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Effect of hydropriming on *Trigonella foenum* callus growth, biochemical traits and phytochemical components under PEG treatment

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Abstract

The induction of secondary metabolites under osmotic stress is well documented. However, cell death is probably due to osmotic stress. This work tries to study the synergetic effect of hydropriming and polyethylene glycol (PEG) on enhancing the secondary metabolites production in fenugreek callus cultures without facing cell death. PEG initiates the stress and the hydropriming increase the plant cell response against the stress. Fenugreek calli were initiated from hypocotyl of two groups of seeds, the first was hydroprimed overnight before germination, the second remained dry. Three months old calli of the two groups were subcultured on media containing two different concentration of PEG (5, 10%). The calli growth, biochemical analysis, secondary metabolism keys, and secondary metabolites were determined after 4 weeks. PE induced oxidative stress, which increased the membrane lipid peroxidation and decreased cell viability and growth. Hydropriming enhanced the activity of antioxidant enzymes, regulating the reactive oxygen species level, accumulating the osmolytes and secondary products. Therefore the primed callus can tolerate the osmotic stress initiated with PEG. Consequently, cell biomass increased and not affected by PEG treatment. On the other hand, the calli from non-primed seeds have a significant decrease in fresh weight, and dry weight under the higher PEG treatment. The hydropriming protected the growth of the cells under PEG treatment with a high content of secondary metabolites and high antioxidant machinery. The synergetic effect of hydropriming and PEG can be used as a simple and low-cost way to produce valuable compounds in commercial industrial bioreactors.

Key message

The synergetic effect of hydropriming and PEG enhances the secondary metabolites production in fenugreek callus. PEG initiates the stress and the hydropriming improves the plant cell response against the stress.

Keywords Antioxidant · Flavonoids · Phenols · Saponin · Shikimic acid · Trigonelline

Introduction

Fenugreek, *Trigonella foenum-graecum* L. is a medicinally and economically important legume. Its cell culture has been reported for the isolation of important bioactive secondary metabolites (Ahmad et al. 2016).

Plant secondary metabolites play an important role in plant protection from various stresses. The synthesis of these compounds is low under normal conditions. Many biotechnological methods have been used to improve secondary metabolites production. One of these methods is the elicitation. It has been considered as the most efficient and easy to apply for increasing the production of desirable secondary metabolites

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from cell, organ and plant systems. Elicitation is the process in which the synthesis of secondary metabolites by the plants is activated to ensure their survival in various conditions (Thakur et al. 2019).

The polyethylene glycol (PEG) is an osmotic agent causing osmotic stress, biochemical and morphological changes. These changes lead to an increase in secondary metabolites production in plant cells and organ cultures (Masoabi et al. 2018). These biochemical changes commonly appeared as an increase in reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide (O^{2-}) and hydroxyl radicals ($OH^.$) causing oxidative stress. This oxidative stress results in dangerous damage at vital molecules in the cell such as degradation of membrane lipids, proteins, and nucleic acids, causing cell death (Guo et al. 2018). Thus, activation of antioxidant machinery is the most important defense response in living cells under stress conditions. This antioxidant machinery consists of enzymatic antioxidants e.g., superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and non-enzymatic antioxidant compounds e.g., ascorbate, tocopherol, carotenoids and other secondary metabolites (flavonoids and phenolics) (Sánchez-Rodríguez et al. 2011).

The induction of secondary metabolites under stress is well documented. However, cell death is probably due to the harmful effects of stress. The combined use of elicitors can be an effective way to enhance the secondary metabolites production in the plant cell, tissue and organ cultures without facing cell death. The first elicitor initiates the stress and the second one can increase the plant cell response against the stress (Sarmadi et al. 2018).

Hydropriming is a proven way in the elicitation of plant defense responses (Naguib 2019). It can probably stimulate secondary metabolites production in fenugreek callus cultures. To date, few studies have investigated the metabolic changes and biochemical responses of fenugreek tissue cultures under osmotic stress and, to the best of our knowledge; the interaction between the hydropriming and osmotic stress on the plant callus culture has not been investigated. The present study tried to gain insight into the effects of hydropriming and different levels of PEG on the growth, physiological, biochemical and metabolic traits of fenugreek callus culture for the first time. Our findings have application in the management and commercial exploitation of controlled fenugreek cell and tissue cultures for the semi-industrial production of the important pharmaceutical compounds.

Materials and methods

Plant material

Trigonella foenum-graecum (cv Giza 30) mature seeds were obtained from local markets in Egypt. The seeds were

botanically authenticated with voucher specimens deposited in the Cairo University Herbarium, Egypt. The seeds were divided into two groups for callus induction. The first remained dry (as it is obtained from the market) and the second was conditioned in between two towel papers wet with distilled water for 12 h for hydropriming and after that seeds were air-dried to avoid excess moisture.

Seed surface sterilization and germination

The two groups of *T. foenum-graecum* seeds were surface sterilized with 10% Formaline for 10 min, then 70% ethanol for 30 s, and then immersed in 95% NaOCl for 30 min with frequent shaking, after that rinsed in sterile distilled water several times. After surface sterilization, the seeds from each group were germinated on sterile moist filter paper at 28 °C in dark for 7 days.

Callus induction and culture establishment

We used the hypocotyls as explants of fenugreek (*T. foenum-graecum*) in this study for callus induction. Three hypocotyls pieces (2–3 mm away from the apex) were cultured on Murashige and Skoog (MS) medium (obtained from Sigma-Aldrich) supplemented with 2 mg/L 2,4-dichlorophenoxy-acetic acid (2,4-D), 0.2 mg/L 6-benzyl amino purine (BAP), 30 g/L sucrose, and 8 g/L agar (obtained from Sigma-Aldrich). The pH of the medium was adjusted to 5.7 ± 0.01. The medium was autoclaved at 120 °C and 108 kPa for 20 min. The explants on the media were incubated at 27 °C under a 16 h photoperiod. The light intensity was 1700 lx at the shelf surface.

