



Exogenously Used Proline Offers Potent Antioxidative and Osmoprotective Strategies to Re-balance Growth and Physio-biochemical Attributes in Herbicide-Stressed *Trigonella foenum-graecum*

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Abstract

The present study was performed to verify that the exogenous application of proline as an antioxidant can effectively reduce the damage of the herbicide Basagran® on fenugreek (*Trigonella foenum-graecum*). Sterilized healthy seeds were soaked in proline (7 mM) with or without the herbicide Basagran® (10⁻⁴ M) applied as a foliar spray. Performance was evaluated based on the plant's physiological and biochemical attributes. Results revealed that herbicide stress caused seedling growth inhibition, which could be due to hydrogen peroxide (H₂O₂) accumulation with an increase in malondialdehyde (MDA) level and electrolyte leakage (EL). As a consequence, proline metabolism was affected, including the activity of proline dehydrogenase (PRODH) and pyrroline-5-carboxylate synthetase (P5CS), and levels of pyrroline-5-carboxylate (P5C) and proline (Pro) as well as glutathione (GSH) level, total antioxidant capacity (TAC), and activity of catalase (CAT) and glutathione-S-transferase (GST) were affirmatively influenced. However, the exogenous application of proline attenuated the harmful effects of Basagran® by improving growth performance, which might be related to enhanced antioxidant activities, total chlorophyll, relative water content (RWC), and GSH levels. Proline treatment also reduced the damage caused by increased MDA levels and reactive oxygen species (ROS) by regulating the enzymatic and non-enzymatic defense systems. Principal component analysis showed that increased oxidative damage and water imbalance were the most important contributors to herbicide stress-induced damage; however, the proline-mediated antioxidant defense was the crucial determinant of herbicide tolerance in fenugreek. Collectively, findings obtained from this study revealed that externally used proline protects against herbicide stress by enhancing cellular defense mechanisms. These results indicate the capacity of proline to improve the tolerance of fenugreek plants subjected to herbicide constraints.

Keywords Exogenous proline · Herbicide · Fenugreek · Growth · Oxidative stress · Antioxidants

Highlights

- Herbicide Basagran inhibited fenugreek growth.
- The herbicide affected fenugreek chlorophyll and carotenoid accumulation.
- MDA and electrolytes leakage increased following treatment with Basagran giving an index of oxidative stress.
- System antioxidant participated in response to the herbicide by changing in catalase CAT and glutathione-S-transferase GST activities.
- Herbicide affected proline metabolism, including the activity of proline dehydrogenase (PRODH) and pyrroline-5-carboxylate synthetase (P5CS), and level of pyrroline-5-carboxylate (P5C) and proline (Pro)
- Externally used proline protects against herbicide stress through enhancing cellular defense mechanisms.

1 Introduction

Herbicides are synthetic molecules used in agriculture to control non-economic weed plants. Their use has significantly improved crop productivity and the sustainability of agricultural systems in many parts of the world (Fang et al. 2015). However, herbicides are rarely completely selective and thus may pose chemical stress to cultivated plants, dampening the plant's resistance to the accumulation of reactive oxygen species (Tissut and Séverin 1984). The effectiveness of the herbicide depends on the specific dose applied, which can vary according to the target plant and the period of application. The spectrum of effectiveness

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corresponds to all species controlled by a product in a given dose (Scalla 1991; Tissut and Séverin 1984).

Herbicides can be classified in distinct ways, including the chemical name, chemical characteristics of the compound, toxicity, or mode of action. There are two principal classes of herbicides, contact herbicides and translocated herbicides (Au 2003).

The herbicide Basagran® [containing 480 g/L of bentazon sodium salt as the active ingredient (a.i.)] is one of the commercial formulations that is used internationally to efficaciously manage broadleaf weeds on several crops, especially in the most economically applicable ones, including rice and maize (Oliveira et al. 2017; Radwan et al. 2019). Basagran® is a contact herbicide that competes with plastoquinone for its binding site on protein D1, which blocks photosystem II (PSII) electron transport, leading to inhibition of photosynthesis and oxidative stress and subsequent weed cell damage (Radwan et al. 2019). It is used only as a post-emergence herbicide to control annual and perennial broadleaf weeds.

All herbicide molecules are supposed to be toxic to all plants. Herbicides generate abiotic stress that can lead to ROS buildup (Kim and Lee 2005; Boulahia et al. 2016). ROS have long been considered phytotoxic because they lead to cell damage due to peroxidation of membrane lipids, oxidation of proteins, inhibition of enzyme activity, and DNA damage. However, it is now recognized that they play an essential role in the life and death of plant cells because they can act as cellular signaling molecules to set up defense mechanisms against stress (Arora et al. 2002; Sewelam et al. 2016), whether of biotic or abiotic origin. These species are partially reduced or activated by oxygen derivatives, which include both free radical superoxide ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and non-radical form of hydrogen peroxide H_2O_2 , which cause cell damage, metabolic problems, senescence, structural and functional lack of cell organelles, and eventually death (Blokhina et al. 2003; Molla et al. 2014). According to Sharma et al. (2012), the toxicity of ROS is not necessarily correlated with their reactivity; in several cases, less ROS can cause significant toxicity due to their long half-life, allowing them to spread and reach sensitive sites where they can react and cause damage over a long distance from a place of production.

Plants contain two components of the defense system, enzymatic and non-enzymatic antioxidants, to cope with stressful conditions of ROS (Apel and Hirt 2004; Hossain et al. 2010; Molla et al., 2014). The main antioxidant enzymes consist of superoxide dismutase (SOD), catalase CAT, glutathione peroxidase (GPX), glutathione S-transferase GST, ascorbate peroxidase (APX), and glutathione reductase (GR) (Hossain et al. 2010; Zouari et al. 2016a, b, c), along with non-enzymatic

antioxidants, including ascorbic acid, tocopherols, polyphenols, carotenoids, and proline (Zouari et al. 2016a, b, c).

The plant defense system may not be sufficient, as plants accumulate osmolytes or compatible solutes along with activation of the enzymatic system to protect the cellular machinery from various environmental stresses (Sharma et al. 2019), so many anti-stress stimulants, including proline (Meena et al. 2019), can be used exogenously. Such products often provide innovative solutions by helping plants cope with chemical stress caused by herbicides. They can act by different mechanisms by stimulating the plant's physiology as well as its antioxidant system. Several studies have shown that proline is the most common compatible solute that accumulates under environmental stresses (Ashraf and Foolad 2007), mainly in the cytosol of cells where it actively participates in the plant's osmotic adjustment. Besides its role as an osmoregulator (Monneveux and Nemmar 1986), proline has a protective function in the cytoplasm by detoxifying free radicals and redox-correcting potential of plant cells (Meena et al. 2019). It can also regulate the cytoplasmic pH or as a carbon and nitrogen reserve for use by the plant during the stress period (Aggarwal et al. 2011).

Thus, exogenous proline applied as seed drenching or priming or as a foliar spray could be a cost-effective strategy to improve stress tolerance under adverse environmental conditions (Ashraf and Foolad 2007). Various reports have shown that exogenous use of proline stimulates tolerance to different abiotic stress through improving the efficiency of various components of plant oxidative defense system (Abdelhamid et al. 2013; Rady et al. 2016; Merwad et al. 2018; Semida et al. 2018; Meena et al. 2019).

