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Pivotal role of fibrous roots in drought tolerance of saffron (*Crocus sativus* L.) and mitigation of oxidative stress by penconazole

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We investigated the effects of PEG-induced drought on the antioxidative mechanisms of *Crocus sativus* L. and the mitigating effects of PEN on drought tolerance. Contents of H_2O_2 and MDA in fibrous roots were considerably lower than those in leaves and were attenuated by PEN pretreatment. Activities of CAT, SOD, and POX were significantly higher in fibrous roots than in leaves. Among antioxidative enzymes, POX activity in fibrous roots was 600–2000-fold higher than that in leaves. The increase in CAT and SOD activities under the influence of PEN in the fibrous roots is the main reason for the decrease in MDA levels and highlights the important role of this organ in the response to drought stress. A somewhat similar increase in CAT activity was also observed in the leaves under drought conditions due to PEN. Under drought, PEN led to a significant increase in carotenoid content and an elevated ratio of *Chl a* to *Chl b* in the leaves. However, in spite of the higher content of non-enzymatic antioxidants in leaves than in fibrous roots, the lower MDA levels in fibrous roots indicated greater efficiency of antioxidative enzymes in controlling membrane lipid peroxidation. In conclusion, the results demonstrate that PEN can mitigate oxidative damage in saffron under drought conditions by enhancing the activity of SOD (up to 275%) and CAT (up to 189%) and by increasing proline content (up to 50%) in fibrous roots.

Keywords *Crocus sativus* L., Drought stress mitigation, Fibrous roots, Penconazole, Antioxidative enzymes, Non-enzymatic antioxidants, Malondialdehyde

Abbreviations

MDA	Malondialdehyde
PEN	Penconazole
PEG	Polyethylene glycol
CAT	Catalase
SOD	Superoxide dismutase
POX	Peroxidase
Chl a	Chlorophyll a
Chl b	Chlorophyll b
ROS	Reactive oxygen species

Saffron (*Crocus sativus* L.) is a triploid, sterile plant that has been utilized as a medicinal plant and a spice for thousands of years. Iran, India, Greece, Morocco, Azerbaijan, Afghanistan and Spain are the main producers of saffron[1]. This plant is a rich source of bio-molecules such as polyphenols and antioxidants[2] and is used as an herbal medicine due to its analgesic and sedative properties[3].

One of the most critical challenges that land plants face is drought, which is the largest single factor limiting crop productivity worldwide[4]. Drought stress induces several physiological and biochemical responses, including inhibition of cell division, stomatal closure, reduction of photosynthesis and induction of reactive oxygen

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species (ROS)[5,6]. The imbalance between the production and removal of ROS leads to oxidative stress, and ROS elimination by antioxidants is a key response to abiotic stresses. To counteract oxidative damage by ROS, plants upregulate enzymatic and non-enzymatic antioxidants[7]. Enzymatic antioxidant defense systems such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO), play pivotal roles in mitigating oxidative damage caused by drought stress[8,9]. Plants also possess non-enzymatic antioxidants such as ascorbic acid, glutathione, carotenoids and secondary metabolites like phenolics, flavonoids, flavonols, and anthocyanins[10,11].

Drought stress also affects carbohydrate metabolism, leading to changes in carbohydrate content. Carbohydrates serve as energy reserves and osmotic regulators during water deficit[12], while proline, a multifunctional amino acid, accumulates to act as osmotic regulator, maintain cellular integrity, and enhance stress tolerance[13].

According to several studies, certain chemicals such as some amino acids and their derivatives[14,15], specific minerals like silicon[16], the fungicide paclobutrazol[17], δ-aminolevulinic acid[18] have been found to mitigate salt stress in plants. The mitigating effects of broad-spectrum systemic fungicide hymexazole on cadmium stress tolerance in *Echinochloa frumentacea* Link have also been reported[19].

Triazoles are a group of compounds with plant growth regulation properties that were originally used as fungicides because they inhibit ergosterol biosynthesis. These compounds known as "plant multiprotectants" have the capacity to induce plant acclimation to abiotic stresses. Triazoles, with effective free-radical scavenging properties and the ability to detoxify ROS, protect plants under stress[20]. Penconazole (PEN) is a triazolic fungicide with plant growth-regulation (PGR) characteristics[21].