Callus proliferation from around the edges of the explants was transferred for the establishment of stock cultures to subculture medium contains the same concentration of phytohormones as in the initiation medium, and maintained at the same induction conditions. Callus tissues were subcultured every 4 weeks on fresh medium for three times till obtaining suitable healthy callus mass for performing the different treatments.

Polyethylene glycol (PEG) treatment

Experimental treatments were prepared by adding different concentrations of PEG 6000 (obtained from Sigma-Aldrich) (5% and 10% w/v) to the subculture media. Homogeneous 3-month-old calli (3 g) as the inoculum were transferred to the baby food jars containing prepared media (25 mL) and incubated at the same previous conditions. The calli groups were arranged and marked as following:

C: Control calli initiated from hypocotyl of non-primed seeds and grow on MS media free of PEG.

P: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media free of PEG.

PEG 5: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 5% PEG.

PEG10: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 10% PEG.

PPEG5: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 5% PEG.

PPEG10: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 10% PEG.

The experiment was done with triplicates per treatment. Totally five jars were considered as experimental units in each replicate. The calli were harvested after 4 weeks for analysis.

Growth of treated calli

The callus samples were separated from the solid medium and weighed using citizen scale CY 204 to calculate their fresh weight (FW). They were then dried at 60 °C till constant weight for dry weight (DW) determination.

The plasma membrane permeability was determined as the electrolyte leakage percent according to Poovaiah and Leopold (1973). Calli pieces (0.1 g) immersed in 10 mL distilled deionized water in closed test tubes and incubated at 32 °C for 2 h. After incubation, the electrical conductivity of the solution (EC1) was measured, and then the tubes were autoclaved at 121 °C for 20 min, after that the electrical conductivity (EC2) was measured after cooling to 25 °C. Plasma membrane permeability % (The electrolyte leakage) was expressed following the formula: Plasma membrane permeability % (EL %) = EC1/EC2 × 100.

Biochemical traits

Reactive oxygen species and antioxidant machinery

Oxidative stress markers H_2O_2 content was determined according to the method of Alexieva et al. (2001). Fresh calli (1 g) was homogenized in 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 3800×g for 10 min. The assay mixture was 3 mL containing 0.5 mL 100 mM K-phosphate buffer (pH 6.8) and 2 mL KI reagent (1 M KI in fresh double-distilled water H_2O) added to 0.5 mL of the extract supernatant. The tubes were incubated in dark at room temperature for 1 h, and then the solution absorbance measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Malonyl dialdehyde (MDA) content (lipid peroxidation product) was evaluated according to the method described by Li (2000). Fresh calli (0.2 g) was homogenized in 1.5 mL 5% TCA. The homogenates were centrifuged at 13,000×g

for 20 min. A reaction mixture of 0.5 mL supernatant and 1 mL 20% TCA and 1 mL 0.5% TBA (thiobarbituric acid) was incubated at 95 °C in a water bath for 25 min. The reaction mixture was cooled immediately and centrifuged at 3800×g for 5 min. The absorbance of the supernatants was determined at 450, 532 and 600 nm, respectively. Calculation of MDA was based on the following formula:

$$MDA\ (\mu m/mL) = 6.45(A532 - A600) - 0.56\ A450$$

Antioxidant enzymes

Enzymes extraction

Fresh calli (3 g) was homogenized in 0.05 M cold phosphate buffer (pH 6.5) containing 1 mM EDTA, Na_2 and centrifuged at 3800×g for 10 min. The supernatant was completed 15 mL volume and used as calli extract and enzymes source (Ma et al. 2012).

Enzymes assays

Superoxide dismutase (SOD) activity was evaluated based on the nitro blue tetrazolium (NBT) reduction method (Beyer and Fridovich 1987). The reaction solution consists of 3 mL assay buffer (comprising of potassium phosphate buffer (50 mM, pH 7.8) and 9.9 mM L-methionine), 60 μL crude enzyme and finally 30 μL riboflavin (added last to initiates the reaction). The reaction solution was incubated under Fluorescent lamps at 25 °C for 7 min. The reaction was stopped by turning off the lamps. The absorbance of the blank and reaction solution was measured at 560 nm. SOD activities were evaluated with the following formula: SOD activity (%) = (1 − A/B) × 100 (A: absorbance of the sample; B: absorbance of blank).

One unit of SOD activity was defined as the amount of the enzyme that inhibited 50% of NBT photoreduction.

Catalase (CAT) activity was measured according to Kar and Mishra (1976). Assay mixture (5 mL) comprising 300 μM of phosphate buffer (pH 6.8), 100 μM of H_2O_2 and 1 mL of the crude extract was incubated at 25 °C for 5 min. The reaction was stopped by the addition of 10 mL 2% H_2SO_4. The residual H_2O_2 was titrated against 0.01 N $KMnO_4$ until a faint purple color persisted for at least 15 s. A blank activity was run at the same time, in which the enzyme activity was stopped at zero time. One unit of CAT activity was defined as the amount of enzyme that destroys 10 μmol H_2O_2.

Peroxidase (POX) was measured according to Upadhyaya et al. (1985). Assay mixture (5 mL) containing 300 μM of phosphate buffer (pH 6.8), 50 μM catechol, 50 μM H_2O_2 and 1 mL of crude enzyme extract was

incubated at 25 °C for 5 min. The reaction was stopped by the addition of 1 mL 10% H_2SO_4. The absorbance of the produced color was measured at 430 nm. One unit of POX activity was defined as the amount of enzyme that causes 0.01 change in the optical density.

Osmolytes determination

Proline content was determined according to the method of Bates et al. (1973). Calli (0.5 g) was homogenized in 10 mL 3% aqueous sulphosalicylic acid. The homogenate was centrifuged at 3800×*g* for 10 min. The reaction mixture consists of 2 mL of glacial acetic acid and 2 mL acid ninhydrin were added to 2 mL of supernatant in a glass test tube. The reaction mixture was incubated in a boiling water bath for 1 h. After incubation, the reaction was stopped by placing the tubes in an ice bath. Toluene (4 mL) was added to the reaction solution and stirred well for 20–30 s. The toluene layer was taken and absorbance of the produced color was measured at 520 nm. The amount of proline is calculated from a standard curve with known concentrations of proline.

