

# Seed Priming with 24-Epibrassinolide Alters Growth and Phenylpropanoid Pathway in Flax in Response to Water Deficit

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## ABSTRACT

The aim of the present study was to determine the effects of seed priming with 24-Epibrassinolide (EBR) ( $10^{-8}$  M) on growth, biochemical, and molecular characteristics of *Linum usitatissimum* L. seedlings under Polyethylene Glycol (PEG) induced drought stress conditions. Imposition of flax seedlings to 6, 12, and 18% PEG declined germination rate, shoot length, root length, fresh and dry weights, and significantly increased phenolic content and PAL (Phenylalanine Ammonia Lyase) enzymatic activity while lowering flavonoid content. Application of EBR significantly increased the germination rate, shoot and root lengths, dry weight, fresh weight, and anthocyanin content whereas reduced phenolic content. At the molecular level, *CHS* (Chalcone Synthase) and *PAL* (Phenylalanine Ammonia Lyase) transcripts were upregulated under drought stress and even more expressed by application of EBR. In conclusion, EBR pre-sowing seed priming considerably alleviated damages caused by drought stress and improved growth parameters in *Linum usitatissimum* L. seedlings.

**Keywords:** Gene expression, *Linum usitatissimum* L., Physiological parameters, Water deficit.

## INTRODUCTION

Drought is a major environmental constraint limiting plant growth and crop production. The percentage of terrestrial areas under drought stress is rising and assumed to be doubled by the end of 21st century. Therefore, plant responses to drought, from perception to transcriptional and physiological changes, must be considered at a global biological level (Farooq *et al.*, 2009; Krishnan and Pereira, 2008). Drought stress promotes generation of secondary metabolites, such as phenylpropanoids, phenolic acids, flavonoids and anthocyanins, which are believed to have powerful radical scavenging ability to reduce photooxidative stress (Caliskan *et al.*, 2017). Phenolics provide structural integrity and scaffolding support to plants and contribute to the

regulation of seed germination and the growth (Kulbat, 2016). Flavonoids are responsible for attraction of pollinators, fruit dispersion, seed germination, growth and development of seedling, and protection of plants from different biotic and abiotic stresses (Samanta *et al.*, 2011). Anthocyanins, a class of flavonoids, are also known for their protective role in plants against various biotic and abiotic stresses, due to their powerful antioxidant properties (Liu *et al.*, 2018). They prevent lipid peroxidation, and maintain membrane integrity to decelerate cell senescence (Jiao *et al.*, 2012).

AT the molecular level, plants also respond to drought stress via alteration of gene expression and formation of complex network leading to modification of target proteins, which are responsible for physiological and biochemical adaptations

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(Tani *et al.*, 2019). PAL is the key enzyme in the phenylpropanoid pathway that converts phenylalanine to trans-cinnamic acid. The enzyme responds to biotic and abiotic stresses as well. PAL gene expression is influenced by a variety of environmental stresses, including UV irradiation, drought, wounding, and pathogen infection (Cass *et al.*, 2015).

Chalcone Synthase (CHS) is representative of plant polyketide synthase superfamily, which produce diverse flavonoids (such as anthocyanins). The enzyme regulates the first step in the flavonoid biosynthesis pathway considered as a part of plant developmental stages whose expression is induced in plants under stress conditions (Dao *et al.*, 2011).

Brassinosteroids (BRs), a class of plant hormones, have been proven to protect plants from abiotic stresses (Sharma *et al.*, 2017). 24-Epibrassinolide (EBR) was shown to significantly improve drought tolerance in plants by acting as signaling compound (Tanveer *et al.*, 2019). The hormone sustains plant growth via carbon assimilation rate enhancement, ROS reduction, solute accumulation and water relations (Tanveer *et al.*, 2018). BR also enhances pigment contents, photosynthetic efficiency, enzymatic, and nonenzymatic antioxidants (Sharma *et al.*, 2016a; Sharma *et al.*, 2016b). The ability of hormone to induce plant tolerance largely results from the interaction with other phytohormones (Bajguz, 2007).

Flax (linseed) belongs to the genus *Linum* (Linaceae family) and is one of the crop plants cultivated for its oil and fiber purposes (Millam *et al.*, 2005). The plant is being considered as a platform for the development of novel bio-products due to its adaptability and product diversity. In 2004, flax was grown in 47 countries (Smith and Jimmerson, 2005) and Canada had the highest production of flax in the world followed by China, USA, India and EU (Statistics Canada, 2006). Several basic nutritional and health- properties have been

attributed to this plant as a functional food (Fitzpatrick, 2007).

To the best knowledge of authors based on literature review, no research has been conducted on the effects of EBR on phenylpropanoid pathway in flax under drought imposition, at seedling stage. In this study, our main interest was to explore the ameliorative impacts of EBR in relation to drought stress through growth parameters and non-enzymatic antioxidants. Additionally, we investigated expression of two important genes involved in phenylpropanoid pathway, CHS and PAL, in ten-day old flax seedlings.

## MATERIALS AND METHODS

### Seed Treatment

Seeds of *Linum usitatissimum* L., TN-97-1 cultivar were obtained from the Agricultural Research, Education and Extension Organization of West Azerbaijan, Urmia, Iran. Seeds were sterilized with 70 % ethanol for 2 min, then with 50% sodium hypochlorite solution for 4 min, and washed four times with sterilized water thoroughly. The seeds were soaked in sterilized water and kept in the refrigerator for 3 days. Then, 1,500 seeds were soaked either in (i) Sterilized water, or in (ii) EBR ( $10^{-8}$  M) (Sigma Alderich, Germany), at  $27\pm 2^{\circ}\text{C}$  for 24 hours under dark conditions. The appropriate concentration of EBR was selected based on previous publications (Rattan *et al.*, 2012; Derevyanchuk *et al.*, 2015). Afterwards, seeds were transferred to two sheets of sterile filter paper moistened with solutions including sterilized water, PEG (6%), PEG (12%) and PEG (18%) in sterile glass bottles (13 cm depth and 10 cm diameter) and allowed to germinate in the dark at  $27\pm 2^{\circ}\text{C}$  for 3 days (Ozdemir *et al.*, 2004). Eight glass bottles were considered per treatment, each contained 20 seeds. To make PEG (6%), 6 g PEG was dissolved in water and volume adjusted to 100 mL. The same method was applied for preparation of

PEG (12%) and PEG (18%). The number of germinated seeds was recorded at the end of 24, 48, and 72 hours after transfer into petri plates. After 3 days, bottles were transferred to light conditions for 7 days. The temperature of the growth chamber was maintained at  $27\pm 2^{\circ}\text{C}$  with light intensity of  $350\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ . Daytime humidity was between 60 and 70%. All measurements were carried out on 10 day old seedlings. Plants were harvested with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  freezer.

### Germination Rate and Growth Parameters

To calculate the germination rate, the number of planted seeds was recorded in each bottle. The number of germinated seeds was recorded after 24, 48, and 72 hours. A fraction comparing the number of germinated seeds to the number of planted seeds was calculated and shown as a percentage:

$$\text{GR} = \text{Germinated seeds} / \text{Total seeds} \times 100$$

Twenty seedlings from each treatment were sampled randomly at day 10, followed by measuring lengths and fresh weight of shoots and roots. For Dry Weight (DW) determination, samples were oven dried at  $70^{\circ}\text{C}$  for 72 hours and then weighed.