The scarcity of water resources and the prevalence of arid conditions in saffron-producing countries present formidable obstacles to saffron cultivation, and finding inexpensive means to alleviate the adverse effect of drought on saffron production seems crucial. Investigating strategies to improve plants' tolerance to drought stress constraints is an important step toward enhancing agricultural production and increasing the yield of valuable pharmaceutical compounds, including antioxidants[22]. Some studies have been conducted the role of PEN in enhancing drought and salt stress tolerance in certain plants[23–33]. However, the effects of PEN on responses of saffron plants and the negative impacts induced by drought stress have never been studied.

In this research, we aimed to evaluate mitigation of drought stress in saffron by PEN and to elucidate the underlying mechanisms of drought tolerance. We hypothesized that the application of PEN could protect saffron by up-regulating the ROS-scavenging enzymes and non-enzymatic antioxidants. To our knowledge, this is the first report on the impact of PEN on saffron plants under drought stress.

Results

Effects of drought and PEN pretreatment on growth and morphological characteristics of *Crocus sativus* L. are shown in Fig. 1. Drought decreased the growth of all organs compared to the control. PEN (15 and 25 mg/L) improved leaf fresh weight (FW) at 15 and 10% PEG, respectively (Fig. 2A). PEN at both concentrations increased fibrous roots FW at 5% PEG. Positive effects of PEN (15 mg/L) on corm dry weight (DW) were observed at 5% and 15% PEG, whereas 25 mg/L PEN improved corm DW at all PEG levels compared with the control. Two-

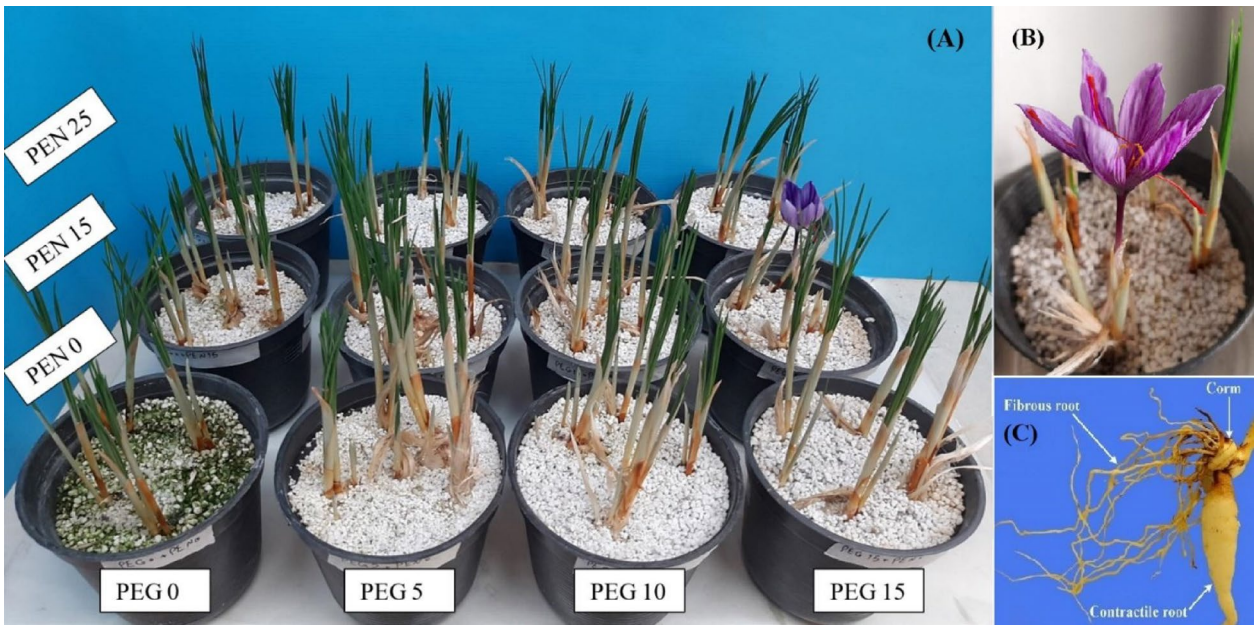

Fig. 1. Effects of PEN (0, 15 and 25 mg/L) pretreatment on growth and morphological appearance of *Crocus sativus* L. under PEG (0, 5, 10 and 15%) induced drought (**A**). Flower (**B**) and underground parts of saffron plant: corm, contractile root, and fibrous root (**C**). Note to the lack of algal growth in samples grown from PEN pretreated corms and under all PEG concentrations.