Glycine betaine (GB) analysis was carried out according to the method of Grieve and Grattan (1983). Calli (0.5 g) was ground in 5 mL 0.05% toluene and mechanically shaken for 24 h at 25 °C, and then the homogenate was centrifuged at 3800×*g* for 10 min. The supernatant (0.5 mL) was mixed with 1 mL of 2 N HCl solution and then add 0.1 mL potassium tri-iodide solution (7.5 g iodine and 10 g KI in 100 mL of 1 N HCl). The reaction mixture was incubated in a shaking ice-cold water bath for 90 min. After incubation 2 mL of ice-cooled water was added, and then 10 mL of 1, 2 dichloroethane (chilled at − 10 °C) was added to it after gentle shaking. A continuous airstream was passed in the tubes for 1–2 min, two layers were separated the optical density of the organic lower layer was recorded at 365 nm. The amount of GB in the test sample as calculated from the standard curve.

Soluble carbohydrates were estimated according to the phenol sulphuric acid method of DuBois et al. (1956).

Calli (1 g) grounded in 5 mL dist. water and incubated in a boiling water bath for 30 min. After incubation, the homogenate was centrifuged at 3800×*g* for 10 min. The reaction solution contains 1 mL supernatant, 1 mL of phenol solution, and 5 mL of concentrated H_2SO_4 to each tube and then shake well, set a blank with 1 mL of water instead of the sample solution. After 10 min shake well and incubate in a water bath at 30 °C for 20 min. The absorbance of the developed color was read at 490 nm. Calculate the amount of total soluble carbohydrate present in the sample solution using a glucose standard curve.

Secondary metabolism keys

The shikimic acid concentration was determined according to Zelaya et al. (2011). Calli (0.2 g) were ground in 2 mL 0.25 M HCl and then centrifuged at 3800×*g* for 30 min. The supernatant (50 µL) reacted with 0.5 mL of a 1% periodic acid and incubated at room temperature for 3 h. After incubation 0.5 mL 1 M NaOH and 0.3 mL 0.1 M glycine were added. The absorbance of the solution was measured at 380 nm. The amount of shikimic acid was determined using the shikimic acid standard curve.

Phenylalanine ammonia lyase (PAL) activity was determined according to Rösler et al. (1997). Enzyme extract (0.5 mL) was added to 2 mL assay buffer (0.2 M sodium borate-NaOH, pH 7.7, containing 61 mM L-Phe, and the mixture was incubated at 40 °C for 1 h. The reaction was finished by the addition of 0.5 mL 6 M HCl. The absorbance of the solution was determined at 280 nm as a measure of the formed cinnamic acid.

Phytochemical components

The tannin, steroids, saponins and alkaloids contents in the calli extract were determined according to Harborne (1973) and modified by Trease and Evans (1996). Total flavonoid content was evaluated according to Pallab et al. (2013). The phenols content in the extract was determined according to Julkunen-Tiitto (1985).

Determination of tannin content Calli extract (2 mL) was pipette out and 0.3 mL 0.1 N $FeCl_3$ in 0.1 N HCl was added to it. Zero-point (0.3 mL) 0.0008 M potassium ferricyanide was also added. The absorbance of the solution was read at 720 nm. The concentration of tannin was calculated from the standard curve.

Determination of steroid content Calli extract (2 mL) was pipetted out and 2 mL of cholesterol color reagent was added. The solution was left to stand for 35 min. After incubation, the absorbance of the solution was taken at 550 nm. The concentration of steroids was calculated from the cholesterol standard curve.

Determination of saponin content Calli extract (0.5 mL) was evaporated to dryness. A fresh solution of vanillin acetic acid (5% w/v, 0.2 mL) and perchloric acid (0.8 mL) was added and kept at 70 °C for 15 min. The sample was cooled on ice for 20 s before adding glacial acetic acid (5 mL). The absorbance was read at 550 nm. The concentration of saponin was calculated from the sapogenin standard curve.

Determination of alkaloid content Calli extract (1 mL) was pipette out and 5 mL 60% H_2SO_4 added. They were mixed

and allowed to stand for 3 h. The absorbance of the resulting solution was taken at 565 nm. The concentration of alkaloids was calculated from the atropine standard curve.

Total flavonoid The assay solution consists of 0.3 mL sodium nitrite solution (5%) was added to 1 mL calli extract and incubated at room temperature for 5 min. After incubation, 0.3 mL aluminum chloride (10%) was added, then 2 mL 1 M NaOH was added. Finally, volume was completed up to 10 mL with distilled H_2O and mix well. The absorbance was recorded at 510 nm. The total flavonoid content of the extract was calculated from the quercetin standard curve.

Estimation of phenolic compound Calli extract (1 mL) mixed with 0.5 mL 2 M Folin-Ciocalteu reagent and 2.5 mL 20% Na_2CO_3. The mixture was kept stand at room temperature for 20 min. The absorbance was measured at 725 nm. The phenol concentration was determined from the pyrogallol standard curve. The data of total phenols of extract were calculated from the pyrogallol standard curve.

Trigonelline content This was determined according to Jyothi et al. (2017). The extract absorbance was measured at 265 nm against blank solution (phosphate buffer pH 6.5) using a UV spectrophotometer.

Statistical analysis

The experiments were carried out in a completely randomized design of 5 replicates per treatment and each treatment repeated in three sets. All results were analyzed by SPSS software (version 14). Data were expressed as mean ± SD. Comparison of mean values of the sample and the control was done using ANOVA test. $P < 0.05$ was considered to be significant.