### Total Phenolic, Flavonoid, and Anthocyanin Contents

The total phenolic content of the extracts was determined using the Folin and Ciocalteu reagent, following the method described by Weidner *et al.* (2009) with slight modifications. Sample and standard readings were made using a spectrophotometer (APEL PD 303 UV-Vis Spectrophotometer, Japanese) at 765 nm against the blank reagent. The polyphenolic extract (125  $\mu\text{L}$ ) was mixed with 1.5 mL of water and 125  $\mu\text{L}$  of Folin-Ciocalteu's

phenol reagent (1:1). After 5 minutes, 1.25 mL of saturated sodium carbonate solution (8% w/v in water) was added to the mixture. The reaction was kept in the dark for 90 minutes and, after centrifugation, absorbance of blue color was measured at 765 nm.

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method (Quettier *et al.*, 2000). The sample contained 1 mL of methanol solution of the extract in the concentration of  $1\ \text{mg mL}^{-1}$  and 1 mL of  $\text{AlCl}_3$  (2%) solution dissolved in methanol. The samples were incubated for one hour at room temperature. The absorbance was determined using spectrophotometer at  $\lambda_{\text{max}} = 415\ \text{nm}$ . The same procedure was repeated for the standard solution of quercetin and the calibration line constructed.

Anthocyanin content was determined using the protocol of Abbas *et al.* (2013). 0.1 g of *Linum usitatissimum* L. samples were extracted for 1 day at  $25^{\circ}\text{C}$  in 1 mL of 1% (v/v) hydrochloric acid in methanol. The mixture was centrifuged at  $13,000 \times g$  for 10 minutes and the absorbance of the supernatant was measured at 530 and 657 nm. Anthocyanin concentrations were calculated using the formula  $[A_{530} - (1/4 \times A_{657})]$ . The anthocyanin content was defined as the product of relative anthocyanin concentration and the extract volume. One anthocyanin unit equals to one absorbance unit  $[A_{530} - (1/4 \times A_{657})]$  in 1 mL of the extraction solution.

#### Intracellular PAL Assay

For PAL assay, 0.1 g of plant sample was ground in ice-cold 0.1M Tris-HCl buffer (pH= 8.8) containing 1% polyvinylpyrrolidone and 1 mM EDTA. The homogenate was centrifuged at  $8,000 \times g$ , at  $4^{\circ}\text{C}$  for 10 minutes and supernatant tested for PAL activity. PAL activity was determined by monitoring the reaction product trans-cinnamate at 290 nm (Hura *et al.*, 2007). The reaction mixture contained 50 mM Tris-HCl (pH= 8.8), 20 mM L-phenylalanine, and enzyme in a total



volume of 2 mL. The reaction was allowed to proceed for 30 min at 30°C and was stopped by the addition of 0.5 mL of trichloroacetic acid (10%). One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.01 min<sup>-1</sup> under assay conditions. The activity was expressed in U g<sup>-1</sup> fresh weight.

### RNA Extraction and Semi-Quantitative RT-PCR

RNA samples were extracted by the CTAB method with slight modification (Gambino *et al.*, 2008). First-strand cDNA was synthesized in a 12 µL reaction system containing 1 µL oligo dT, 2 µL total RNA and 9 µL DEPC water at 65°C for 5 minutes. This was followed by addition of 2 µL dNTP, 1 µL reverse transcriptase, 1 µL RNase inhibitor and 4 µL reaction buffer at 42°C for 1 hour and 70°C for 5 minutes. RT-PCR reaction was performed according to the manufacturer's instructions, with gene specific primers (Table 1). The LuPAL and LuCHS primers were designed based on gene bank sequences. PCR reaction was conducted in 25 µL volumes containing 12.5 µL master mix, 1 µL cDNA, 0.75 µM of each of the primers (Table 1) and 10 µL H<sub>2</sub>O. The reactions were initiated at 95 °C for 3 min, followed by 28–30 cycle of 95°C for 25 seconds, 58–62°C for 30 seconds, 72°C for 25 seconds and a final extension at 72°C for 7 minutes. The intensity of PCR amplified bands was visualized under UV and measured using gel documentation system (Ingenius 3, Syngene, UK).

### DATA ANALYSIS

Data were analyzed using Image J, Excel 2010 and SPSS 22.0 softwares. Analysis of Variance (ANOVA) was conducted to compare the mean values among different treatments ( $P \leq 0.05$ ).

### RESULTS

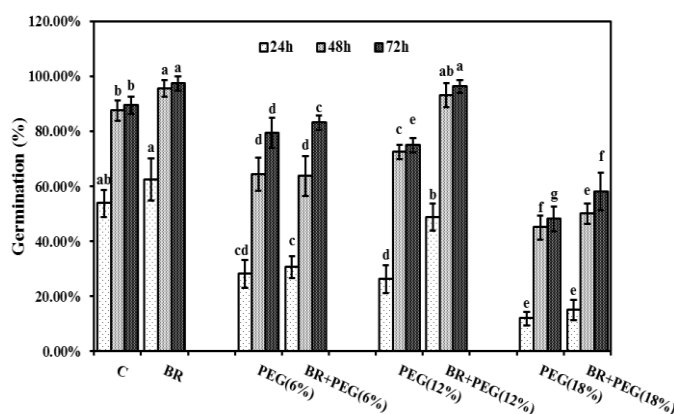
#### Germination Rate and Growth Parameters

The effect of osmotic stress induced by PEG on the germination rate of linseed is presented in Figure 1. The germination rate decreased 10, 14.37 and 41.25% under (6%), (12%) and (18%) PEG impositions, respectively, compared to the control. At PEG (18%), a drastic reduction in germination rate was noted. However, EBR application alleviated the inhibitory effect of PEG on germination of flax seeds (Figure 1).

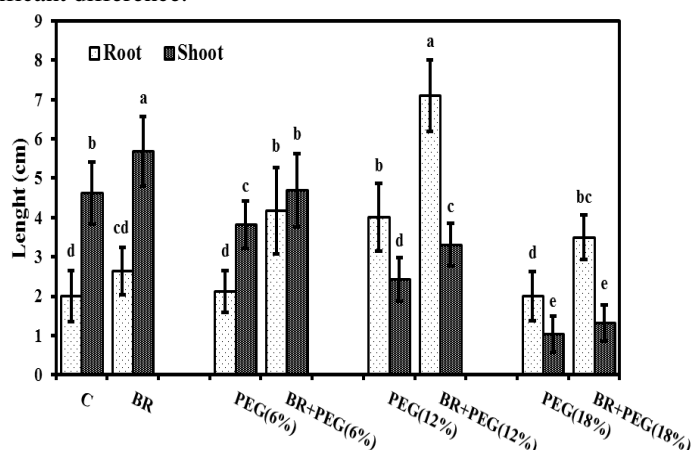
A strong negative correlation coefficient was noted between PEG concentration and shoot length, which decreased to 3.8, 2.4, and 1cm by exposing the seedlings to 6, 12, and 18% PEG, respectively, compared to the controls (4.6 cm). Whereas, EBR (10<sup>-8</sup>M) application improved the growth of linseed shoot to 4.6, 3.3, and 1.3 cm in BR+PEG (6%), BR+PEG (12%) and BR+PEG (18%) (Figure 2). Water deficit induced by PEG increased root length to 2.1 cm and 4 cm and decreased to 2 cm in seedlings exposed to

