*, 25%) of third group were upregulated in PEG treatment but the expression showed irregular and insignificantly up- or downregulation in treated-Spd plants. Expression of *VfNAC* was significantly downregulated PEG + 2 mM Spd-treated plants, while *VfMYB* demonstrated significantly low expression levels in PEG + 1.5 mM and PEG + 2 mM Spd-treated plants. The lowest expression of *VfERF* showed in PEG + 0.5 mM Spd and PEG + 2 mM Spd-treated plants. On the other hand, the highest transcript levels of *VfNAC*, *VfMYB* and *VfERF* were recorded in PEG + 1 mM Spd, PEG + 0.5 mM Spd and PEG + 2 mM Spd-treated plants, respectively. The two members (*VfHSP* and *VfDHN*, 16%) of group 4 showed no induction under PEG + 2 mM Spd treatment. Expression of *VfHSP* was significantly upregulated under all other treatments compared to the control, whereas the expression of *VfDHN* was markedly downregulated.

Based on the hierarchical classification, heat map representation of the expression of selected genes in the roots of Badii revealed three groups (Fig. 6). Group 1 contains seven members (*VfWRKY*, *VfAPX*, *VfSOD*, *VfCAT*, *VfNAC*, *VfERF* and *VfRD22*, 58.5%). Overall, transcript levels of these genes significantly upregulated due to PEG and Spd application. The highest transcript levels of *VfNAC* and *VfRD22* was observed in PEG + 1 mM Spd, PEG + 1.5 mM and PEG + 2 mM Spd-treated plants. Under drought stress, 1 mM and 2 mM Spd-treated plants had significantly higher expression levels of *VfSOD* and *VfCAT*. Moreover, when plants were subjected to drought stress Spd-treated plants at 0.5 mM and 1.5 mM showed significantly higher expression of *VfWRKY*, *VfAPX* and *VfERF*. Group 2 mainly consists of three genes (*VfNCED*, *VfHSP* and *VfLEA*, 25%) which were widely upregulated under different treatments. The lowest transcript accumulation of *VfNCED* and *VfHSP* was recorded in plants treated with 0.5 mM Spd, whereas similar data was obtained for *VfLEA* by application of 2 mM Spd. Group 3 contained *VfDHN* and *VfMYB* (16.5%) which are slightly upregulated under PEG, PEG + 0.5 mM Spd, PEG + 1 mM Spd and PEG + 1.5 mM treatments, but slightly downregulated under the PEG + 2 mM Spd treatment.

## Discussion

In this study, PEG treatment markedly reduced photosynthetic parameters (A, gs, E and Ci) in Badii seedlings. Previous studies in faba bean under drought stress reported that stomatal and non-stomatal limitation were responsible for the A reduction (Abid *et al.* 2017). In maize (*Zea mays* L.), Spd application showed improved drought-stressed seedling growth by effective alleviation of both stomatal and non-stomatal limitations of photosynthesis (Li *et al.* 2018*b*). In the current study, under drought stress conditions, gs, E and Ci decreased, indicating that drought stress induced inhibition of photosynthesis in Badii was dominated by stomatal factors. The photosynthetic capability of PEG-treated Badii seedlings was more seriously affected than Spd-treated seedlings expressed by the changes in A, indicating that Spd application could alleviate the reduction in gs, E and Ci of Badii leaves under drought stress, which may have improved A. It suggests that Spd is able to influence A through increasing gs, and E levels under drought stress. These data are consistent with the findings of Shu *et al.* (2014) and Khoshbakht *et al.* (2018), revealing that application of exogenous Spd significantly increased A in cucumber (*Cucumis sativus* L.) and mandarin Bakraii (*Citrus reticulate* × *Citrus limetta*) plants under salt and stress. Moreover, Torabian *et al.* (2018) and Farooq *et al.* (2009) reported that exogenous application of Spd improves gs, Ci, E and A, thereby enhancing the drought tolerance of common bean (*Phasolus vulgaris* L.) and rice (*Oryza sativa* L.) plants, respectively.