Results

Effect of hydropriming on the growth of treated calli

Results in Table 1 showed the positive effect of hydropriming on the growth of the calli either under osmotic stress or not. The primed callus showed significantly higher growth than the control under normal conditions.

Under osmotic stress primed protected the growth of the calli. The primed calli group under polyethylene glycol (PEG) treatment showed a non-significantly decrease in the fresh and dry weight compared to that of the control group.

Priming interestingly protects the plasma membrane stability of the calli cells under osmotic stress. The primed calli group has a plasma membrane stability index non-significantly lower than that in the control (Table 1).

Effect of hydropriming on biochemical traits under PEG treatment

### Effect of hydropriming on reactive oxygen species and antioxidant machinery under PEG treatment

Oxidative stress markers Priming significantly decreased the H_2O_2 content in the non-treated primed calli group than that in the control. Priming can protect the calli from the oxidative stress under PEG treatment. Under PEG treatment H_2O_2 content in the primed calli group was non significantly higher than that in the control. On the other hand, H_2O_2 content in the non-primed group significantly increased under PEG treatment compared to that in the control (Fig. 1).

Interestingly malonyl dialdehyde (MDA) content as a measure for the lipid peroxidation showed a similar trend to the H_2O_2 content. under non-treatment conditions, the MDA content was significantly lower in the primed group than that in the control. MDA content under the PEG treatment increased significantly in the non-primed calli compared to that in the control. On the other hand under the

Table 1 The effect of hydropriming on calli growth and plasma membrane permeability under PEG treatment

Calli sample	PEG %	Fresh weight (g)	Dry weight (g)	Plasma membrane permeability (%)
Non-primed Calli	0% PEG	5.289 ± 0.483a	1.780 ± 0.293a	89.520 ± 1.020a
	5% PEG	3.450 ± 0.392b	1.510 ± 0.201b	63.520 ± 1.930b
	10% PEG	2.050 ± 0.572c	1.420 ± 0.332b	37.940 ± 2.930c
Primed Calli	0% PEG	9.450 ± 0.048d	2.540 ± 0.102c	91.730 ± 0.994a
	5% PEG	6.490 ± 0.190a	1.920 ± 0.092a	89.440 ± 1.024a
	10% PEG	5.540 ± 0.103a	1.690 ± 0.083a	88.560 ± 1.396a

Values are given as means of three replicates ± SD. Within a row, means followed by different letters are significantly different according to the ANOVA test
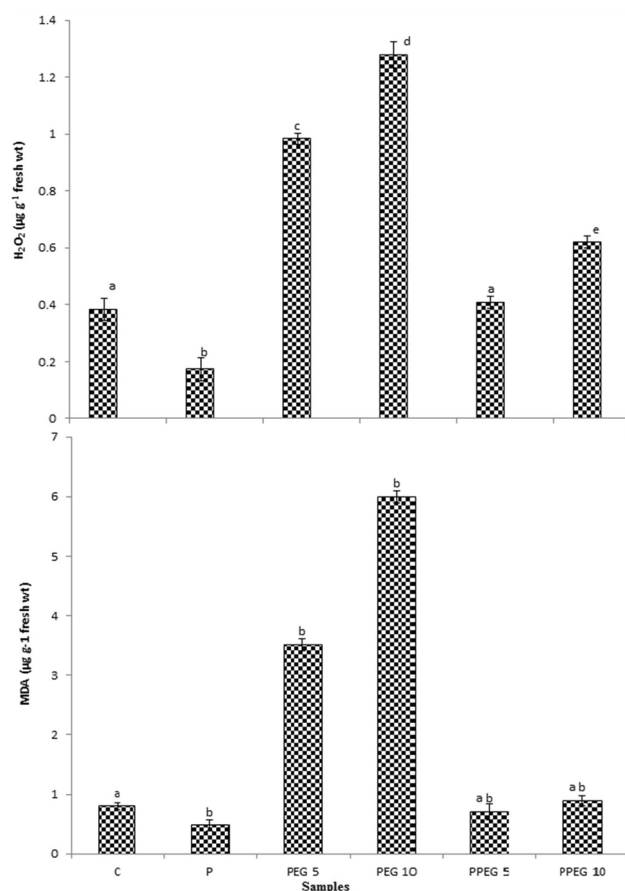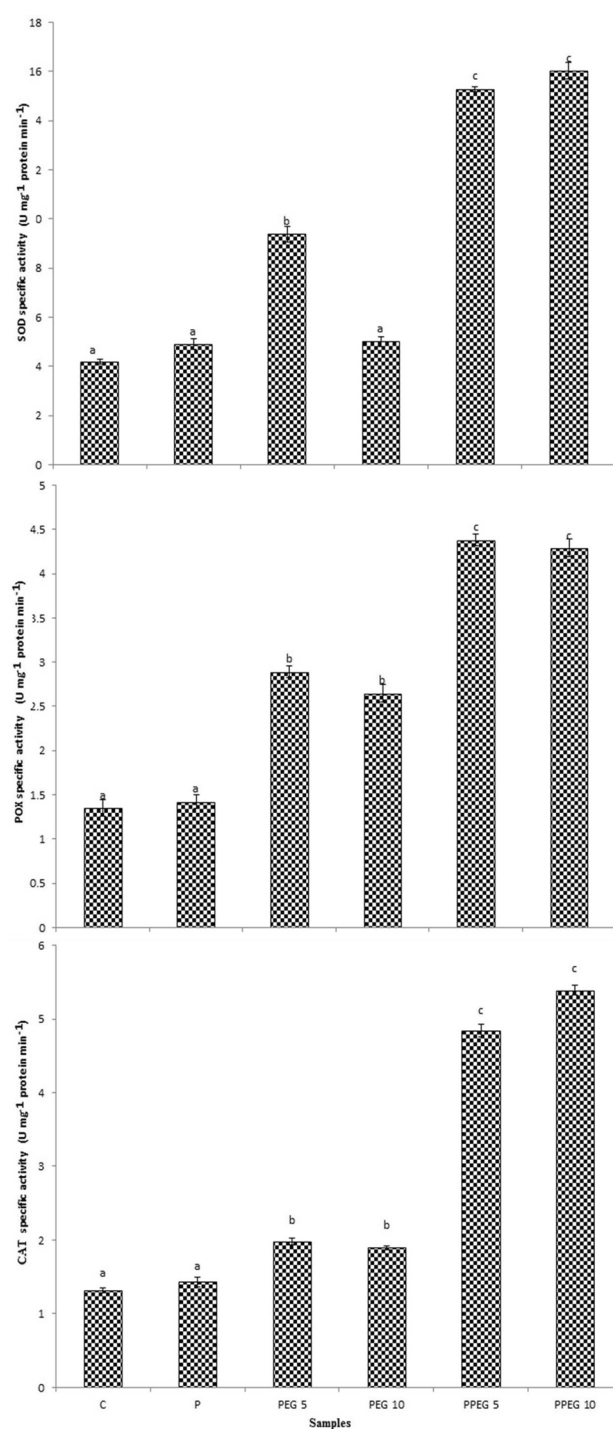
Fig. 1 The effect of hydropriming on H_2O_2 and MDA content in calli under PEG treatment. C: Control calli initiated from hypocotyl of non-primed seeds and grow on MS media free of PEG. P: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media free of PEG. PEG 5: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 5% PEG. PEG10: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 10% PEG. PPEG5: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 5% PEG. PPEG10: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 10% PEG