This approach, which has a significant role in increasing plant performance in stressful environments, has now gained the attention of many researchers. To our knowledge, there have been no studies concerning the exogenous use of proline under herbicide stress conditions.

Much research has been carried out on the effects of herbicides on various cultivated plants (Rutherford and Krieger-Liszkay 2001; Xiao et al. 2008; Fayez et al. 2011; Boulahia et al. 2016). Regarding our study, fenugreek, an annual legume widely cultivated in most world regions, was chosen for its medicinal value. Fenugreek has strong adaptability to diverse climatic and soil conditions, making it suitable for cultivation in various habitats, including more than 20 Asian countries, Africa, Europe, America, and some regions of Australia; it promotes weight gain. Fenugreek, in certain forms, acts as an anti-inflammatory and is also known for its soothing properties and other essential uses (Chaudhary et al. 2018; Belmecheri-Cherifi et al. 2019; Mohammad et al. 2019).

The present study aimed to demonstrate the protective effect of exogenous proline applied as a seed soaking solution against the oxidative stress induced by the herbicide

Basagran® by altering the activity of antioxidant enzymes and the amount of antioxidant metabolites in fenugreek (*Trigonella foenum-graecum*) seedlings. Growth and physiological parameters as well as antioxidant (e.g., CAT and GST) activities were measured. The effect of the herbicide and proline on photosynthesis (chlorophyll, carotenoids) and proline metabolism was also analyzed concerning effects on ROS and lipid peroxidation (evaluated as malondialdehyde, MDA).

2 Material and Methods

2.1 Plant Materials and Experimental Layout

Basagran® (bentazone 48%) [3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide] belongs to the Benzothiadiazinone class of herbicides. According to the company's instructions, the herbicide was purchased from Syngenta Crop Protection, which is recommended for use in the field at the rate of 4L ha⁻¹. Fenugreek (*Trigonella foenum-graecum* L.) seeds were uniformly selected and sterilized for 3 min with a 1% sodium hypochlorite solution before being washed with distilled water. The seeds were then soaked in distilled water or 7 mM proline for 12 h at 26 ± 1 °C. Then, 50 proline-soaked seeds were placed on Petri dishes (10 dishes for each treatment) with water-moistened paper, and the same procedure was achieved for the same number of distilled water-soaked seeds. The Petri dishes of each treatment were placed in an incubator at 28 ± 1 °C for 2 days. After germination, fenugreek seedlings were transplanted into plastic containers containing peat and then installed in a growing room at 25 °C, photoperiod of 16 h/8 h. Distilled water was used to irrigate these containers. Three weeks later, identical seedlings were selected, grouped into four classes, and the groups were classified as follows:

Group 1: Control (C) treatment, in which the seeds were soaked in distilled water

Group 2: Proline (P) control treatment, in which the seeds were soaked in proline solution (7 mM) for 12 h

Group 3: Herbicide (H) treatment, in which the seedlings were sprayed with Basagran® at a concentration of 0.4 mL L⁻¹ for one time

Group 4: Herbicide + proline (HP) treatment, in which the seeds were soaked in proline solution (7 mM) for 12 h + the seedlings were sprayed with Basagran® at a concentration of 0.4 mL L⁻¹ for one time

The herbicide Basagran® solution was efficiently sprayed on both the upper and lower surfaces of the seedling leaves until saturation using a 2-L hand sprayer.

2.2 Growth Parameters

Four seedlings were selected indiscriminately from each of the four experimental groups to assess some growth parameters. The lengths of the above-ground and underground parts (expressed in cm) were measured with a ruler from crown to terminal bud and then from crown to root tip, respectively. Seedling roots were thoroughly washed with distilled water, quickly dried with absorbent paper, and separated from the aerial parts. The two separated parts were weighed, and the biomass obtained, which corresponds to the fresh matter, was expressed in g. The dry matter was obtained after steaming at 65 °C for 72 h (until the dry matter weight was stabilized).

2.3 Photosynthetic Pigment Contents

Photosynthetic pigments were assayed according to the method described by Lichtenthaler (1987). A 0.1-g sample of fresh plant leaf material was mashed in 80% acetone, and the extract was centrifuged at 5000 rpm for 15 min. The supernatant was collected for spectrophotometric determination of pigment contents by recording the readings at 663, 647, and 470 nm. All pigment contents were expressed as mg mL⁻¹ of extract solution.

2.4 MDA and H₂O₂ Content

Lipid peroxidation was determined by measuring the level of malondialdehyde. MDA was determined spectrophotometrically according to Alia and Saradhi (1991). A 0.1-g sample of fresh plant leaf tissue was cold-milled and homogenized in 2 mL of 0.1% TCA (trichloroacetic acid) solution. After centrifugation at 13,000 rpm for 15 min at 4 °C, 0.5 mL of the supernatant obtained was supplemented with 0.5 mL of TCA-TBA solution. All samples were placed directly into a water bath at 95 °C for 30 min, cooled on ice for 10 min, and then centrifuged at 10,000 rpm for 10 min. The optical densities of the TBA-MDA complex were measured at two wavelengths: 532 nm and 600 nm. The content of MDA is calculated by using an extinction coefficient ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$).

H₂O₂ content ($\mu\text{mol g}^{-1} \text{ FW}$) was determined according to Loreto and Velikova (2001). A 5-mL sample of 5% TCA was used to homogenize 0.25 g of fresh leaves. The homogenates were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and applied to a reaction medium of 10 mM potassium phosphate buffer (pH7.0) + 1 M KI. The absorbance was measured, spectrophotometrically, at 390 nm against H₂O₂ as a standard.

2.5 Electrolyte Leakage Assessment

Membrane integrity was assessed by the evaluation of relative EL. The electrical conductivity of leaf fragments of about 8 mm diameter was measured according to Dionisio-Sese and Tobita (1998). These fragments were taken from the same leaf for each treatment, these fragments were floated in 20 ml of distilled water, and their electrical conductivity (EC1) was measured after 60 min. They were then heated in a water bath for 5 min. After cooling, a second electrical conductivity reading (EC2) was taken.

$$EL(\%) = (EC1/EC2) \times 100$$

2.6 Estimation of Relative Water Content

The water status of the seedlings was assessed by measuring the RWC. For this purpose, the leaf fragment samples were weighted, indicating the initial fresh weight (FW). The fragments were then placed in Petri dishes containing 20 mL of distilled water and kept in the dark and cold. After 24 h, the leaf fragments were collected and gently wiped from the surface with absorbent paper and weighed again, indicating the weight in the case of full turgidity (TW). Finally, the samples were dried in an oven set at 80 °C for 48 h and weighed to register their dry weight (DW). The RWC is calculated according to the formula of Clarke and McCaig (1982):

$$RWC(\%) = [(FW - DW)/(TW - DW)] \times 100$$

2.7 Assessments of Proline and Pyrroline-5-Carboxylate

Similar to proline, P5C reacts with ninhydrin (Kim et al. 1994) in the widely used proline spectrophotometric method of Bates (1973). Purified P5C and L-proline were quantified using separate ninhydrin reactions. The proline-P5C values measured by the ninhydrin reaction were subtracted from the P5C values determined by the *O*-amino-benzaldehyde assay as follows: Leaf samples were homogenized in 3% (w/v) sulfosalicylic acid and then centrifuged for 10 min. Half of the supernatant was used for proline determination, and the other half was used to determine P5C by the *O*-amino-benzaldehyde assay at 440 nm (Mezl and Knox 1976). Known concentrations of L-proline and DL-P5C as 2,4-dinitrophenylhydrazine-hydrochloride-double salt were used to estimate proline and P5C concentrations in plant extracts (Miller et al. 2009). The free proline concentration in the sample was measured on a dry weight basis to obtain the content in $\mu\text{mol g}^{-1}$ DW.