**Table 1.** List of primers used in RT-PCR study.

Gene name	Primer sequence (5'→3')	Gene bank	Size(bp)	Reference
<i>LuPAL</i>	FWD: CGGAACAATCACAGCTTCCGG REV: TCCTAGCCATTGAGGCGAAGTC	AY837828.1	294	This study
<i>LuCHS</i>	FWD: GGAGTGGACATGCCCGGAGC REV: CAGTATGGCAGGCCACCAGC	AY837832.1	543	This study
<i>LuEF1α</i>	FWD: GCTGCCAACTTCACATCTCA REW: GATCGCCTGTCAATCTTGGT	GR508911	140	(Huis <i>et al.</i> , 2010)



**Figure 1.** Germination percentage in *Linum usitatissimum* L. under drought stress with or without EBR application (C: Control). Values are mean±SE based on twenty replicates (n= 20). Different letters on the columns represent significant difference.



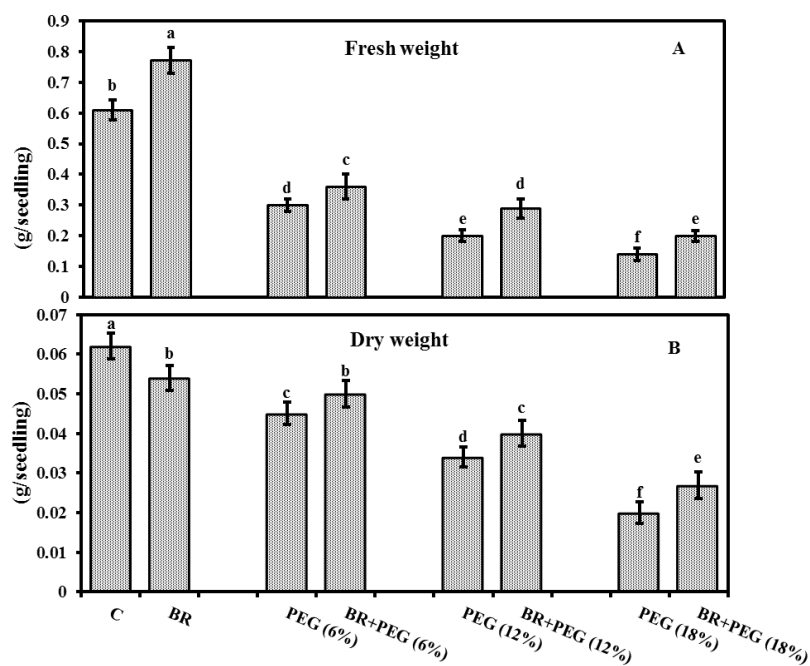
**Figure 2.** Root and shoot lengths (cm) in *Linum usitatissimum* L. under drought stress with or without EBR application (C: Control). Values are mean±SE based on twenty replicates (n= 20). Different letters on the columns represent significant difference.

PEG (6%), PEG (12%) and PEG (18%), respectively, compared to the control group (2 cm). However, EBR seed priming promoted root growth in BR 2.6 cm, BR+PEG (6%) 4.1 cm, BR+PEG (12%) 7 cm, and BR+PEG (18%) 3.5 cm (Figure 2).

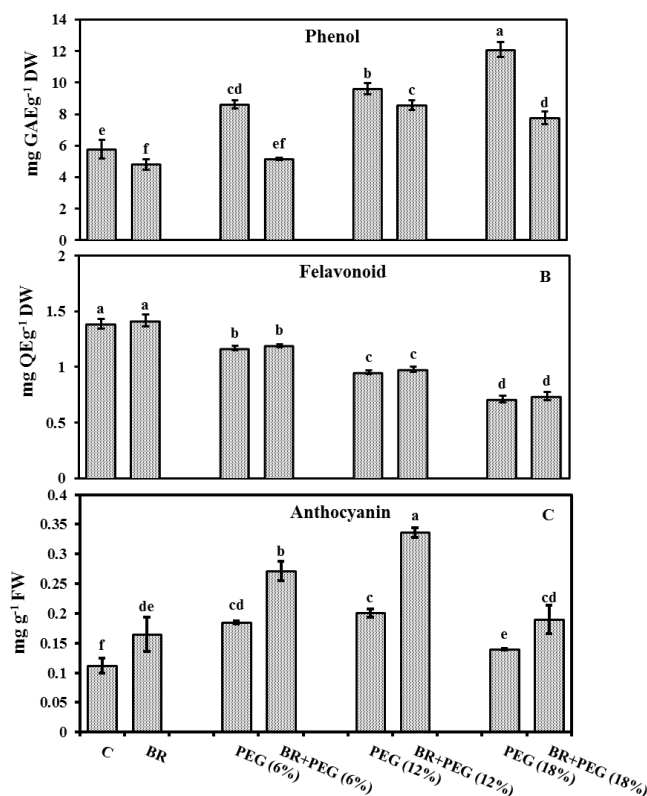
PEG- induced drought stress caused reduction in both FW and DW of seedlings, while EBR presoaking seed elevated FW and DW compared with the seedlings treated with PEG alone. The highest FW was recorded in BR (0.77 g) and the lowest was in PEG (18%) (0.14 g) (Figure 3-A). In case of DW, control with 0.062 g and PEG (18%) with 0.02 g had the highest and lowest amounts among treatments (Figure 3-B).

#### Determination of Total Phenolic, Flavonoid and Anthocyanin Contents

Figure 4-A shows the total phenolic content under different PEG treatments. Total phenolic content enhanced to 8.6 mg GAE g<sup>-1</sup><sub>FW</sub>, 9.6 mg GAE g<sup>-1</sup><sub>FW</sub>, and 12 mg GAE g<sup>-1</sup><sub>FW</sub> in PEG (6%), PEG (12%), and PEG (18%), respectively. The highest phenolic content was detected under PEG (18%), and the lowest observed under BR (4.80 mg GAE g<sup>-1</sup><sub>FW</sub>). Treatment with EBR reduced phenolic content to 5.1 mg GAE g<sup>-1</sup><sub>FW</sub> at BR+PEG (6%), 8.5 mg GAE g<sup>-1</sup><sub>FW</sub> at



**Figure 3.** (A) Fresh weight and (B) Dry weight (g) in *Linum usitatissimum* L. seedlings under drought stress, with or without EBR (BR) application (C: Control). Values are mean $\pm$ SE based on twenty replicates (n= 20). Different letters on the columns represent significant difference.