PEG treatment, MDA content in the primed group showed a non-significant increase compared to that in control (Fig. 1).

Antioxidant enzymes Priming enhanced the activity of the studied antioxidant enzymes under PEG treatment. The anti-oxidant enzymes showed the highest activity in the primed calli group under 10% PEG treatment (Fig. 2).

Effect of priming on osmolytes accumulation under PEG treatment

Priming increased the accumulation of the osmolytes (pro-line, glycine betaine, and soluble carbohydrates) in calli under PEG treatment (Fig. 3). The primed calli showed

Fig. 2 The effect of hydropriming on antioxidant enzymes [super-oxide dismutase (SOD), peroxidase (POX), catalase (CAT)] in calli under PEG treatment. C: Control calli initiated from hypocotyl of non-primed seeds and grow on MS media free of PEG. P: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media free of PEG. PEG 5: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 5% PEG. PEG10: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 10% PEG. PPEG5: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 5% PEG. PPEG10: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 10% PEG
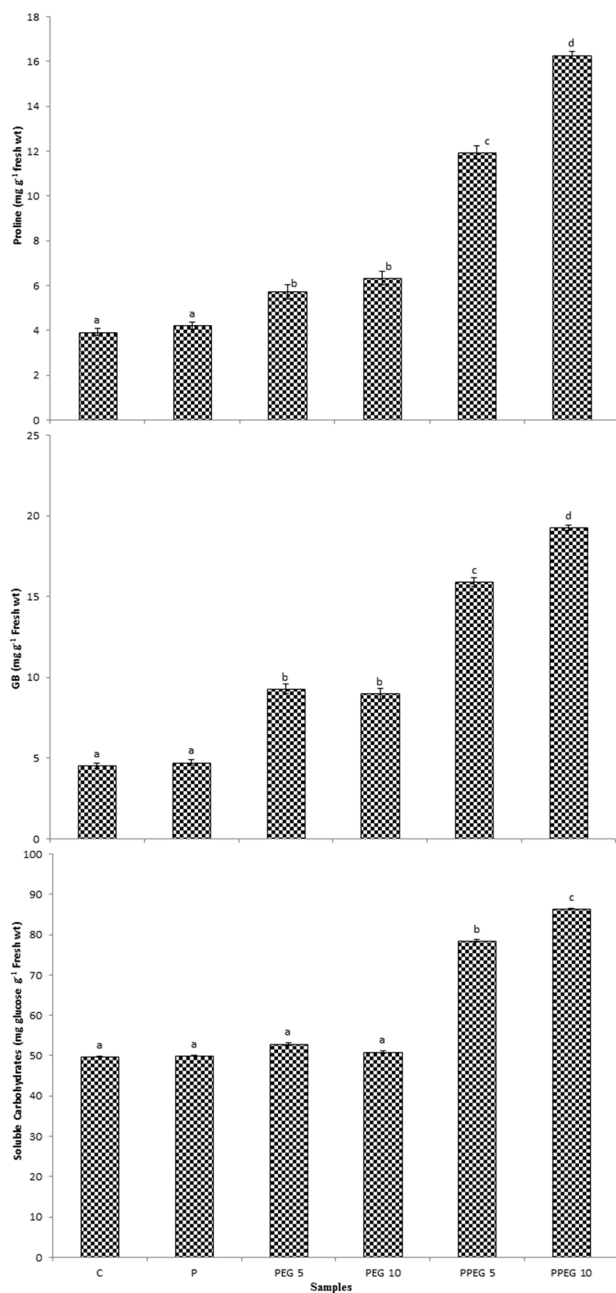
Fig. 3 The effect of hydropriming on osmolytes [proline, glycine betaine (GB) and soluble carbohydrates] content in calli under PEG treatment. C: Control calli initiated from hypocotyl of non-primed seeds and grow on MS media free of PEG. P: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media free of PEG. PEG 5: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 5% PEG. PEG10: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 10% PEG. PPEG5: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 5% PEG. PPEG10: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 10% PEG

the highest accumulation ratio about (315.116, 326.615, 73.650%) for proline, glycine betaine and carbohydrates respectively compared to that in the control. On the other hand in the non-primed calli, the accumulation of the osmolytes was in a low ratio in comparison to that in the control. The non-primed group accumulation ratio of proline and glycine betaine was 61.300 and 98.890% respectively, while the accumulation of carbohydrates was non-significant (about 2.330%).