RWC significantly decreased in PEG-treated *Vicia faba* plants which could be the consequence of a decrease in Ci due to stomatal closure. Under drought stress, exogenous application of Spd (1.5 mM) increased RWC compared to non-Spd treated plants, suggesting that Spd application considerably improved the water relations of Badii drought-stressed plants. Farooq *et al.* (2009) reported that exogenous Spd treatment increased the RWC in rice under osmotic stress, which was consistent with the results in this study. Similar data were stated by Li *et al.* (2014) in white clover (*Trifolium repens* L.) by application of spermine, suggesting the contribution of PAs in osmotic adjustment under osmotic stress (Talaat and Shawky 2016). On the other hand, application of Spd did not improve the water relations of cucumber (Kubiś *et al.* 2014) and finger millet (*Eleusine coracana* L. Gaertn.) (Satish *et al.* 2018) drought-stressed plants. Interestingly, the decrease in total chlorophyll (Chlt) content was lower in plants treated with Spd (1.5 mM), suggesting their better tolerance to drought stress than other treatments due to greater capacity to maintain Chlt degradation. In agreement with this observation, application of Spd alleviated the inhibitory effect of drought stress on maize leaf photosynthetic pigments such as Chla, Chlb, Chlt and Chla/Chlb (Li *et al.* 2018b). These results were similar to previous studies in creeping bentgrass (*Agrostis stolonifera*) and valerian (*Valeriana officinalis* L.) under drought stress (Li *et al.* 2015*a* and Mustafavi *et al.* 2016, respectively), in which Spd effectively alleviated the decline of Chlt content under drought stress. The results presented here suggest that exogenous Spd (1.5 mM) may play a protective role in chloroplasts, resulting in Chlt retention under osmotic stress through promoting the synthesis of chlorophyll and preventing its degradation (Hu *et al.* 2016).

Electrolyte leakage (EL), which is used to assess the integrity and stability of cell membrane, has been broadly exploited as a potential physiological marker of drought stress tolerance (Parkash and Singh 2020). Spd-treated plants showed lower EL level than untreated under drought stress, which indicates the involvement of Spd in the stabilisation of cellular membranes. Moreover, our results show that in comparison to PEG-treated plants, Spd application (1 and 1.5 mM) decreased MDA content, suggesting protection of membrane integrity. These data are in agreement with several previous studies in finger millet (Satish *et al.* 2018), rice (Farooq *et al.* 2009), valerian (Mustafavi *et al.* 2016), white clover (Li *et al.* 2014) and bentgrass (Li *et al.* 2015*a*), where exogenous Spd was reported to mitigate stress-induced growth inhibition possibly due to stabilisation and protection of membranes and minimisation of oxidative damage.

The present experiment also revealed that drought challenge increased the proline concentration in leaves of Badii. In this regard, Misra and Gupta (2005) reported that accumulation of proline in plants has been correlated with drought stress tolerance. Spd application increased drought-induced proline accumulation in leaves of Badii, suggesting that drought stress may be partially alleviated with the accumulation of proline. These data are in line with the results of Farooq *et al.* (2009), Kubiś *et al.* (2014), Mustafavi *et al.* (2016) and Satish *et al.* (2018) who reported that exogenous Spd increased proline accumulation in rice, cucumber, valerian and finger millet, respectively exposed to osmotic stress.

The level of osmolytes such as soluble sugars was also increased by drought stress and the highest level was reported in Spd-treated plants, suggesting that Spd, as an important osmoprotectant, could regulate osmotic adjustment in Badii plants under drought stress, resulting in reduced water loss. A similar result was also observed in drought-stressed wheat (Marcińska *et al.* 2020). These results also suggest that Spd might act as metabolite signalling molecules which modulate the accumulation of soluble sugars in faba bean under drought stress through activation of various genes involved in the regulation of different processes ($CO_2$ assimilation, photosynthesis, Calvin cycle, sugar processing), leading to drought tolerance.

Reduction of photosynthetic pigment content (Chlt) and decrease in photosynthetic activity under drought stress might be associated with ROS generation which is reflected from the increased $H_2O_2$ content in leaves of drought-stressed plants over controls. Hence, the use of exogenous Spd decreased $H_2O_2$ content suggesting that Spd application might increase the tolerance to drought by controlling the production of $H_2O_2$. Reduction $H_2O_2$ induces oxidative stress by exogenous PAs application was reported in other studies under different abiotic stress (Farooq *et al.* 2009; Hasan *et al.* 2021). At low concentrations, $H_2O_2$ acts as a signal molecule involved in activation of calcium ($Ca^{2+}$)-permeable channels as an important part of the mechanism for abscisic acid (ABA)-induced stomatal closure, leading to plant response to drought stress (Li *et al.* 2015*b*).