**Figure 4.** (A) Phenol (mg GAE g<sup>-1</sup> FW), (B) Flavonoid (mg QE g<sup>-1</sup> FW) and (C) Anthocyanin (mg g<sup>-1</sup> FW) in *Linum usitatissimum* L. Seedlings under drought stress with or without EBR (BR) application (C: Control). Values are mean $\pm$ SE based on six replicates (n = 6). Different letters on the columns represent significant difference.

BR+PEG (12%), and 7.7 mg GAE g<sup>-1</sup><sub>FW</sub> in BR+PEG (18%).

Flavonoid production was significantly lowered to 1.16, 0.95, and 0.71 mg QE g<sup>-1</sup><sub>FW</sub> by exposing seedlings to PEG (6%), PEG (12%), and PEG (18%), respectively, compared to the control group (1.4 mg QE g<sup>-1</sup><sub>FW</sub>) (Figure 4-B). However, application of EBR did not have much effect on flavonoid content and was reported as 1.41 mg QE g<sup>-1</sup><sub>FW</sub> in BR, 1.19 mg QE g<sup>-1</sup><sub>FW</sub> in BR+PEG (6%), 0.97 mg QE g<sup>-1</sup><sub>FW</sub> in BR+PEG (12%), and 0.73 mg QE g<sup>-1</sup><sub>FW</sub> in PEG (18%) +BR.

Seedlings grown under drought stress possessed comparatively higher anthocyanin level compared to the control seedlings (Figure 4-C). This value was reported 1.5, 2, and 1.2 fold higher under, respectively, PEG (6%), PEG (12%), and PEG (18%), compared to the control group. However, EBR application elevated the anthocyanin content with the highest identified in the BR+PEG (12%)-treated seedlings (0.33 mg g<sup>-1</sup><sub>FW</sub>).

#### PAL Enzymatic Activity

Seedlings raised with PEG exhibited a significant increase in PAL activity under PEG (6%) (67.5 U g<sup>-1</sup><sub>FW</sub>), PEG (12%) (76.8 U g<sup>-1</sup><sub>FW</sub>), and specially PEG (18%) (131.8 U g<sup>-1</sup><sub>FW</sub>) compared with the control group (39 U g<sup>-1</sup><sub>FW</sub>) (Figure 5). However, seed priming with EBR declined the PAL activity in BR+PEG (6%) to 61.16 U g<sup>-1</sup><sub>FW</sub>, BR+PEG (12%) to 66 U g<sup>-1</sup><sub>FW</sub> and BR+PEG (18%) to 119 U g<sup>-1</sup><sub>FW</sub>. EBR seed priming increased the enzyme activity in BR group to 52.8 U g<sup>-1</sup><sub>FW</sub>.

#### Gene Expression Analyses

The *CHS* transcript level was enhanced in PEG (6%), PEG (12%), and PEG (18%) to, respectively, 4.5, 5, and 2.8 fold, compared to the control group (Figure 7A). EBR seed priming significantly increased *CHS* gene expression in BR+PEG (6%) but decreased its mRNA level in BR+PEG (12%) and

BR+PEG (18%) groups. The highest *CHS* expression was observed in PEG (12%) group and the lowest was detected in the control group (Figure 6).

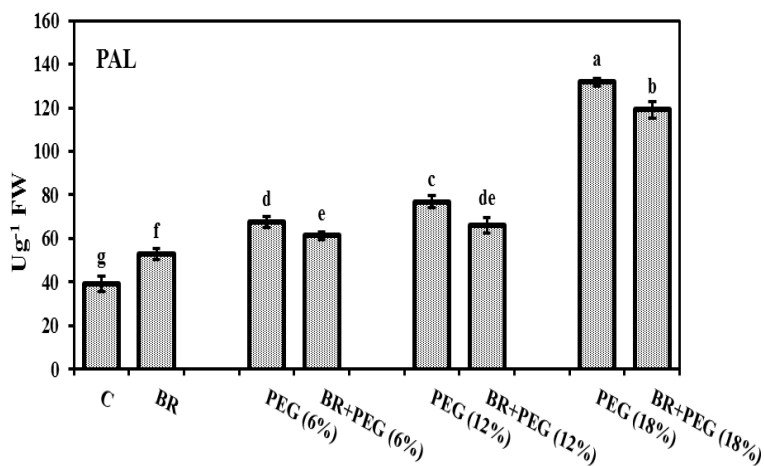
*PAL* gene expression increased 1.3 and 1.5 fold under PEG (6%) and PEG (18%), respectively, but did not alter under PEG (12%), compared to the control group (Figure 7B). However, EBR seed priming pretreatment enhanced *PAL* mRNA level in PEG (12%) and PEG (18%), but did not change *PAL* transcripts in PEG (6%). The highest *PAL* expression was observed in BR and BR+PEG (18%) groups and the lowest was found in the control group (Figure 6).

## DISCUSSION

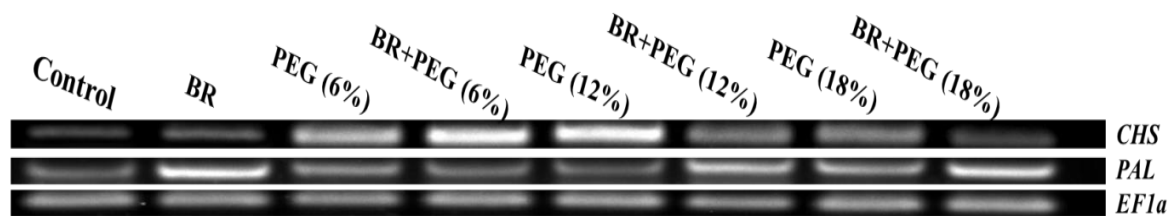
### Germination Rate and Growth Parameters

Germination is potentially the most critical stage of exposure to drought stress (Ahmad *et al.*, 2009). Drought reduces the osmotic potential of the seed and subsequently declines the germination rate (Shitole and Dhumal, 2012). In this study, drought stress resulted in delay and decrease of seed germination in flax. However, supplementation of EBR to PEG induced drought stressed seedlings enhance the seed germination showing the stimulatory role of EBR on seed germination promotion, which could be due to gibberellic acid biosynthesis (Steber and Mccourt, 2001).