Effect of priming on secondary metabolism keys under PEG treatment

The shikimic acid content significantly decreased under PEG treatment either in primed or non-primed calli, but the decrease ratio in the primed calli (about -80.6%) was significantly higher than that in the non-primed group (about -56.6%) (Fig. 4).

Phenylalanine ammonia lyase (PAL) activity showed a significant increase in the two groups under PEG treatment. The PAL activity in the primed group increased in a higher ratio (about 130.2%) than that in the non-primed group (about 47.4%) (Fig. 4).

Effect of hydropriming on phytochemical components under PEG treatment

Hydropriming significantly increased the phytochemical components in fenugreek calli under normal conditions. Under PEG treatment the phytochemical components significantly increased in both calli groups (primed and non-primed). In the case of the non-primed group, this significant increase was only under the low concentration of PEG. On the other hand in the primed calli the phytochemical components significantly increased under the two PEG concentrations (Fig. 5).

Trigonelline is an important active component in the fenugreek, hydropriming increased its production either under normal conditions or under the PEG concentrations (Fig. 6). Hydro priming increased its content under normal conditions about (70.6%) and under 5% and 10% PEG concentration about (239.3 and 244.4% respectively). On the other hand trigonelline content in the non-primed calli significantly increased only under 5% PEG treatment (about 70.6%) while under 10% PEG treatment its increase was non-significant (about 13.7%).

Discussion

The positive effect of hydropriming on activating the metabolic status of plant cells is well reported (Naguib 2019). This positive effect is ensured in the present study as the
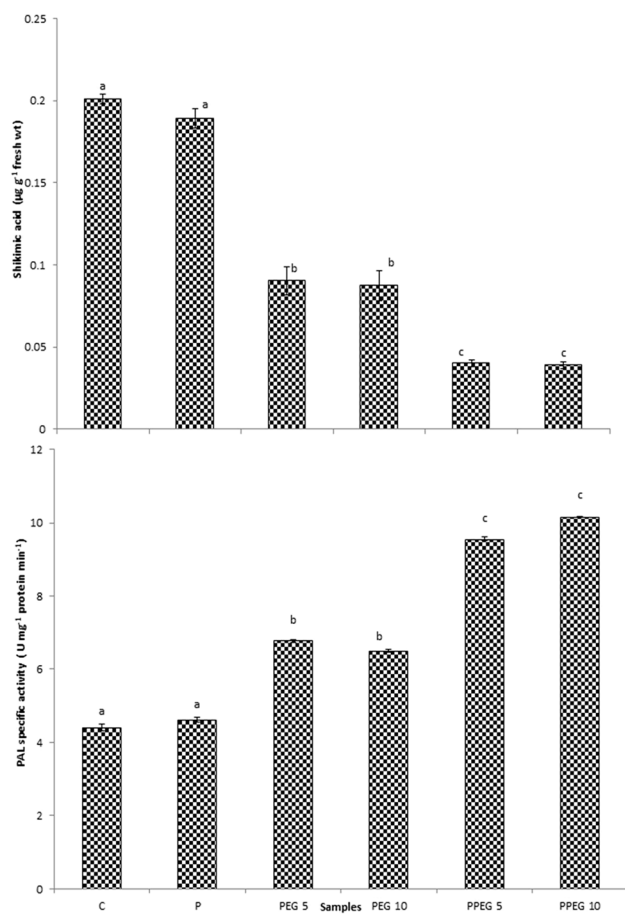
Fig. 4 The effect of hydropriming on secondary metabolism Keys [shikimic acid and phenylalanine ammonia lyase (PAL)] content in calli under PEG treatment. C: Control calli initiated from hypocotyl of non-primed seeds and grow on MS media free of PEG. P: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media free of PEG. PEG 5: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 5% PEG. PEG10: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 10% PEG. PPEG5: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 5% PEG. PPEG10: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 10% PEG

growth of calli initiated from primed seeds was higher than that of the calli initiated from dry seeds either under normal conditions or under polyethylene glycol (PEG) treatment. Calli growth under PEG treatment significantly decreased. This decrease in growth under drought stress can be attributed to the decrease in cell division due to oxidative stress. PEG treatment results in an increase in the reactive oxygen species (ROS) which results in the degradation of many vital biomolecules such as protein, DNA, lipids in membranes (Guo et al. 2018). The oxidative stress in the non-primed calli under PEG treatments is ensured with our results (Fig. 1). The H_2O_2 content highly significant increases in these calli under the two PEG concentrations. The harmful

effect of ROS accumulation has appeared in a significant increase in the malonyl dialdehyde (MDA) content (Fig. 1). Drought increases the level of free radicals and ROS causing peroxidation and bio-membrane degradation, which initiates oxidative stress (Karimi and Souri 2016). The severity of membrane damage is directly related to the intensity of the oxidative stress and the level of ROS, including H_2O_2 (Sarmadi et al. 2018). The membrane damage is significantly shown in the non-primed calli under PEG treatment (Table1). This damage increases the water and cell component losing and so cells lose its viability and die (Akula and Ravishankar 2011).

Priming induced PEG tolerance in priming calli. Priming can protect the calli cells from oxidative stress. This appeared in the non-significant increase in the H_2O_2 and MDA (Fig. 1), which helped in the protection of the membrane stability (Table 1). This is agreed with Rao and FTZ (2013) who reported that PEG tolerant callus line maintained membrane integrity under drought stress, on the other hand, sensitive callus line loosed its membrane integrity under PEG stress. This caused lower growth and metabolic disturbance. Lipid peroxidation activation under PEG treatment is also reported in other species like the bean (Yasar et al. ; 2010), blueberry callus (Ming et al. 2009).