2.8 Assays of P5CS and ProDH Activities

Protein was extracted for the P5CS enzyme activity assay according to Hayzer and Leisinger (1980) with a small modification (Huang et al. 2013). Briefly, a 0.2-g leaf sample was homogenized in 3.0 mL of ice-cold extraction buffer containing 0.5 M Tris-HCl (pH 7.5), 10 mM MgCl_2 , 2 mM phenylmethylsulfonyl fluoride, and 2% polyvinyl-pyrrolidone. The homogenate was centrifuged at $20,000 \times g$ for 20 min at 4 °C. The supernatant was extracted as a crude extract. A 1 mL crude extract was added to 3-mL reaction medium containing 50 mM Tris-HCl (pH 7.0), 20 mM MgCl_2 , 10 mM ATP, 100 mM hydroxamate HCl, and 50 mM L-glutamate to start the reaction. A 3 mL terminating solution containing FeCl_3 (5%, w/v) and trichloroacetic acid (12%, w/v) in 5 M HCl was added to stop the reaction after 30 min at 37 °C. The supernatant was recorded at 535 nm against the tube without ATP as a control. The enzyme activity was expressed as U g^{-1} FW. One U was defined for the enzyme content producing 1 μmol γ -glutamine in 1 min.

ProDH activity was assayed according to Lutts et al. (1999). Briefly, a 0.2-g leaf sample was homogenized in 3.0 mL ice-cold extraction buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM β -mercaptoethanol, 7 mM MgCl_2 , 3 mM EDTA (ethylenediaminetetraacetic acid), and 0.6 M KCl. The extract was centrifuged at $12,000 \times g$ for 20 min at 4 °C. As a crude extract, the supernatant was collected to determine the enzyme activity. A 0.2 mL crude extract was mixed with 0.15 mM $\text{NaCO}_3\text{-HCl}$ (pH 10.3), 15 mM L-proline, and 1.5 mM NAD^+ in a reaction mixture buffer. The result was given as U g^{-1} FW. One U was defined as the reduction rate of 0.01 A_{340} per min.

2.9 Assessment of Non-enzymatic Antioxidant Compounds and Total Antioxidant Capacity

The colorimetric approach was used to assay the GSH (Ellman 1959). The reaction of GSH with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) served as the basis for the principle, thus releasing the 5-thio-2-nitrobenzoic acid (TNB) absorbing at 412 nm. For this experiment, a 0.2 g fresh leaf sample was homogenized in 3 volumes of a 5% sulfosalicylic acid and then centrifuged at 13,000 rpm at 4 °C for 10 min. A 200 μL supernatant was dissolved in 1 mL of 0.2 M phosphate buffer (pH 8) and 100 μL of Ellman's reagent (0.04%) prepared in phosphate buffer. The resultant mixture was incubated at room temperature for 10 min. Absorbance at 412 nm was recorded using a spectrophotometer apparatus. The concentrations were deducted from a standard curve established with GSH.

The total antioxidant capacity of seedling extracts was determined according to the method of phosphomolybdenum. This method relies on the reduction of molybdenum:

Mo (VI) present in the form of molybdate ions (MoO_4^{2-}) to Mo (V) MoO^{2+} in the presence of the extract to form a green phosphate/Mo (V) complex at an acidic pH (Prieto et al. 1999). A 0.3 mL of every methanolic extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were pierced and incubated for 90 min at 95 °C. After cooling, the absorbance was recorded at 695 nm compared to the blank containing 3 mL of the reagent solution and 0.3 mL of methanol incubated under the same conditions as the sample. The total antioxidant capacity was expressed in mg equivalents of ascorbic acid by a gm of dry matter (mg EAA/g DM).

2.10 Determination of Enzymatic Antioxidants Activities

The catalase activity (CAT; EC 1.11.1.6) was determined by measuring the disappearance of H_2O_2 from the assay mixture using the method of Dorey et al. (1998). Extraction was carried out in 50 mM phosphate buffer (pH = 7.0). A UV–VIS spectrophotometer was used to calculate the disappearance of H_2O_2 . One CAT unit is specified as the enzyme amount required to decompose $1 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$ under the assay mentioned above conditions ($\epsilon = 36 \text{ M}^{-1} \text{ cm}^{-1}$).

Glutathione S-transferase (GST; EC 2.5.1.18) activity was measured according to the protocol defined by Habig et al. (1974). GST was reacted with 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of GSH, and the activity was estimated at 340 nm with a coefficient of molar extinction ($\epsilon = 9.6 \text{ M}^{-1} \text{ cm}^{-1}$).

2.11 Statistical Analysis

All treatments were conducted with at least four replicates. Results were expressed as means \pm standard deviation (SD). Data were subjected to a one-way analysis of variance (ANOVA). The Tukey's test (HSD) was used for significance analysis. Differences between treatments were

significant when the P value was less than 0.05 ($P < 0.05$). The *heatmap3* function of the heatmap package of R 3.6.1 was used for heatmap and hierarchical clustering considering Euclidean distances' preparation with the normalized mean values. XLSTAT 2016 was used to perform PCA and visualize the PCA as a biplot.

3 Results

3.1 Exogenous Proline Improves Growth Performance and Photosynthetic Pigment Content in Herbicide-Stressed Seedlings

The use of Basagran® herbicide negatively affected fenugreek growth parameters. However, the exogenously used proline significantly reduced the toxic effects of the herbicide (Table 1). Fenugreek seedlings treated with herbicide (without proline pretreatment) showed a significant reduction in SL, RL, SFW, RFW, SDW, and RDW (by 56.43%, 55.78%, 23.41%, 33.34%, 88.89%, and 85.72%, respectively), compared with the control seedlings. However, treatment of seeds with proline (group 4) relieved the toxic effects of herbicide stress and significantly increased SL and RL by 117.83% and 171.89%, respectively. Besides, SFW, SDW, RFW, and RDW were markedly increased by 108.3%, 50%, 100%, and 100%, respectively, compared with the corresponding control (the herbicide-stressed seedlings without proline, Table 1).