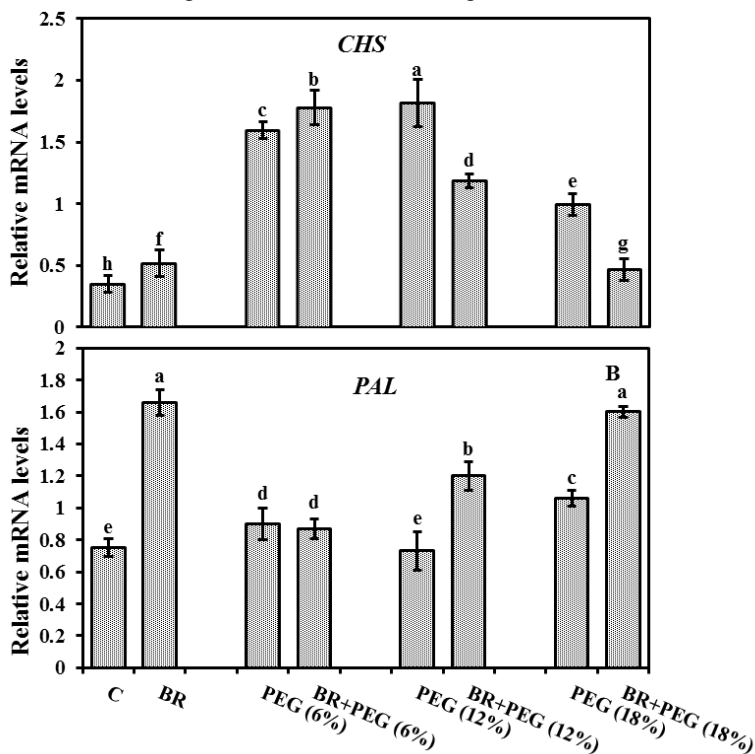
In the present study, growth of seedlings was severely impaired under drought stress, as reflected in reduced root and shoot lengths, whereas pre-sowing seed application of EBR improved seedling growth. BRs impinge root growth based on meristematic cell proliferation and elongation (González-García *et al.*, 2011). The crucial role of BR as key regulator in the optimal control of cell cycle progression (González-García *et al.*, 2011) and cell expansion (Chaiwanon and Wang, 2015) has already been shown. The shoot growth promotion of BR has also been attributed to enhancement in size and number of leaf epidermal and mesophyll cells since BR



**Figure 5.** PAL enzyme activity ( $U\ g^{-1}\ FW$ ) in *Linum usitatissimum* L. seedlings under PEG induced drought stress with or without EBR (BR) application (C: Control). Values are mean $\pm$ SE based on 6 replicates ( $n=6$ ). Different letters on the columns represent significant difference.



**Figure 6.** RT-PCR gene expressions of *Chalcone Synthase (CHS)*, *Phenylalanine Ammonia Lyase (PAL)* and *EF1a* in *Linum usitatissimum* L. seedlings under PEG induced drought stress with or without EBR (BR) application.



**Figure 7.** Relative mRNA levels of (A) *Chalcone Synthase (CHS)* and (B) *Phenylalanine Ammonia Lyase (PAL)* in *Linum usitatissimum* L. seedlings under PEG induced drought stress with or without EBR (BR) application.



loss-of-function mutants displayed significant reduction in cell size, leaf epidermal, and mesophyll cell number (Anwar *et al.*, 2018).

Drought stress is one of the most important factors affecting fresh and dry weights in plants. Under drought stress, accumulation of dry matter in all plant organs is reduced and leads to a loss in dry weight of the plant, although different plant organs show different degrees of decline (Asrar and Elhindi, 2011). Reducing dry matter may be due to a significant reduction in photosynthesis, decrease in plant growth (Shao *et al.*, 2008), and changing the amount of water or nutrients (Zokaee-Khosroshahi *et al.*, 2014). However, EBR seed priming enhanced fresh and dry weights under drought stress, which could be attributed to chlorophyll accumulation, amylase activity, stomatal conductance, photosynthesis (Anwar *et al.*, 2018), total protein contents, and membrane stability (Anwar *et al.*, 2018; Aghaee and Rahmani, 2019).

#### Total Phenolic, Flavonoid and Anthocyanin Contents

Phenolic compounds play an important role in the detoxification of free radicals. Thus, a common response to drought stress is an increase in synthesis of phenolic compounds (Ksouri *et al.*, 2007). In this study, phenolic compounds were accumulated in higher amounts in *Linum usitatissimum* L. seedlings under drought stress, as compared with the controls. This induction could be related to antioxidative role of phenolic compounds to scavenge ROS in plants under stresses (Fayez and Bazaid, 2014). However, phenolic content tended to decrease to almost control seedlings range under EBR application, which is in consistency with growth promotion due to decrease of oxidative stress and increase of antioxidative enzymes activity (Jiroutova *et al.*, 2018; Aghaee and Rahmani, 2019).

Flavonoids are plant secondary metabolites with important roles in plant physiology (Pourcel *et al.*, 2007). Various biotic and abiotic stress conditions affect the accumulation of flavonoids in plant vegetative tissues and organs (Braidot *et al.*, 2008). They are known as efficient metal chelators, and plants alleviate stress via formation of chelate complexes with flavonoids (Lachman *et al.*, 2005). In this study, phytochemical analysis revealed lower accumulation of total flavonoid content under drought stress. A uniform mechanism for flavonoid production and secretion has not been illustrated under stress due to the fact that the flavonoid accumulation is dependent on several parameters such as stress condition, frequency, duration, timing and plant species (Cetinkaya *et al.*, 2017). In the present study, EBR did not exert any influence on flavonoid accumulation under PEG-induced drought stress, indicating that ameliorative impacts of EBR are not induced via flavonoid production in flax. The mechanism of BR relevance in flavonoid content has largely remained unknown (Li *et al.*, 2017).

Anthocyanins are the colored water-soluble pigments in glycosylated forms, belonging to the phenolic group (Hock *et al.*, 2017). Anthocyanin biosynthesis, as branch of flavonoid pathway, includes genes such as *Chalcone Synthase (CHS)*, *Chalcone Isomerase (CHI)*, *Dihydroflavonol Reductase (DFR)*, *Leucoanthocyanidin dioxygenase (LDOX)*, and *UDP-glucose: Flavonoid-3-O-Glucosyl Transferase (UF3GT)* (Grotewold, 2006). Anthocyanins, as antioxidants, play an important role in water homeostasis (Sperdoui and Moustakas, 2012). They scavenge ROS and prevent oxidative damage in various plant species under stresses (Chen and Murat, 2002). The accumulation of anthocyanin pigments has been reported in response to various stresses such as drought, salinity, high light, chilling, UV-exposure, wounding, pathogen infection, pollution, osmotic stress, and



nutrient deficiency (Kovinich *et al.*, 2015; Winkel-Shirley, 2001). In the present study, drought stress increased anthocyanin content and EBR seed priming caused even more anthocyanin accumulation, revealing better oxidative stress management under hormone application. EBR enhancement of total anthocyanins content has also been shown in two varieties of pepper (*Capsicum annuum*) under salinity stress (Abbas *et al.* 2013).

### PAL Enzymatic Activity

Phenylalanine Ammonia Lyase (PAL) is a key enzyme of the phenylpropanoid pathway catalyzing the deamination of phenylalanine to trans-cinnamic acid (Cass *et al.*, 2015). Moreover, Phimchan *et al.* (2014) reported that under drought stress, PAL enzyme activity is enhanced as compared to the non-drought-stressed plants. In this study, PAL activity was enhanced to overcome detrimental impacts of drought stress via enhancing phenolics and anthocyanin contents. However, application of EBR decreased its activity and returned it to about the range of the control plants, indicating less oxidative stress upon hormone pretreatment. Our data shows consistency between fluctuation of PAL enzymatic activity and phenolic content. The phenolics are synthesized via phenylpropanoid pathway, in which PAL is a key and primary enzyme. Considering elevation in the anthocyanin content and reduction in phenolics upon EBR treatment in this study, it can be concluded that measured phenolics are related to phenolic compounds not including anthocyanins (Ayub *et al.*, 2018).