The cross talk between calcium message systems and $H_2O_2$ may be also involved in stress-induced antioxidant enzyme gene expression, resulting in increasing antioxidant enzyme activities and thereby controlling free radical production (Hasan *et al.* 2021). Previous research indicated that application of exogenous Spd could increase tolerance of ginseng (*Panax ginseng* C. A. Meyer) seedlings to salt stress by elevating the activities of antioxidant enzymes which ultimately led to enhanced ROS scavenging ability (Parvin *et al.* 2014). These results are consistent with those of a study in the roots of cucumber under salt stress (Wu *et al.* 2018). Previous studies have reported a high activity of CAT, SOD, APX and GPX in leaves and roots of faba bean drought-stressed plants was associated with expression of related genes, suggesting a potential role in reducing the damage of ROS to plants under water deficit (Abid *et al.* 2017, 2021). Interestingly, a similar result was witnessed in this study. In addition to that, Spd-treated plants showed higher activity of antioxidant enzymes compared to untreated plants; suggesting that activity of CAT, SOD, APX and GPX in leaves of Badii may be substantially induced by Spd application. Exogenous application of Spd resulted in considerable elevation of CAT, SOD and APX and the subsequent reduction of ROS in rice (Farooq *et al.* 2009), valerian (Mustafavi *et al.* 2016), maize (Li *et al.* 2018*a*) and creeping bentgrass (Li *et al.* 2015*a*), which is in accordance with our results.

It is evident that Spd exhibits an antioxidant effect by increasing the activities of antioxidant enzymes which lead to control of ROS production in faba bean Spd-treated plants and thereby result in improving cell membrane stability and properties, as showed by lower MDA, $H_2O_2$ and electrolyte leakage. Taken together, these data reveal that Spd could be involved in the regulation of $H_2O_2$ messenger systems, resulting in tolerance to drought stress associated with antioxidant defence in leaves of faba bean. A similar protective effect was reported in drought-stressed rice (Sohag *et al.* 2020) and pearl millet (Awan *et al.* 2021) by exogenous application of plant growth regulators such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA). Indeed, a significant improvement was observed in the plant defence system resulting from increased activities of antioxidative enzymes due to exogenous ABA, SA and JA under PEG, which consequently lowered the level of $H_2O_2$ in drought-stressed plants. These data suggest that Spd act as phytohormones or regulatory molecules in faba bean drought stress responses.

Several studies reported that using Spd was more effective than other plant regulators in enhanced tolerance to drought in plants because PAs have the advantage of being naturally occurring compounds, which are non-toxic to plants and

mammals at the effective concentrations applied. Moreover, PAs can regulate various physiological processes such as photosynthesis activity, osmolyte accumulation, redox homeostasis, growth, biomass production and antioxidant defences under drought which lead to increased crop yield and quality without any negative effect for crops or the environment (Alcázar *et al.* 2020).

In various plant species, abiotic stresses modulated the accumulation of endogenous PAs by influencing the expression of main related genes such as *ADC*, *SAMDC*, *SPDS* and *SPMS* (Chen *et al.* 2019). To provide evidence for the influence of exogenous application of Spd on PAs biosynthesis, the expression pattern of genes involved in this metabolism including *VfADC*, *VfSAMDC*, *VfSPDS* and *VfSPMS* was analysed in the leaves of control and Spd-treated seedlings under drought stress.

Drought stress increased the expression of *ADC1*, *ADC2*, *SPDS1*, *SPDS2*, *SPMS* and *SAMDC1* and increased Put, Spd and Spm levels which are associated with drought tolerance in *Arabidopsis thaliana* (Alcázar *et al.* 2011). Similar data were reported by Bae *et al.* (2008) in *Theobroma cacao*. Indeed, water deficit induced the expression of *TcODC*, *TcADC* and *TcSAMDC*, resulting in increased Spd and Spm levels. In wheat (*Triticum aestivum* var. Sakha 94), water deficit augmented the content of Put and Spm, decreased the level of Spd in shoots and showed accumulation of *ADC*, *SPDS* and *SAMDC*, whereas *SPMS* and *ODC* expression was not changed (Ebeed *et al.* 2017). In the present study, differential expression of *VfADC*, *VfSAMDC*, *VfSPDS* and *VfSPMS* in Badii has been reported under PEG-treatment. The expression of of *VfADC*, *VfSAMDC* and *VfSPDS* is strongly induced by drought stress, while *VfSPMS* is slightly increased compared to the control. This could lead to the accumulation of PAs in leaves of drought-stressed Badii plants. These data corroborate other studies on the effect of drought stress, which reported a significant upregulation of *ADC*, *SAMDC* and *SPDS* (Alcázar *et al.* 2011). In addition, under drought application expressions of *VfADC*, *VfSAMDC*, *VfSPDS* and *VfSPMS* were irregular and significantly up- or downregulated in Spd-treated plants compared to non-Spd-treated plants. Overall, Spd treatment positively impacts the expression of PAs biosynthesis related-genes which could coincide with a relative accumulation of PAs during drought stress. Taken together, PAs accumulation could not be explained by the expression of PAs biosynthesis related-genes, because endogenous PAs level is regulated by complex processes including biosynthesis, degradation, conjugation and back-conversion (Fu *et al.* 2016).