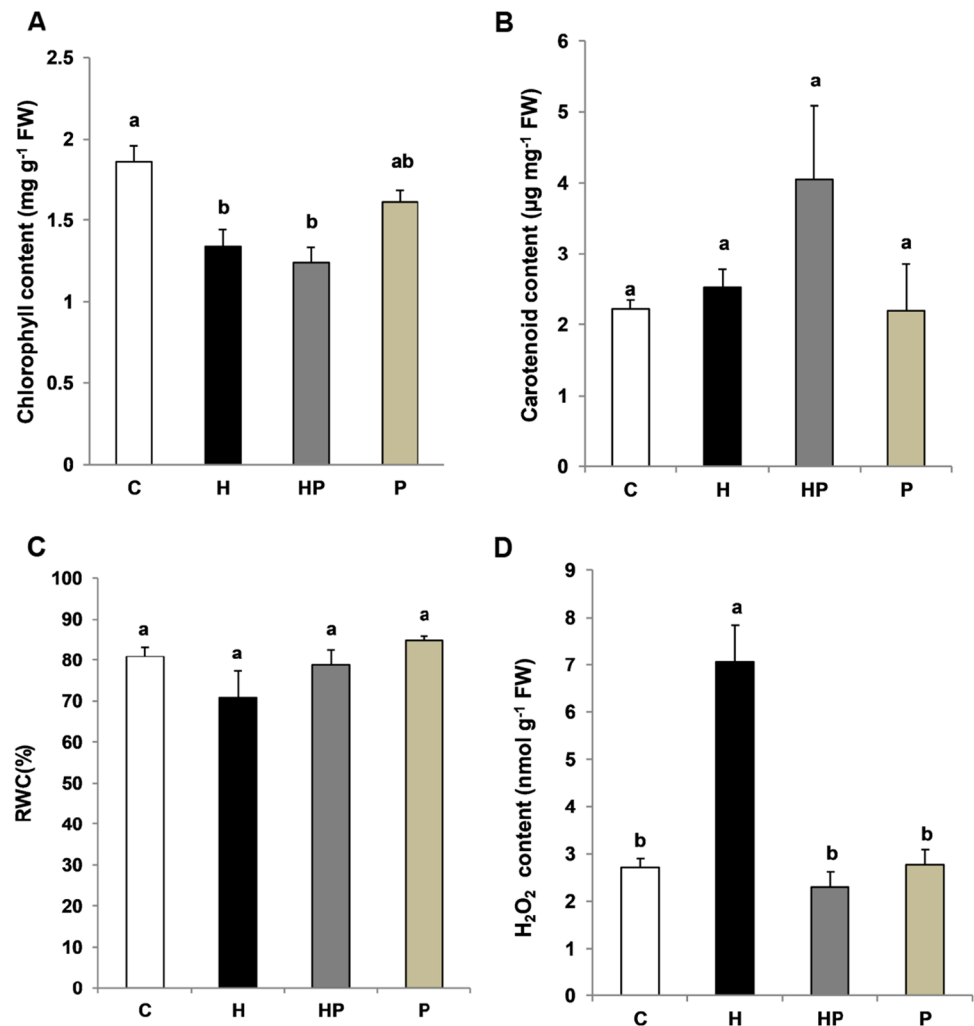
The use of Basagran® herbicide negatively affected fenugreek chlorophyll content, which reduced by 39.84% compared to the control (Fig. 1a). On the contrary, seed treatment with proline mitigated the effects of the herbicide and significantly improved the content of chlorophylls (Fig. 1a). In control plants, the carotenoid content was increased by 13%, while it was further increased by 39.84% in proline-pretreated seedlings that were sprayed with the herbicide compared to the herbicide-treated seedlings without proline treatment (Fig. 1b).

Table 1 Impact of externally applied proline 7 mM on roots and shoots length, roots, and shoots fresh and dry weight of *Trigonella foenum-graecum* seedlings stressed by Basagran® 10^{-4} M

Treatments	Parameters					
	Shoot length (cm)	Root length	Shoot fresh weight (mg)	Shoot dry weight	Root fresh weight (mg)	Root dry weight
Control	10 ± 1.08^b	8.63 ± 1.80^b	0.47 ± 0.08^b	0.032 ± 0.003^a	0.10 ± 0.01^b	0.008 ± 0.002^a
Herbicide	6.25 ± 2.33^b	5.38 ± 1.44^c	0.36 ± 0.11^c	0.021 ± 0.002^b	0.02 ± 0.01^b	0.002 ± 0.001^a
Pro + herbicide	13.025 ± 0.96^a	14.63 ± 2.36^a	0.75 ± 0.18^a	0.034 ± 0.011^a	0.23 ± 0.04^a	0.011 ± 0.003^a
Proline (Pro)	11.75 ± 1.19^a	12.88 ± 1.55^{ab}	0.43 ± 0.17^b	0.029 ± 0.005^a	0.14 ± 0.02^b	0.029 ± 0.043^a

Exogenous proline was applied by seeds soaking. Data presented are the means of 4 replicate determinations \pm SE. Values specified in different lowercase letters in each single column indicate significant differences according to Tukey's test ($P \leq 0.05$)

Fig. 1 Impact of externally applied proline 7 mM on contents of total chlorophyll (A), carotenoids (B), relative water content (RWC) (C), and H_2O_2 (D) of *Trigonella foenum-graecum* seedlings stressed by Basagran® 10^{-4} M. Control (C), herbicide-stressed (H), herbicide-stressed + proline treated (HP), proline control (P). Exogenous proline was applied to seeds by soaking. Data presented are the means of 4 replicate determinations. Vertical bars indicate standard errors. Different letters represent significant differences according to Tukey's test ($P \leq 0.05$)



3.2 Exogenous Proline Adjusts ROS Accumulation, Lipid Peroxidation, Electrolytes Leakage, and Relative Water Content under Chemical Stress

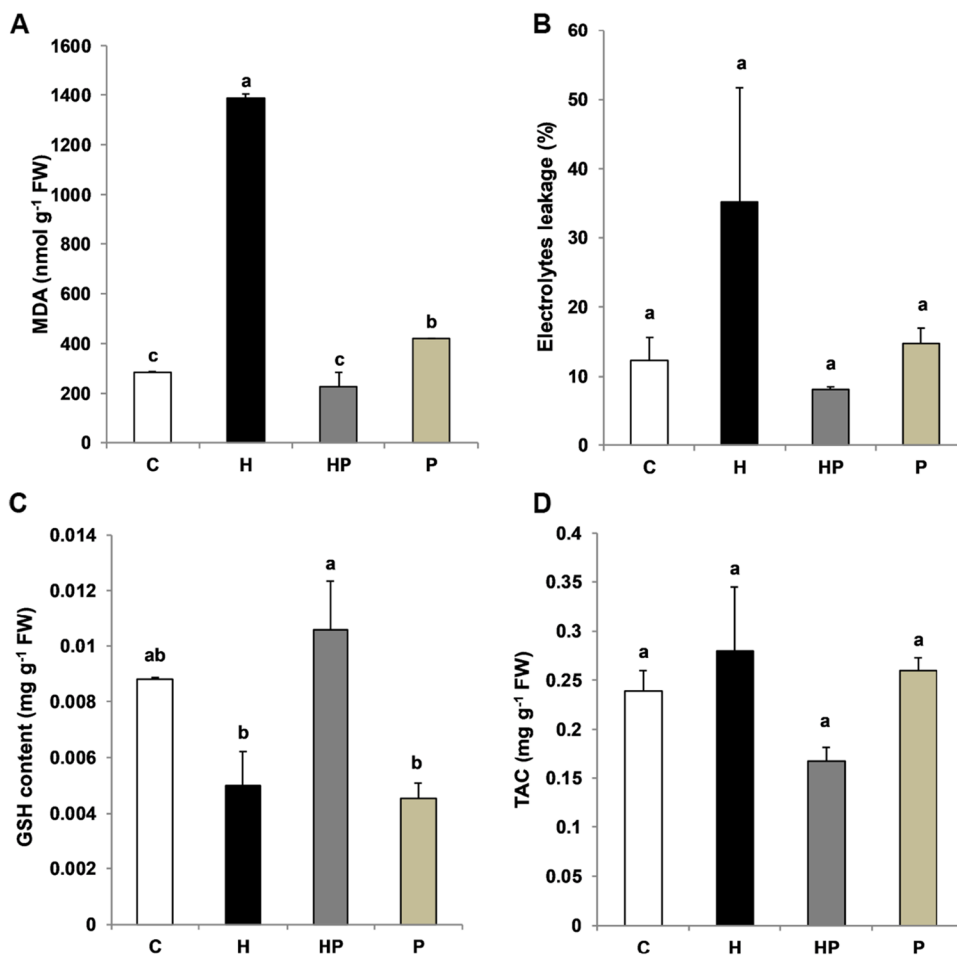
MDA and H_2O_2 production increased significantly by 79.43% and 64%, respectively, in fenugreek seedlings treated with Basagran® herbicide only compared to the control (Fig. 2a). Pre-supply of proline to fenugreek seedlings stressed with the herbicide decreased MDA and H_2O_2 production by 83.58 and 67.51%, respectively, compared with seedlings treated with the herbicide only (Fig. 2). Under Basagran® stress-free conditions, pre-supply of proline did not exhibit a significant increase in MDA and H_2O_2 levels. MDA generated by ROS caused a loss of membrane integrity and increased electrolyte leakage by 65.2% under the adverse conditions of herbicide stress. However, electrolyte leakage was decreased by 76.85% with proline pretreatment of the herbicide-stressed seedlings compared with seedlings stressed with the herbicide only (Fig. 1a). Herbicide stress