### Gene Expression

One of the most important enzymes in flavonoid/anthocyanin biosynthesis pathway is Chalcone Synthase (CHS) (Sanchez, 2008). *CHS* expression is often induced by biotic and abiotic stresses like elicitor,

pathogen, UV irradiation, and post-harvest wilting procedures (Vannozzi *et al.*, 2012; Versari *et al.*, 2001). In the present study, the expression of *CHS* was enhanced under PEG conditions indicating its drought responsiveness and showed consistency with anthocyanin content. However, application of EBR led to less *CHS* transcript level, which does not support higher accumulation of anthocyanine content. Since the activity of enzymes correlates strongly with the metabolite concentration, it can be concluded that regulation of anthocyanin synthesis in flax occurs at the post-transcriptional, translational or post-translational levels (Niu *et al.*, 2017).

Phenylalanine Ammonia Lyase (PAL, EC 4.3.1.5) is the key enzyme of the phenylpropanoid biosynthetic pathway (Cass *et al.*, 2015). In this study, expression of *PAL* was enhanced under drought stress, especially under PEG (18%) to increase phenolics production. Accumulation of phenolics as antioxidant compounds is one of the general responses to abiotic stresses in plants (Winkel-Shirley, 2001). EBR pre-sowing seed priming elevated *PAL* transcript level indicating that EBR is positive regulator of the *PAL* gene. However, there was a lack of correlation between PAL enzymatic activity and PAL transcript level under EBR application revealing that PAL regulation occurs at both transcriptional and post-transcriptional levels (Promyou *et al.* 2007).

Expression of *CHS* and *PAL* genes were reported to be associated together (Yuan *et al.*, 2012). However, our data on transcript levels of *CHS* and *PAL* support upregulation of *PAL* gene with downregulation of *CHS* mRNA level under application of EBR hormone (Figures 6 and 7). Interestingly, fluctuation in *CHS* transcript expression supports anthocyanin content.

### CONCLUSIONS

The present study showed that EBR seed priming improves seed germination, fresh

weight, dry weight, shoot and root lengths, with reduction in phenolic content and PAL enzymatic activity and induction of anthocyanin accumulation revealing less oxidative damage occurring under drought stress in flax plants. Since, water deficit adversely affects the global crop production, and impacts are getting more serious in the recent decades, BR treatment seems to be a promising solution to reduce the harmful effects of drought stress. Understanding BR's mechanism for reducing the severity of drought requires more research.

## REFERENCES

1. Abbas, S., Latif, H. H. and Elsherbiny, E. A. 2013. Effect of 24-Epibrassinolide on the Physiological and Genetic Changes on Two Varieties of Pepper under Salt Stress Conditions. *Pak. J. Bot.*, **45**: 1273–1284.
2. Aghae, P. and Rahmani, F. 2019. Biochemical and Molecular Responses of Flax to 24-Epibrassinolide Seed Priming under Drought Stress. *J. Plant Interact.*, **14**(1): 242-253.
3. Ahmad, S., Ahmad, R., Ashraf, M. Y., Ashraf, M. and Waraich, E. A. 2009. Sunflower (*Helianthus annuus* L.) Response to Drought Stress at Germination and Seedling Growth Stages. *Pak. J. Bot.*, **41**: 647–654.
4. Anwar, A., Liu, Y., Dong, R., Bai, L., Yu, X. and Li, Y. 2018. The Physiological and Molecular Mechanism of Brassinosteroid in Response to Stress: A Review. *Biol Res.*, **51**: 46.
5. Asrar, A. W. and Elhindi, K. M. 2011. Alleviation of Drought Stress of Marigold (*Tagetes erecta*) Plants by Using Arbuscular Mycorrhizal Fungi. *Saudi J. Biol. Sci.*, **18**: 93–98.
6. Ayub, R. A., Reis, L., Lopes, P. Z. and Bosetto, L. 2018. Ethylene and Brassinosteroid Effect on Strawberry Ripening after Field Spray. *Rev. Bras. Frutic.*, **40**(3): e-544.
7. Bajguz, A. 2007. Metabolism of Brassinosteroids in Plants. *Plant Physiol. Biochem.*, **45**: 95-107.
8. Braidot, E., Zancani, M., Petrusa, E., Peresson, C. and Bertolini, A. 2008. Transport and Accumulation of Flavonoids in Grapevine (*Vitis vinifera* L.). *Plant Signal. Behav.*, **3**: 626–632.
9. Caliskan, O., Radusiene, J., Temizel, K. E., Staunis, Z., Cirak, C., Kurt, D. and Odabas, M. S. 2017. The Effects of Salt and Drought Stress on Phenolic Accumulation in Greenhouse-Grown *Hypericum pruinatum*. *Ital. J. Agron.*, **12**(3).
10. Cass, C. L., Peraldi, A., Dowd, P. F., Mottiar, Y., Santoro, N., Karlen, S. D., Bukhman, Y. V., Foster, C. E., Thrower, N., Bruno, L. C., Moskvin, O. V., Johnson, E. T., Willhoit, M. E., Phutane, M., Ralph, J., Mansfield, S. D., Nicholson, P. and Sedbrook J. C. 2015. Effects of Phenylalanine Ammonia Lyase (PAL) Knockdown on Cell Wall Composition, Biomass Digestibility, and Biotic and Abiotic Stress Responses in Brachypodium. *J. Exp. Bot.*, **66**(14): 4317–4335.
11. Cetinkaya, H., Kulak, M., Karaman, M., Karaman, H. S. and Kocer, F. 2017. "Flavonoids from Biosynthesis to Human Health: Flavonoid Accumulation Behavior in Response to the Abiotic Stress: Can a Uniform Mechanism Be Illustrated for All Plants?" (EDs): Gonçalo Deira Duarte de Campos Justino and Pedro Boto Pereira Franco Pinheir, Publisher: Intech.
12. Chaiwanon, J. and Wang, Z. Y. 2015. Spatiotemporal Brassinosteroid Signaling and Antagonism with Auxin Pattern Stem Cell Dynamics in *Arabidopsis* Roots. *Curr. Biol.*, **25**: 1031–1042.
13. Chen, T. H. and Murata, N. 2002. Enhancement of Tolerance of Abiotic Stress by Metabolic Engineering of Betaines and Other Compatible Solutes. *Curr. Opin. Plant Bio.*, **5**: 250–257.
14. Dao, T. TH., Linthorost, H. J. and Verpoorte, R. 2011. Chalcone Synthase and Its Functions in Plant Resistance. *Phytochem. Rev.*, **10**: 397-412.
15. Derevyanchuk, M., Litvinovskaya, R., Khripach, V., Martinec, J. and Kravets, V. 2015. Effect of 24-Epibrassinolide on *Arabidopsis thaliana* Alternative Respiratory Pathway under Salt Stress. *Acta Physiol. Plant.*, **37**: 215.