Several studies reported that exogenous PAs treatment modulated the expression of various abiotic-stress response genes including genes encoding antioxidant enzymes like *CAT*, *SOD* and *APX*, resulting in better abiotic stress tolerance (Li *et al.* 2014; Marco *et al.* 2019). In the present study, 12 drought stress response genes including *VfCAT*, *VfSOD* and *VfAPX* were differentially expressed under drought stress in Spd-treated leaves and root tissue, suggesting that Spd could modulate the expression of studied genes, resulting in drought stress tolerance.

The results from qRT-PCR analysis in leaf and root tissue showed that PEG treatment significantly affected *VfCAT*, *VfSOD* and *VfAPX* transcript levels and these antioxidant enzymes exhibited different expression patterns. Moreover, under drought stress, application of some Spd concentrations strongly affected gene transcript levels encoding antioxidant enzymes and significantly increased their expression compared to non-Spd treated plants. These data suggested that increased expression of *VfCAT*, *VfSOD* and *VfAPX* could have partly influenced or improved CAT, SOD and APX activities, which resulted in lower generation of $H_2O_2$ and MDA content and improved cell membrane stability by lower electrolyte leakage (EL), thereby alleviating oxidative damage and consequently lead to better drought tolerance od Spd-treated plants. The data is consistent with the findings of Li *et al.* (2014) which reported that exogenous application of Spd induced the expression of genes encoding antioxidant enzymes (*Cu/ZnSOD*, *CAT*, *POD* and *APX*) with improving tolerance to drought stress.

It is well known that the plant hormone ABA acts as a regulator of plant responses to environmental stresses including drought. Marco *et al.* (2011) reported that PAs-overproducer plants overexpress some ABA-responsive genes. Recently, Marco *et al.* (2019) found that Spm plays a key role in modulating *Arabidopsis* salt stress responses through induction of ABA-responsive genes such as 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) which in turn modulates ABA levels. Moreover, several studies reported a positive correlation between the expression level of *NCED* gene and ABA content (Qin *et al.* 2019). Exogenous Spd significantly increased *VfNCED* levels in leaf and root tissue, which could lead to the increase of ABA levels in Spd-treated plants. These results suggested that Spd could improve faba bean tolerance to drought stress by regulation of ABA metabolism at its transcriptional level.

Several drought-responsive genes are regulated by ABA-dependent and independent pathways. The plant-specific *RD22*, an ABA-dependent drought-related gene was induced under drought stress in various plant species such as *Arabidopsis thaliana* (Harshavardhan *et al.* 2014). Thereby, *AtRD22* was used as a marker for drought stress tolerance. In *Arabidopsis*, the expression of PAs biosynthesis genes (*ADC1*, *ADC2*, *SPDS1*, *SPDS2*, *SPMS*, *ACL5*, *SAMDC1* and *SAMDC2*) and drought inducible genes *RD29A* and *RD22* was upregulated suggesting that under drought stress, the transcriptional regulation of PAs biosynthesis was modulated by ABA level (Alcázar *et al.* 2011). PEG-treatment induced *VfRD22* gene in leaves and roots tissue compare to control and Spd-treated tissues exhibited the highest increase in expression suggesting that increased

ABA levels, to which *VfRD22* respond, has occurred more in Spd-treated than untreated tissues.

Paul and Roychoudhury (2017) reported that seed priming using PAs (Spm and Spd) is a potential approach for improved rice tolerance to salt stress through regulation of expression of salt stress-response genes involved in various metabolic pathways including late embryogenesis abundant (LEA) proteins encoding a large group of hydrophilic proteins with a major role in drought and other abiotic stress tolerances in plants. In higher plants, LEA proteins can be divided into eight subgroups including dehydrins (DHNs). Upregulation of *LEA* and *DHN* genes could confer drought stress tolerance in several plant species (Guo *et al.* 2019; Aduse Poku *et al.* 2020). Heatmap results clearly showed differential transcript abundance of *VfLEA* and *VfDHN* in leaves and roots of Spd-treated seedlings upon exposure to drought stress. In general, the expression levels of *VfLEA* gene were reported to be upregulated in leaves and roots, while the expression of *VfDHN* was significantly downregulated in leaf tissue and slightly induced in root tissue. Together, these results showed that Spd-induced *VfLEA* protein in faba bean could be responsible for improved drought tolerance. On the other hand, Spd-induced antioxidant defence and *VfDHN* expression might be decreased by scavengers of $H_2O_2$ (Li *et al.* 2015*b*).