The most important defense response against oxidative stress is the activation of the antioxidant machinery including, antioxidant enzymes such as superoxide dismutase (SOD), peroxidases (POX), catalase (CAT), glutathione reductase (Guo et al. 2018). Rao and Jabeen (2013) reported the enhancement of the CAT, POX and SOD activity in PEG tolerant sugarcane callus cultures. Others also reported the significant increase in activity of antioxidant enzymes in callus derived from drought tolerant explants, such as maize, tobacco (Bueno et al. 1998; Li and van Staden 1998). The decrease in the H_2O_2 content in the primed calli combined with the higher increase in the studied antioxidant enzymes (SOD, PPO, and POX) than that in the non-primed calli (Fig. 2). This is in harmony with the fact that the balance between production and elimination of reactive oxygen species at the intracellular level is an urgent need for cell survival under stress (Guo et al. 2018; Naguib 2018).

Besides, the regulation of antioxidant defense mechanisms in living cells to fight osmotic stress, plants have developed various mechanisms to counteract osmotic stress. One of these mechanisms is osmolytes or osmoprotectants accumulation in the plant cells in response to osmotic stress. Many osmotically active molecules such as sugars, proline, glycine betaine (GB), and organic acids get accumulated to balance the water relations under osmotic stress. The aggregation of these solutes in the cells under water stress results in highly negative osmotic potential, which causes endosmosis and protects the cell turgidity (Anjum et al. 2017; Sharma et al. 2019; Tanveer
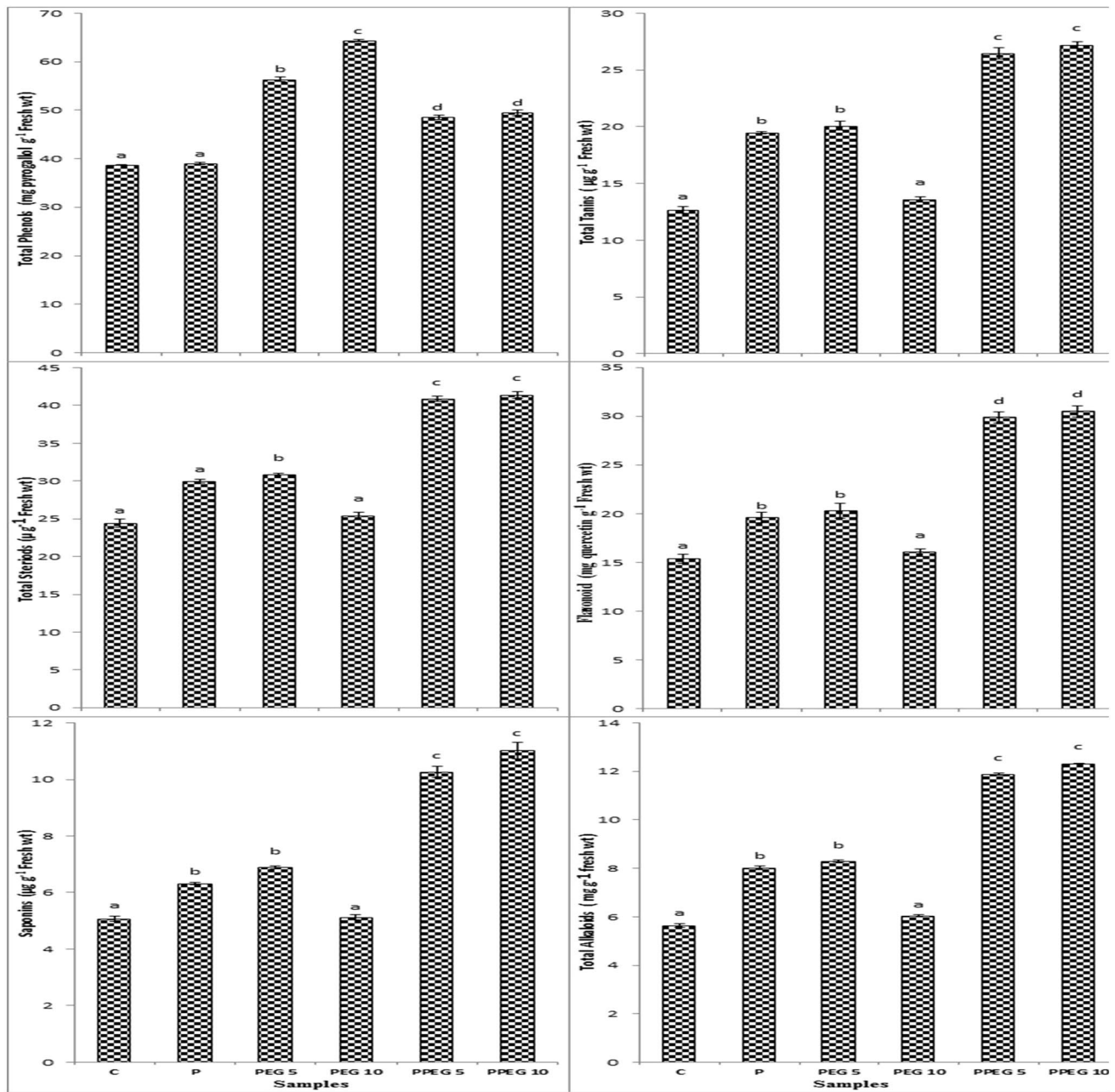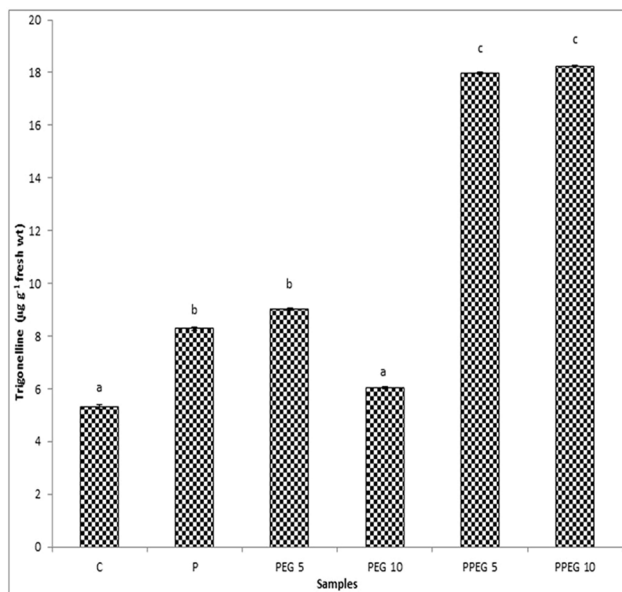
Fig. 5 The effect of hydropriming on Phytochemical components content in calli under PEG treatment. C: Control calli initiated from hypocotyl of non-primed seeds and grow on MS media free of PEG. P: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media free of PEG. PEG 5: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 5% PEG.