16. Farooq, M., Basra, S. M. A., Wahid, A. and Rehman, H. 2009. Exogenously Applied Nitric Oxide Enhances the Drought Tolerance in Fine Grain Aromatic Rice (*Oryza sativa* L.). *J. Agron. Crop Sci.*, **195**: 254–61.
17. Fayez, K. A. and Bazaid, S. A. 2014. Improving Drought and Salinity Tolerance in Barley by Application of Salicylic Acid and Potassium Nitrate. *J. Saudi Soc. Agric. Sci.*, **13**: 45–55.
18. Fitzpatrick, K. 2007. Innovation in Western Canadian Functional Food. *Cereal Foods World.*, **52**: 289–290.
19. Gambino, S., Perrone, I. and Gribauto, I. 2008. A Rapid and Effective Method for RNA Extraction from Different Tissues of Grapevine and other Woody Plants. *Phytochem. Anal.*, **19**: 520–525. 20.
20. González-García, M. P., Vilarrasa-Blasi, J., Zhiponova, M., Divol, F., Mora-García, S., Russinova, E. and Caño-Delgado, A. I. 2011. Brassinosteroids Control Meristem Size by Promoting Cell Cycle Progression in *Arabidopsis* Roots. *Development*, **138**: 849–859.
21. Grotewold, E. 2006. The Genetics and Biochemistry of Floral Pigments. *Ann. Rev. Plant Biol.*, **57**: 761–780.
22. Hock, E., Azrina, A., Sou, T. T. and See, M. L. 2017. Anthocyanidins and Anthocyanins: Colored Pigments as Food, Pharmaceutical Ingredients, and the Potential Health Benefits. *Food. Nutr. Res.*, **61**(1): 1361779.
23. Huis, R., Hawkins, S. and Neutelings, G. 2010. Selection of Reference Genes for Quantitative Gene Expression Normalization in Flax (*Linum usitatissimum* L.). *BMC Plant. Biol.*, **10**: 71.
24. Hura, T., Grzesiak, S., Hura, K., Thiemt, E. and Tokarz, K. 2007. Wedzony M. Physiological and Biochemical Tools Useful in Drought-Tolerance Detection in Genotypes of Winter Triticale: Accumulation of Ferulic acid Correlates with Drought Tolerance. *Ann. Bot.*, **100**: 767–775.
25. Jiao, Y., Jiang, Y., Zhai, W. and Yang, Z. 2012. Studies on Antioxidant Capacity of Anthocyanin Extract from Purple Sweet Potato (*Ipomoea batatas* L.). *Afr. J. Biotechnol.*, **11**: 7046–7054.
26. Jiroutova, P., Oklestkova, J. and Strnad, M. 2018. Crosstalk between Brassinosteroids and Ethylene during Plant Growth and under Abiotic Stress Conditions. *Int. J. Mol. Sci.*, **19**: 3283.
27. Kovinich, N., Kayanja, G., Chanoca, A., Otegui, M. S. and Grotewold, E. 2015. Abiotic Stresses Induce Different Localizations of Anthocyanins in *Arabidopsis*. *Plant Signal. Behav.*, **10**(7).
28. Krishnan, A. and Pereira, A. 2008. Integrative Approaches for Mining Transcriptional Regulatory Programs in *Arabidopsis*. *Brief. Funct. Genomic. Proteomic.*, **7**: 264–274.
29. Ksouri, R., Megdiche, W., Debez, A., Falleh, H., Grignon, C. and Abdelly, C. 2007. Salinity Effects on Polyphenol Content and Antioxidant Activities in Leaves of the Halophyte *Cakile maritima*. *Plant Physiol. Biochem.*, **45**: 244–249.
30. Kulbat, K. 2016. The Role of Phenolic Compounds in Plant Resistance. *Biotechnol. Food Sci.*, **80**(2): 97–108.
31. Lachman, J., Dudjak, J., Miholová, D., Kolihová, D. and Pivec, V. 2005. Effect of Cadmium on Flavonoid Content in Young Barley (*Hordeum sativum* L.) Plants. *Plant Soil Environ.*, **51**(11): 513–516.
32. Li, X., Zhang, L., Ahammed, G.J., Li, Z.X., Wei, J. P., Shen, C., Yan, P., Zhang, L. P. and Han, W. Y. 2017. Nitric Oxide Mediates Brassinosteroid-Induced Flavonoid Biosynthesis in *Camellia sinensis* L. *J. Plant Physiol.*, **214**: 145–151.
33. Liu, Y., Tikunov, Y., Schouten, R. E., Marcelis, L. F. M., Visser, R. G. F. and Bovy, A. 2018. Anthocyanin Biosynthesis and Degradation Mechanisms in Solanaceous Vegetables: A Review. *Front. Chem.*, **6**: 52.
34. Millam, S., Bohus, O. and Anna, P. 2005. Plant Cell and Biotechnology Studies in *Linum usitatissimum* L.: A Review. *Plant Cell. Tissue. Organ Cult.*, **82**: 93–103.
35. Niu, J., Zhang, G., Zhang, W., Goltsev, V., Sun, S., Wang, J., Li, P. and Ma, F. 2017. Anthocyanin Concentration Depends on the Counterbalance Between its Synthesis and Degradation in Plum Fruit at High Temperature. *Sci. Rep.*, **7**: 7684.
36. Ozdemir, F., Bor, M., Demiral, T. and Turkan, I. 2004. Effect of 24-Epibrassinolide on Seed Germination, Seedling Growth, Lipid Peroxidation, Proline Content and Antioxidant System of Rice (*Oryza sativa* L.) under Salinity Stress. *Plant Growth Regul.*, **41**: 1–9. 37.