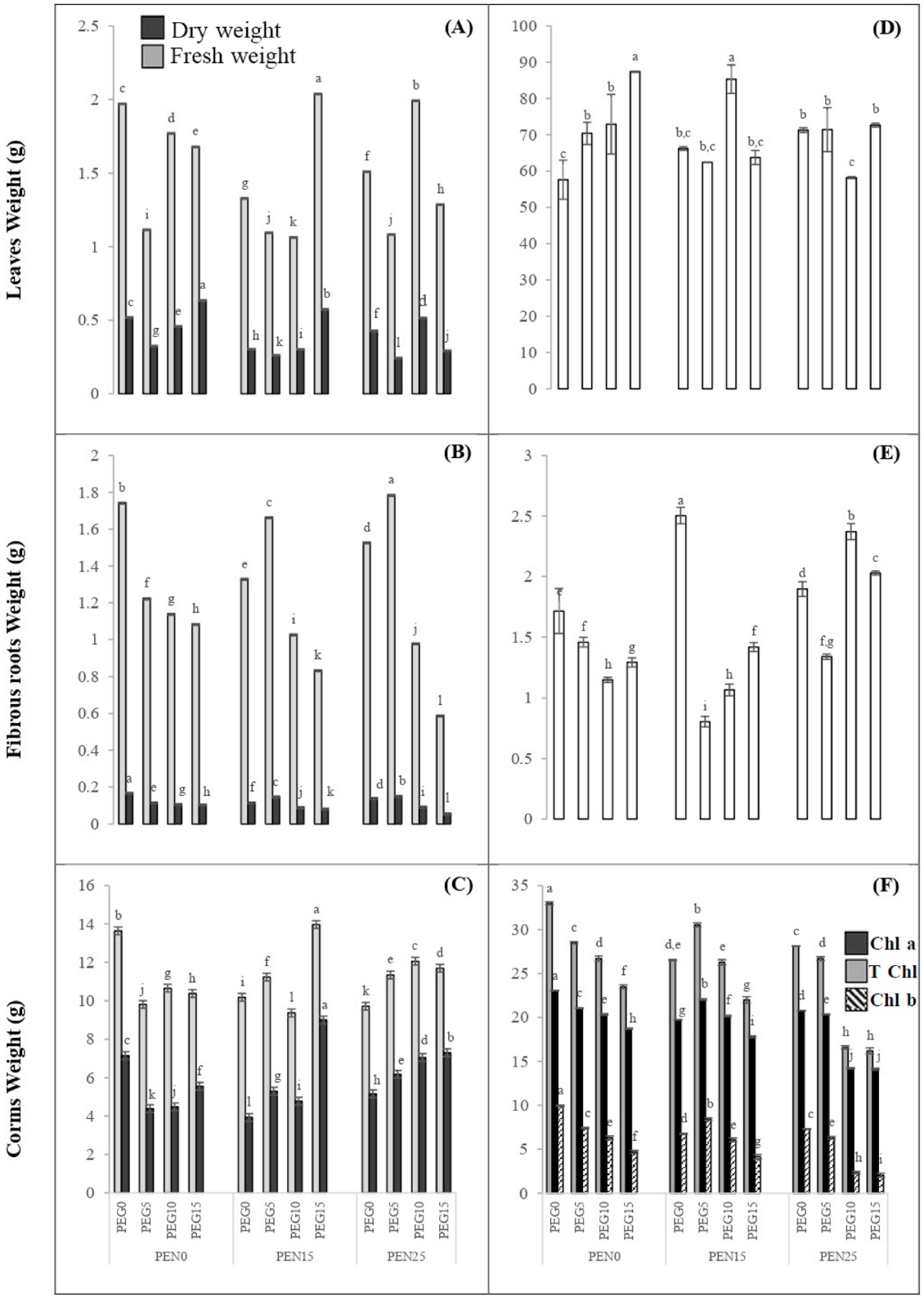

Fig. 2. Effects of PEN (0, 15 and 25 mg/L) pretreatment on fresh weight and dry weight of leaves (**A**), fibrous roots (**B**), and corms (**C**), leaves relative water content (RWC) (**D**), leaf carotenoids content (**E**), and leaves chlorophylls (Total *Chl*, *Chl* a, *Chl* b) content (**F**) of *C. sativus* L. under PEG (0, 5, 10 and 15%) induced drought. Dry weight of PEN treated corms increased significantly with increasing drought intensity. Vertical bars indicate means ± SE based on three replicates. Different letters above columns indicate a significant difference at $P < 0.05$ using Duncan multiple range test.

Dependent variables	Independent variables		
	Drought	PEN	Drought × PEN
Fresh weight	6765.148 ***	2117.033 ***	4446.775 ***
Dry weight	7,845,451 ***	5692918.75 ***	4003718.75 ***
Protein	51.673 ***	64.786 ***	7.837 ***
POX	31.183 ***	0.718 ^{ns}	35.791 ***
PPO	59.401 ***	6.359 **	9.499 ***
CAT	11.997 ***	30.905 ***	3.988 *
SOD	72.748 ***	13.071 ***	30.726 ***
Phenolics	2680.014 ***	23849.717 ***	8695.696 ***
Flavonoids	1343.635 ***	1142.442 ***	1170.051 ***
Flavonols	4987.02 ***	1454.018 ***	2871.462 ***
DPPH	9.441 ***	7.081 **	25.402 ***
Anthocyanins	11.354 ***	20.771 ***	73.13 ***
H_2O_2	332.979 ***	564.782 ***	426.248 ***
Carbohydrates	71.905 ***	36.086 ***	209.322 ***
Proline	398.468 ***	24.968 ***	110.371 ***
MDA	148.873 ***	25.391 ***	80.773 ***
Chla	9162.237 ***	8398.985 ***	1976.471 ***
Chlb	1392.097 ***	785.963 ***	140.54 ***
ChlT	2894.901 ***	2128.966 ***	431.753 ***
Carotenoids	226.008 ***	198.222 ***	141.03 ***