PEG10: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 10% PEG. PPEG5: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 5% PEG. PPEG10: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 10% PEG

et al. 2019). The importance of osmolytes accumulation such as proline, Gb and soluble carbohydrates has significantly appeared in the present study. Priming calli showing higher fresh and dry weight than that in non-primed one (Table 1), also showed higher content of osmolytes proline, GB and soluble carbohydrates. These results are

in correlation with Yamada et al. (2005) who showed that drought-tolerant variety of *Petunia hybrida* accumulated more free proline than the sensitive one under drought stress. Rao and Jabeen (2013) showed that proline accumulated in the PEG tolerant lines of sugarcane than that in the sensitive one. Similarly GB and soluble carbohydrates

Fig. 6 The effect of hydropriming on trigonelline content in calli under PEG treatment. C: Control calli initiated from hypocotyl of non-primed seeds and grow on MS media free of PEG. P: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media free of PEG. PEG 5: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 5% PEG. PEG10: Calli initiated from hypocotyl of non-primed seeds and grow on MS media with 10% PEG. PPEG5: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 5% PEG. PPEG10: Primed calli initiated from hypocotyl of hydroprimed seeds and grow on MS media with 10% PEG

accumulated in the tolerant varieties mores than in the sensitive one (Sharma et al. 2019).

Secondary metabolism is activated under stress factors to produce secondary metabolites such as phenolics flavonoids, saponins, alkaloids (Thakur et al. 2019; de Castro et al. 2020).

Shikimic acid and phenylalanine ammonia lyase (PAL) are considered as the key for the gate of secondary metabolism in plant cells (Sharma et al. 2019). Shikimic acid accumulates in inert metabolically tissues. Also, the shikimic acid decreases under stress as it is used in the synthesis of phenolic compounds which help in the resistance against the stress (Bochkov et al. 2012; Naguib 2018; Naguib and Abdalla 2019). The shikimic acid content decreased in the two calli groups under PEG treatment but in the case of non-primed samples, the shikimic acid content was significantly higher than that in the primed one (Fig. 4). The high significant decrease in the shikimic acid content in the primed calli under PEG treatment ensured the highest metabolic status of the primed calli cells as priming increases the metabolic activity of plant cells (Naguib 2018, 2019).

PAL has a critical role in converting the metabolism of phenylalanine from the primary to the secondary

phenylpropanoid/phenolic metabolism in plant cells, through catalyzing deamination reaction of this amino acid to form trans-cinnamic acid. This is the first and committed step in the phenylpropanoid pathway. Therefore the PAL has an extremely important role to run the resistance mechanisms against the toxic effects of stress factors (Gholizadeh and Kohnehrouz 2010). Here we showed that PEG treatment significantly increased the PAL activity in both calli groups. The PAL activity was significantly higher in the primed calli than that in the non-primed (Fig. 4). The results are in harmony with those obtained by Jóźwiak-Żurek et al. (2011) who confirmed that PAL activity increased under stress, but the response of the cucumber genotypes varied in intensity; it was higher in the more tolerant than in the sensitive genotype.

Accumulation of secondary metabolites occurs in the plant cells grown under various stresses, elicitors or signal molecules (Thakur et al. 2019). The results of the present study ensured the previous fact as both calli group under PEG treatment showed higher content of secondary metabolites (phenolics, saponin, tannins, steroids, alkaloids, and flavonoid) (Fig. 5). Primed calli showed higher content of these secondary metabolites. The highest content of secondary metabolites in the primed calli is due to its highest metabolic activity appeared in the lower content in the shikimic acid and the enhanced activity of PAL (Naguib 2019; Abas and Naguib 2019). The enhancement of secondary metabolites under PEG treatment is well reported by various works (Yamaner and Erdage 2013; Gupta et al. 2015; Sarmadi et al. 2018).

In general, fenugreek contains two important phytochemical constituents with medicinal value; (1) diosgenin, a kind of steroidal sapogenin and (2) trigonelline, a kind of N-alkaloid. The fenugreek anti-diabetic and hypocholesterolemic properties are attributed to the trigonelline content (Zhou et al. 2012). Priming significantly increased the trigonelline content either under normal conditions or under PEG treatment (Fig. 6). This correlated with Bitarafan et al. (2019) who reported the increase in the trigonelline in fenugreek under drought stress. Trigonelline is accumulating upon stress and acting as an osmoprotectant. Moreover, trigonelline has been found to function as a hormone that is involved in the control of the cell cycle in plants (Minorsky 2002).

Conclusion

This study considered being the first report for studying the synergetic effect of PEG and hydro priming treatments on fenugreek callus culture to enhance secondary metabolites production. PEG induced oxidative stress and enhanced free radicals like H_2O_2, the PEG treatment increased the membrane lipid peroxidation and decreased cell viability. As a

defense response to the deleterious effects of PEG treatment, the levels of secondary metabolites and trigonelline elevated, along with antioxidant enzyme activity and osmolytes. Hydropriming enhanced the activity of antioxidant enzymes, regulating the ROS level, accumulating the osmolytes and secondary products, thus improved the callus tolerance to drought stress initiated with PEG treatment; consequently, the cell biomass increased. The effects of hydropriming and PEG can be used as a simple and low-cost way to produce these valuable compounds in suspension cultures and commercial industrial bioreactors.

Data availability All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest We declare that we have no competing interests.

Ethical approval This research article does not contain any studies with human participants or animals performed by any of the authors.

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