decreased the RWC of fenugreek seedlings by 14.33% compared with non-stressed seedlings (Fig. 1c). However, proline pretreatment of the herbicide-sprayed seedlings showed an increase in RWC by 11.4% (Fig. 1c).

3.3 Exogenous Proline Modulates Accumulation of Endogenous Proline and P5C and Proline Metabolism Enzyme

The application of Basagran® herbicide (as foliar spraying) to *Trigonella foenum-graecum* markedly increased the contents of endogenous proline and P5C by 47.66% and 23.18%, respectively, and the activity of P5CS by 84.18%, while the activity of ProDH was decreased significantly by 14.88% (Fig. 3). However, the combined application of proline + Basagran® markedly reduced the contents of endogenous proline and P5C by 67.83 and 51.04%, respectively, and the activity of P5CS by 57.27%, while the activity of ProDH was markedly increased by 53%.

Fig. 2 Impact of externally applied proline 7 mM on malonaldehyde (MDA) content (A), electrolytes leakage (B), glutathione (GSH) content (C), and total antioxidant capacity (TAC) (D) of *Trigonella foenum-graecum* seedlings stressed by Basagran® 10⁻⁴ M. Control (C), herbicide-stressed (H), herbicide-stressed + proline treated (HP), proline control (P). Exogenous proline was applied to seeds by soaking. Data presented are the means of 4 replicate determinations. Vertical bars indicate standard errors. Different letters represent significant differences according to Tukey's test ($P \leq 0.05$)



3.4 Exogenous Proline Enhances Non-enzymatic Antioxidants Under Herbicide Stress

The application of Basagran® herbicide significantly increased the content of GSH by 37.5% compared with the control. Further, seed treatment with proline increased GSH content by 100 and 25% in the herbicide-treated seedlings compared to the herbicide-treated seedlings and the control seedlings, respectively (Fig. 2c). The total antioxidant capacity was increased by 17% in Basagran®-stressed fenugreek seedlings. The combined treatment (proline and Basagran®) significantly decreased the total antioxidant capacity by 40% compared to the single treatment of herbicide (Fig. 2d).

3.5 Exogenous Proline Modulates Enzymatic Antioxidant Activities Under Herbicide Stress

To evaluate the effects of exogenously used proline on enzymatic antioxidants, the activities of CAT and GST were measured (Fig. 4). In response to Basagran® stress, CAT and GST were considerably increased by 86.25% and 68.33%, respectively, compared to the control seedlings. In contrast, pre-supply of proline to the herbicide-stressed

seedlings diminished CAT and GST activity by 86.75 and 83.33%, respectively, compared to seedlings treated only with the herbicide Basagran®. Moreover, in non-stressed seedlings, pre-supply of proline increased GST activity by 75% compared to the control seedlings (Fig. 4b).

3.6 PCA and Hierarchical Clustering Heatmap of Proline-Treated Fenugreek Seedlings Under Normal and Chemical Stress Conditions

Mean values of all growth, biochemical, and physiological data were set to perform PCA and hierarchical clustering heatmap. Three clusters (clusters A, B, and C) were formed (Fig. 5a). Cluster A covered MDA, H₂O₂, EL, P5C, Pro, P5CS, GST, Car, and CAT activity. Compared to the non-stressed treatment, cluster A parameters demonstrated an increased model in herbicide-treated fenugreek seedlings only, while they showed a decreased pattern in proline-pretreated seedlings and proline-pretreated herbicide-stressed (HP) seedlings. Cluster B comprised RWC, TAC, and photosynthetic pigments (Chl) of fenugreek seedlings, which showed a moderate increase in herbicide-stressed (H) seedlings compared with the control. Cluster C represented

Fig. 3 Impact of externally applied proline 7 mM on leaf free proline content (A), pyrroline-5-carboxylate (P5C) content (B), pyrroline-5-carboxylate synthase (P5CS) (C), and proline dehydrogenase (ProDH) (D) activities of *Trigonella foenum-graecum* seedlings stressed by Basagran® 10⁻⁴ M. Control (C), herbicide-stressed (H), herbicide-stressed+proline treated (HP), proline control (P). Exogenous proline was applied to seeds by soaking. Data presented are the means of 4 replicate determinations. Vertical bars indicate standard errors. Different letters represent significant differences according to Tukey's test ($P \leq 0.05$)