Several previous studies reported that Spd induced the expression of stress-response genes in response to abiotic stress, which led to abiotic stress tolerance through activation of stress protection mechanisms in plants (Kasukabe *et al.* 2004; Cheng *et al.* 2012). Indeed, Spd acts as a stress-protecting compound and stress-signalling regulator. Among the stress-related genes, the transcription factors (TFs) like WRKY, ERF, NAC, MYB, DREB and bZIP regulate expression of functional genes.

Overexpression of *NAC*, *ERF*, *WRKY* and *MYB* transcription factor leads to enhanced drought stress tolerance in several plant species including wheat (Xue *et al.* 2011; Rong *et al.* 2014; Gao *et al.* 2018; Li *et al.* 2019). Overexpression of the *SPMS* gene leads to increased Spd content in leaves of *Arabidopsis thaliana* transgenic lines (Kasukabe *et al.* 2004). Interestingly, transgenic plants exhibited a significant increase in the expression level of *NAC*, *WRKY* and *MYB* transcription factors resulting in enhanced tolerance to multiple environmental stresses including drought. Moreover, exogenous application of Spd enhanced tolerance of tomato plants to high temperature stress during the ripening stage, which is associated with the up-regulation of some genes involved in stress response including *ERF* (Cheng *et al.* 2012). In this context, Liu *et al.* (2015*b*) reported that the arginine decarboxylase (*ADC*) gene, a member of PAs biosynthesis pathway, may be modulated by different transcription factor families, including WRKY and MYB proteins.

Under drought stress, treated Badii seedlings with Spd at some concentrations showed higher expression of *VfNAC*, *VfERF*, *VfWRKY* and *VfMYB* in leaf and root tissue compared to stress alone. These results could suggest that Spd as a signalling regulator might play an important role in regulation of Badii response to drought stress through modulation of *VfNAC*, *VfERF*, *VfWRKY* and *VfMYB* expression.

Heat shock proteins (HSPs) are a group of proteins serving as molecular chaperones playing an important role in protecting plants from damage induced by multiple environmental stresses such as drought. In several plant species, the expression pattern of HSPs was investigated under drought stress and the expression of several HSP members was found to be upregulated suggesting that HSPs were involved in the acclimation to drought stress (Aneja *et al.* 2015). In rice, *OsHSP50.2* showed drought tolerance in transgenic lines suggesting that HSPs play a pivotal role in drought stress response and tolerance (Xiang *et al.* 2018). According to Sagor *et al.* (2013), heat-stressed *Arabidopsis thaliana* showed accumulated PAs such as Spd, Put and Spm but not thermospermine (T-Spm). Therefore, exogenous application of PAs (Spm) significantly increased the expression of heat shock-related genes (*HSP101*, *HSP90*, *HSP70* and *HSP17.6*), ultimately leading to protection of *Arabidopsis* plants from heat stress-induced damage. In the current study, *VfHSP* was upregulated in leaves and roots of Badii drought-stressed plants compared to controls. However, its transcripts' abundance becomes higher in Spd-treated plants under drought stress, suggesting that Spd increases the accumulation of *VfHSP* transcripts triggered by drought stress, thus might increase tolerance to stress damage.

## Conclusion

This study showed that exogenous application of Spd could alleviate oxidative damage and has multiple roles in improving drought stress tolerance of Badii. The improved cell water status and photosynthetic performance and alleviation of oxidative damage of cell membranes demonstrated in the present work support that conclusion. Moreover, Spd showed induced accumulation of proline and soluble sugars which play a key role in osmotic adjustment leading to improving drought tolerance. Likewise, enhanced antioxidant enzyme activities (CAT, SOD, APX and GPX) and expression of related genes could ameliorate the deleterious effects of drought stress by scavenging ROS. The higher expression of ABA-responsive genes and other stress-related genes in leaf and root tissue suggested that Spd could be involved in ABA-dependent and independent stress response pathways. Taken together, these data supported the protective mechanism of Spd in *Vicia faba* under drought stress. Indeed, Spd applied at 1.5 mM was the most effective concentration in providing Badii seedlings drought tolerance.

However, before recommending this to farmers as an effective measure for mitigating drought injury, field experiments are required in order to investigate the Spd concentration for growth and productivity improvement in faba bean crop under drought stress.

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