37. Phimchan, P., Chanthai, S., Bosland, P. and Techawongstien, W. S. 2014. Enzymatic Changes in Phenylalanine Ammonia-Lyase, Cinnamic-4-hydroxylase, Capsaicin Synthase, and Peroxidase Activities in Capsicum under Drought Stress. *J. Agric. Food Chem.*, **62**: 7057–7062.
38. Pourcel, L., Routaboul, J. M. and Cheynier, V. 2007. Flavonoid Oxidation in Plants: from Biochemical Properties to Physiological Functions. *Trends Plant Sci.*, **12**(1): 29-36.
39. Promyou, S., Ketsa, S. and Van-Doorn, W. G. 2007. Effect of Surface Coating on Ripening and Early Peel Spotting in 'Sucrier' Banana (*Musa acuminata*). *New Zeal. J. Crop Hort.*, **35**: 259-265.
40. Quettier, D. C., Gressier, B., Vasseur, J., Dine, T., Brunet, C., Luyckx, M. C., Cayin, J. C., Bailleul, F. and Trotin, F. 2000. Phenolic Compounds and Antioxidant Activities of Buck Wheat (*Fagopyrum esculentum* Moench) Hulls and Flour. *J. Ethnopharmacol.*, **72**: 35-42.
41. Rattan, A., Kapoor, N. and Bhardwaj, R. 2012. Role of Brassinosteroids in Osmolytes Accumulation under Salinity Stress in *Zea mays* Plants. *Int. J. Sci. Res.*, **3**: 1822-7.
42. Samanta, A., Das, G. and Das, S. K. 2011. Roles of Flavonoids in Plants. *Int. J. Pharm. Sci. Tech.*, **6**(1): 12-35.
43. Sanchez, I. J. F. 2008. Polyketide Synthase in *Cannabis sativa* L. PhD. Thesis, Leiden University, Leiden, The Netherlands.
44. Shao, H. B., Chu, L. Y., Abdul-Jaleel, C. and Zhao, C. X. 2008. Water-Deficit Stress-Induced Anatomical Changes in Higher Plants. *C. R. Biol.*, **331**: 215-225.
45. Sharma, A., Kumar, V., Thukral, A.K. and Bhardwaj, R. 2016a. Epibrassinolide-Imidacloprid Interaction Enhances Non-Enzymatic Antioxidants in *Brassica juncea* L. *Indian J. Plant Physiol.*, **21** (1): 70-75.
46. Sharma, A., Thakur, S., Kumar, V., Kanwar, M. K., Kesavan, A. K., Thukral, A. K., Bhardwaj, R., Alam, P. and Ahmad, P. 2016b. Pre-Sowing Seed Treatment with 24-Epibrassinolide Ameliorates Pesticide Stress in *Brassica juncea* L. through the Modulation of Stress Markers. *Front. Plant Sci.*, **7**: 1569.
47. Sharma, A., Thakur, S., Kumar, V., Kesavan, A.K., Thukral, A. K. and Bhardwaj, R. 2017. 24-Epibrassinolide Stimulates Imidacloprid Detoxification by Modulating the Gene Expression of *Brassica juncea* L. *BMC Plant Biol.*, **17**: 56.
48. Shitole, S. M. and Dhumal, K. N. 2012. Effect of Water Stress by Polyethylene Glycol 6000 and Sodium Chloride on Seed Germination and Seedling Growth of *Cassia angustifolia*. *J. Pharm. Sci. Res.*, **3**: 528-531.
49. Smith, H. V. and Jimmerson, J. 2005. *Briefing*. Agricultural Marketing Policy Center, Montana State University, MO, USA.
50. Sperdouli, I. and Moustakas, M. 2012. Interaction of Proline, Sugars, and Anthocyanins During Photosynthetic Acclimation of *Arabidopsis thaliana* to Drought Stress. *J. Plant Physiol.*, **169**: 577–585.
51. Statistics Canada. 2006. Production Data of Field and Specialty Crops. Accessed: February 17, 2007. <http://www40.statcan.ca/101/cst01/prim11b.htm>, Ottawa, Ontario, Canada.
52. Steber, C. M. and McCourt, P. 2001. A Role for Brassinosteroids in Germination in *Arabidopsis*. *Plant Physiol.*, **125**: 763-769.
53. Tani, E., Chronopoulou, E. G., Labrou, N. E., Sarri, E., Goufa, M., Vaharidi, X., Tornesaki, A., Psychogiou, M., Bebeli, P.J. and Abraham, E. M. 2019. Growth, Physiological, Biochemical, and Transcriptional Responses to Drought Stress in Seedlings of *Medicago sativa* L., *Medicago arborea* L. and Their Hybrid (Alborea). *Agron. J.*, **9**: 38.
54. Tanveer, M., Shahzad, B., Sharma, A., Biju, S. and Bhardwaj, R. 2018. 24-Epibrassinolide; An Active Brassinolide and Its Role in Salt Stress Tolerance in Plants: A Review. *Plant Physiol. Biochem.*, **130**: 69-79.
55. Tanveer, M., Shahzad, B., Sharma, A. and Khan, E. A. 2019. 24-Epibrassinolide Application in Plants: An Implication for Improving Drought Stress Tolerance in Plants. *Plant Physiol. Biochem.*, **135**: 295-303.
56. Vannozzi, A., Dry, I. B., Fasoli, M., Zenoni, S. and Lucchin, M. 2012. Genomewide Analysis of the Grapevine Stilbene Synthase Multigenic Family: Genomic Organization and Expression Profiles upon Biotic and Abiotic Stresses. *BMC Plant Biol.*, **12**: 130.
57. Versari, A., Parpinello, G. P., Tornielli, G. B., Ferrarini, R. and Giulivo, C. 2001.



- Stilbene Compounds and Stilbene Synthase Expression during Ripening, Wilting, and UV Treatment in Grape cv. Corvina. *J. Agric. Food Chem.*, **49**: 5531–5536.
58. Weidner, S., Karolak, M., Karamac, M., Kosin'ska, A. and Amarowicz, R. 2009. Phenolic Compounds and Properties of Antioxidants in Grapevine Roots (*Vitis vinifera*) under Drought Stress Followed by Regeneration. *Acta Soc. Bot. Pol.*, **78**: 97–103.
59. Winkel-Shirley, B. 2001. Flavonoid Biosynthesis, a Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. *J. Plant Physiol.*, **126**: 485–93.
60. Yuan, Y., Liu, Y., Wu, C., Chen, S. and Wang, Z. 2012. Water Deficit Affected Flavonoid Accumulation by Regulating Hormone Metabolism in *Scutellaria baicalensis* Georgi Roots. *PLOS One*, **7**(10).
61. Zokaee-Khosroshahi, M., Esna-Ashari, M., Ershadi, A. and Imani, A. 2014. Morphological Changes in Response to Drought Stress in Cultivated and Wild Almond Species. *J. Sci. Technol.*, **1**(1): 79–92.

## تیمار دانه با هورمون ۲۴-اپی براسینولید رشد و مسیر فنیل پروپانویید را در کتان در پاسخ به تنش آب تغییر می دهد

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### چکیده

مطالعه حاضر با هدف تعیین تاثیر تیمار بذر با هورمون ۲۴-اپی براسینولید (EBR) ( $10^{-8}$  مولار) بر رشد، پارامترهای بیوشیمیایی و مولکولی دانه رست های کتان (*Linum usitatissimum* L.) تحت تنش خشکی ناشی از پلی اتیلن گلیکول (PEG) انجام شد. براساس نتایج به دست آمده در دانه رست های کتان تحت تنش خشکی (6%), (12%), (18%) PEG، جوانه زنی، طول ساقه و ریشه، وزن خشک و وزن تر کاهش یافت در حالی که افزایش معنی داری در میزان ترکیبات فنلی و فعالیت آنزیمی PAL (فنیل آلانین آمونیا لیاز) و کاهش در مقدار فلاونوئیدها مشاهده شد. استفاده از EBR سبب افزایش سرعت جوانه زنی، طول ساقه و ریشه، وزن خشک، وزن تر و محتوای آنتوسیانین و کاهش محتوای فنولی گردید. در سطح مولکولی، تحت تنش خشکی، رونویسی از ژن های CHS (چلکون سینتاز) و PAL (فنیل الانین آمونیا لیاز) افزایش یافت و استفاده از EBR سبب افزایش هر چه بیشتر بیان این دو ژن شد. به طور کلی، خیساندن قبل از کاشت بذر با EBR به طور قابل توجهی سبب کاهش خسارات ناشی از تنش خشکی شده و سبب افزایش پارامترهای رشدی دانه رست های کتان تحت تنش خشکی می گردد.