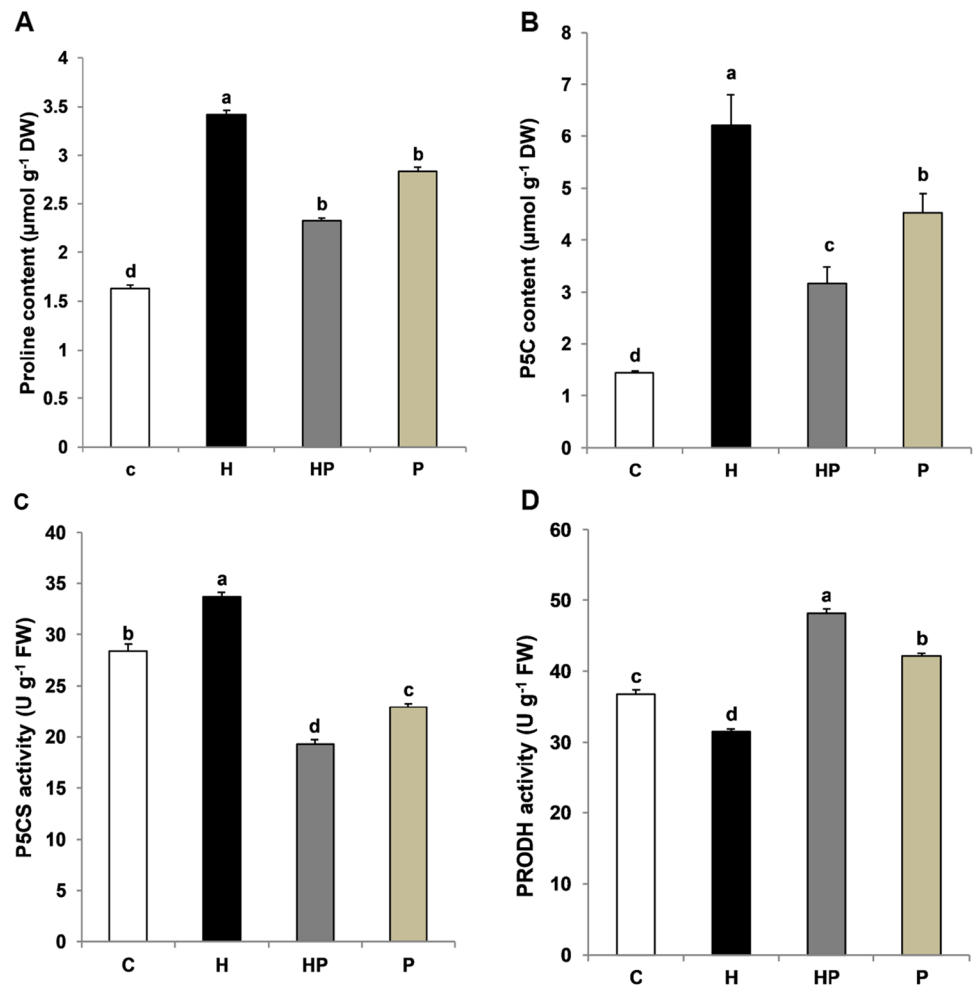
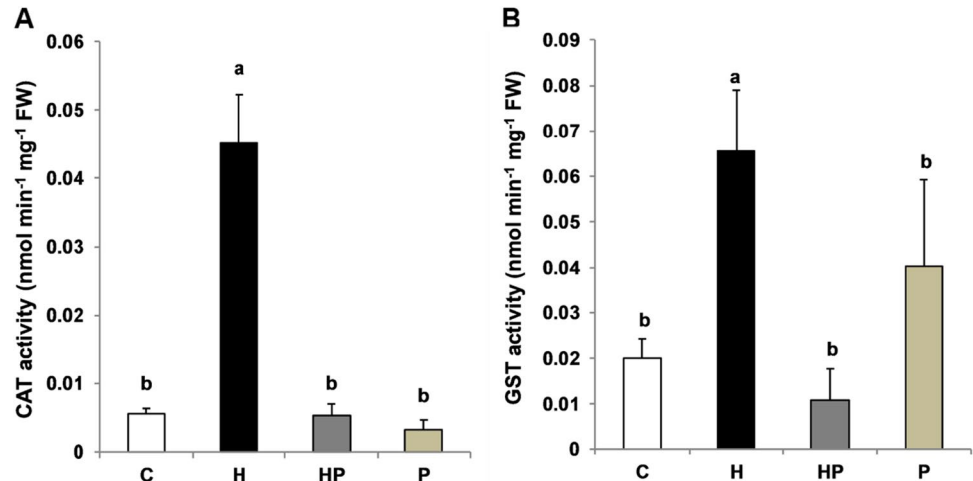


Fig. 4 Impact of externally applied proline 7 mM on leaf catalase (CAT) (A) and glutathione-s-transferase (GST) (B) activities of *Trigonella foenum-graecum* seedlings stressed by Basagran® 10⁻⁴ M. Control (C), herbicide-stressed (H), herbicide-stressed+proline treated (HP), proline control (P). Exogenous proline was applied to seeds by soaking. Data presented are the means of 4 replicate determinations. Vertical bars indicate standard errors. Different letters represent significant differences according to Tukey's test ($P \leq 0.05$)

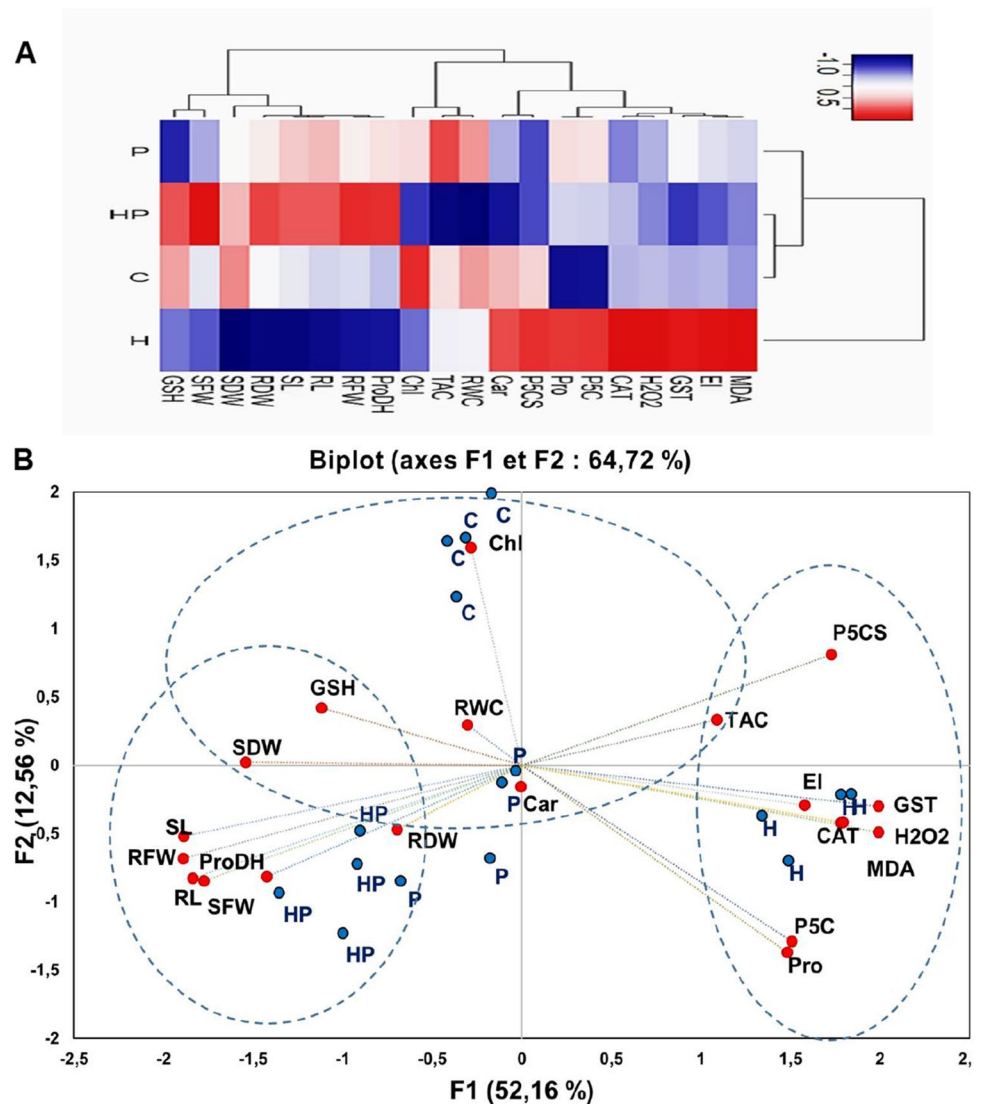


ProDH, GSH, and growth parameters (SFW, SDW, RDW, SL, RL, and RFW). Compared with herbicide-stressed seedlings without pretreatment with proline, cluster C parameters showed the highest increase in proline-pretreated herbicide-stressed (HP) fenugreek seedlings indicating a positive role of exogenous proline in enhancing fenugreek performance

under chemical stress (Fig. 5a). Finally, PCA was performed to unravel the association of physiological and biochemical parameters with the treatment groups (C, H, HP, P) (Fig. 5b). The two F1 and F2 combinedly displayed 64.72% of data variability (Fig. 5b). Results of PCA showed that cluster A variables were strongly associated with herbicide

Fig. 5 **A** Hierarchical clustering with heatmap and **B** principal component analysis (PCA) show the treatment-variable relationships. In hierarchical clustering and heatmap, the mean values of various parameters obtained in this study were normalized and clustered. Three distinct clusters (clusters A, B, and C) were identified at the variable level. The color scale displays the intensity of normalized mean values of different parameters. The entire dataset was analyzed using PCA.

The variables included total chlorophyll (Chl), carotenoid (Car), root length (RL), shoot length (SL), root fresh weight (RFW), root dry weight (RDW), shoot fresh weight (SFW), shoot dry weight (SDW), relative water (RWC), electrolyte leakage (EL), malondialdehyde (MDA), glutathione (GSH), total antioxidant capacity (TAC), proline (Pro), pyrroline-5-carboxylate (P5C), pyrroline-5-carboxylate synthase (P5CS), proline dehydrogenase (ProDH), hydrogen peroxide (H₂O₂), catalase (CAT), and glutathione-s-transferase (GST). Control (C), herbicide-stressed (H), herbicide-stressed + proline treated (HP), proline control (P)



treatment (H). Heatmap cluster B variables were associated with proline pretreatment and control (P and C), whereas cluster C variables were closely interlinked to proline pretreatment followed by herbicide stress (HP) (Fig. 5b). When Basagran® treatments were compared with other treatments, PCA separated the four treatments into notably different groups (Fig. 5).

4 Discussion

The present work has focused on the impact of exogenously used proline as a soaking solution for fenugreek seeds in mitigating the herbicide-related damage to seedlings. In previous reports, proline contributes less than 5% of the overall amount of free amino acids in plants under normal conditions; however, the concentration of the amino acid pool increases up to 80% in several plants under various

types of stress (Shahbaz et al. 2013). Biosynthesis regulates intracellular L-proline levels in plants, catabolism, and transport inside and from cells and cell compartments. L-proline is mainly derived from glutamate and tends to be the most commonly dispersed osmolyte accumulated under stress conditions in plants. It is an osmoprotectant that protects subcellular structure enzymes and increases cellular osmolarity (turgor pressure), which provides the turgor needed for cell expansion under stress conditions (Kumar et al. 2010). It also plays a role in buffering cellular redox potential and scavenging free radicals under stress (Sánchez et al. 2007). Other functions for proline accumulation have been suggested. It acts as a sink of energy, a nitrogen-storage compound, and a signaling molecule (Verbruggen and Hermans 2008). Our research gives for the first time the role of exogenous proline, used as a seed soaking solution (7 mM concentration), in mitigating the harm of Basagran® herbicide stress.

The totality of the research indicated that pollutants such as herbicides and other toxic organic substances in the environment are capable of inducing overproduction of reactive oxygen species in plant tissues, causing oxidative stress that can have multiple damaging effects on cellular components (Rutherford and Krieger-Liszkay 2001; Arora et al. 2002). This study depicted that proline effectively restricted the damaging impact of Basagran® herbicide (10^{-4} M concentration) in Fenugreek (*Trigonella foenum-graecum*) seedlings.

The use of Basagran® 10^{-4} M in our study as a stress agent on *Trigonella foenum-graecum* seedlings caused a significant decrease in total chlorophyll content. Many authors have recorded the same results in other species under herbicidal stress conditions (Abrous-Belbachir et al. 2009; Boulahia et al. 2016; Radwan et al. 2019). Basagran® blocks the transfer of electrons at the photosystem II (PSII) level, as it binds to the quinone B (QB) binding site on the thylakoid protein D1 of the PSII reaction center, preventing the reoxidation of quinone A (QA) (Fayez 2000; Galhano et al. 2009) and, thus, inhibition of photosynthesis. Alikhani et al. (2011) consider that reducing photosynthetic pigments can be a strategy of adaptation to stress. In this case, the amount of light captured can be reduced, thus reducing the formation of active oxygen to limit any possibility of damaging the photosynthetic machinery (Azzedine et al. 2011). The group of plants pretreated with exogenous proline and stressed with Basagran® showed significantly increased chlorophyll content results (Fig. 1). Various research showed that exogenously used proline is well known to protect the cellular components under stress (Ben Ahmed et al. 2010), such as mitochondrial ETS complex (Hamilton and Heckathorn 2001), enzymes like RuBisCO, membranes, and proteins (Meena et al. 2019; Rady et al. 2019a; Semida et al. 2020).

To confirm the state of stress generated in fenugreek seedlings, malondialdehyde level was measured as an indicator of lipid peroxidation during abiotic stress (Pompella et al. 1987). MDA is the product of lipid peroxidation reactions that form when polyunsaturated lipids are attacked by ROS generated under stressful conditions. Thus, the hydroperoxides break down into radical intermediates and aldehydes; one of the most reactive representatives is MDA (Pardha Saradhi et al. 1995). MDA is a high-performance alkylating agent capable of reacting with biological macromolecules. The dosage of this compound is therefore of definite interest in plants subjected to multiple constraints. Our results showed increased MDA concentrations and relative electrolyte leakage in fenugreek seedlings subjected to chemical stress. This can be explained by lipid peroxidation inside leaf cells, which reflects the loss of membrane integrity through oxidative stress. Similar results demonstrated that abiotic stresses such as herbicides cause molecular damage due to ROS overproduction in plants (Fayez et al. 2011; Boulahia

et al. 2016; Radwan et al. 2019). In herbicide-stressed seedlings pretreated with proline, the MDA content is reduced, indicating the antioxidant effect of proline, which explains its role in osmotic adjustment (Rady et al. 2019a; Trovato et al. 2019), protecting membranes from desiccation (Ashraf and Foolad 2007; Ben Ahmed et al. 2010) and in preserving membrane integrity. Pretreatment (seed soaking) with proline considerably reduced electrolyte leakage in Basagran®-exposed seedlings, bringing the electrolyte leakage rate close to that of control seedlings. Ami et al. (2020) observed similar results by applying proline to plants subjected to saline stress.

The negative impact of abiotic stress on the plant can be assessed by measuring growth parameters (Hasanuzzaman et al. 2018). Our results showed a significant reduction in shoot and root lengths and shoot and root fresh and dry weights of herbicide-stressed seedlings compared to control seedlings (Table 1), which may occur as a result of ROS accumulation thus lipid peroxidation (Fig. 1). The same results were observed (Ceballos et al. 2009; Boulahia et al. 2016). Moreover, the exogenous application of proline improved all growth parameters (Table 1) due to the antioxidant properties of proline, which can enhance the scavenging of ROS and alleviate stress damage (Sadak and Mostafa 2015; Yaqoob et al. 2019). The same results have been reported (Ben Ahmed et al. 2010; Hayat et al. 2012; Rady et al. 2019a).

The water status of plants is generally estimated by measuring relative water content, which several researchers consider a good indicator of a plant's water status (Singh et al. 2016; Sohag et al. 2020). Our results for this parameter indicated that chemical stress caused a decrease in the water content of fenugreek leaves (Fig. 1d). The application of proline restored the level of RWC in seedlings subjected to the herbicide Basagran®, with better effect when applied by imbibing the seeds. According to Khedr et al. (2003), the ability of exogenously used proline to maintain high water content in severely stressed young plants could be attributed to its contribution to osmotic adjustment directly by increasing the endogenous proline content and indirectly by increasing the intrinsic internal content of other amino acids. Verbruggen et al. (1996) admit a linear and direct correlation between the water content of the different organs of *Arabidopsis* and their free proline content.

In this study, chemical (Basagran® herbicide)-stressed *foenum-graecum* seedlings showed a notable increase in proline content, which may be accumulated to reduce the herbicide negative effects (Fig. 3) or to provide an additional N source to the plant (de Freitas et al. 2018; Rady et al. 2019a). In conjunction with this increase in proline content, the activity of P5CS was increased to provide further proline biosynthesis. This correlation between the activity of P5CS and proline accumulation was observed

during salt stress (Trovato et al. 2008; Wang et al. 2011; de Freitas et al. 2018). On the other hand, Basagran® reduced the ProDH activity (Fig. 3d). The exogenous application of proline decreased the proline content and P5CS activity (Fig. 3c). The drop in proline concentration and P5CS activity while relieving stress indicates that an excess concentration of proline is not needed in this case, and this demonstrates our discovery of the role of exogenously used proline in reducing the stress damage caused by Basagran®. The same results were observed (Miller et al. 2009; Rady et al. 2019a, 2019b; Trovato et al. 2019).

On the contrary, exogenously used proline provided a substantial increase in ProDH activity, which the drop of proline content can explain; the catabolic pathway seems to be the only way for plants to degrade excess proline (Trovato et al. 2008). Therefore, the obtained findings illustrate the complexity of the response to pretreatment (seed soaking) with proline in *foenum-graecum* seedlings, indicating that further researches are needed to determine the precise role of proline in its metabolism in plants under chemical stress conditions. Among the functions of proline in stressed plants, the contribution to osmotic adjustments and protection of plant cells from the generation of ROS and injury to cellular membranes (Zouari et al. 2016a; Soliman and Shanan 2017; Meena et al. 2019; Application and El-Akhdar 2020).

Catalase is one of the antioxidant enzymes that catalyze the dismutation of hydrogen peroxide H_2O_2 into H_2O and oxygen O_2 (Gill et al. 2011). Our results showed that CAT activity was increased in seedlings treated with the herbicide Basagran® (Fig. 4a). According to Schneider et al. (2020), plants develop specific strategies to cope with the adverse effects of herbicides and thus alleviate their phytotoxicity via numerous detoxification systems. Several studies on transgenic rice plants have revealed that CAT causes tolerance to various abiotic stresses due to H_2O_2 neutralization (Gill and Tuteja 2010; Khalifa et al. 2011). The results obtained in this investigation showed that CAT activity was increased in seedlings stressed by the herbicide. The same results obtained indicate a significant reduction of herbicide damage in proline-pretreated seedlings (Ozden et al. 2009). Our results showed that H_2O_2 content was increased in the leaves of fenugreek seedlings when subjected to chemical stress (Fig. 1d). However, proline pretreatment displayed a suppression in the H_2O_2 level. Our PCA results showed a strong correlation between H_2O_2 , MDA, EL, and CAT with herbicide-stressed fenugreek seedlings without pre-supply of proline (Fig. 5b). Moreover, we found a negative correlation between herbicide-stressed fenugreek seedlings pretreated with proline, indicating that the exogenously applied proline reduced the oxidative damage caused by Basagran® herbicide stress.

Our findings showed that Basagran® stress induced an increase in the total antioxidant capacity TAC (Fig. 2d) in response to the accumulation of ROS, which could explain the accumulated levels of ROS. The exogenous application of proline decreased not only the ROS but also the TAC (Fig. 2d). The PCA results confirmed our results by a strong correlation between TAC and herbicide-stressed seedlings (Fig. 5b).

Glutathione is a cysteine tripeptide whose reductive and nucleophilic properties play a significant role in protecting against oxidative damage to lipids, proteins, and nucleic acids (Penninckx 2000). The synthesis of GSH is stimulated during different stress situations, and its accumulation often coincides with ROS accumulation (Noctor and Foyer 1998). It plays an essential role in chloroplasts as it helps protect them from damage caused by oxidative stress (Gill and Tuteja 2010). In this experiment, lower amounts of GSH were observed in Basagran®-treated leaves than in control (Fig. 2c). Its implication can explain the decrease in GSH content in the metabolism of xenobiotics through glutathione S-transferases (Noctor and Foyer 1998). Pretreatment of proline combined with Basagran® herbicide treatment increases the GSH content of leaves compared to the untreated control and only stressed seedlings (Fig. 2c). Several studies showed that exogenous proline has a crucial role in increasing GSH level (Xu et al. 2009; Hossain et al. 2010; Hossain and Fujita 2010). Glutathione S-transferases are part of a dimeric and multifunctional family of enzymes ubiquitously found in all aerobic organisms (Habig et al. 1974; Ahmad et al. 2010). They are also involved in the detoxification of products resulting from lipid peroxidation. They protect the cell from ROS and stress damage (Nemat Alla and Hassan 2006). The role attributed to GST is supported by our results, which indicate an increase in GST activity in stressed leaves (Fig. 4b). The combination of the xenobiotic with GSH can be explained by the increased use of GST in the conjugation reaction. Several studies confirm an increase in antioxidant activity under the effect of herbicides (Xiao et al. 2008; Miteva et al. 2010; Jin et al. 2012). Our results exhibited that proline pretreatment decreases the GST activity. The same results were observed by Hasanuzzaman et al. (2014). Molla et al. (2014) reported that the pre-supply of proline up-regulates the GST activity by increasing the GSH content.

5 Conclusions

Our study offers new evidence of exogenously used proline-mediated herbicide stress tolerance mechanisms in fenugreek seedlings. The positive effect of proline pretreatment might be due to the reduction of reactive oxygen species (ROS) accumulation caused by chemical (Basagran® herbicide)

stress, probably by increasing the activities of ROS-scavenging enzymatic antioxidants glutathione S-transferases (GST) and catalase (CAT) and non-enzymatic antioxidants glutathione (GSH), carotenoids, and total antioxidant capacity (TAC). Our findings suggest that the pre-supply of proline may be a practical and efficient strategy for reducing the harmful effects of herbicide stress. More in-depth molecular studies are required to understand better the detailed mechanisms of proline-induced herbicide stress tolerance in plants.

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Author Contribution OC and AO conceived and designed the experiments. OC, BK, and MR performed the experiments. OC, ME, and MR analyzed the data. ME and MR contributed reagents/materials/analysis tools. OC wrote the paper. MR, DR, and AO revised the paper. All authors read and approved the final manuscript.

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Declarations

Conflict of Interest The authors declare no competing interests.

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