RWC	6.989 **	25.884 ***	23.281 ***

Table 1. Results of two-way analysis of variance (ANOVA) of drought, PEN and their interaction (drought × PEN) in *Crocus sativus* L. Leaf, for protein, POX, PPO, CAT, SOD, phenolics, flavonoids, flavonols, DPPH, anthocyanins, H_2O_2, carbohydrates, proline, MDA, Chl *a*, *b*, and total Chl, carotenoid, and RWC. Numbers represent F value. ^{ns} not significant. * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$.

Dependent variables	Independent variables		
	Drought	PEN	Drought × PEN
Fresh weight	116181727.3 ***	2611319.25 ***	18987863.58 ***
Protein	39.563 ***	33.175 ***	25.761 ***
POX	8.397 **	2.579 ^{ns}	3.953 *
PPO	12.554 ***	1.41 ^{ns}	25.387 ***
CAT	7.697 **	36.028 ***	22.984 ***
SOD	151.203 ***	182.725 ***	69.536 ***
Phenolics	4874.557 ***	981.589 ***	88.114 ***
Flavonoids	19827.812 ***	8361.851 ***	5140.03 ***
Flavonols	2216.576 ***	2912.507 ***	1567.295 ***
DPPH	100.289 ***	376.815 ***	88.016 ***
Anthocyanins	148.544 ***	212.078 ***	70.535 ***
H_2O_2	14.298 ***	25.676 ***	7.486 ***
Carbohydrates	289.478 ***	37.123 ***	83.171 ***
Proline	682.606 ***	511.021 ***	1065.88 ***
MDA	130.238 ***	166.057 ***	69.295 ***

Table 2. Results of two-way analysis of variance (ANOVA) of drought, PEN and their interaction (drought × PEN) in *Crocus sativus* L. fibrous root, for protein, POX, PPO, CAT, SOD, phenolics, flavonoids, flavonols, DPPH, anthocyanins, H_2O_2, carbohydrates, proline, and MDA. Numbers represent F value. ^{ns} not significant. * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$.

way ANOVA indicated a significant main effect of drought and PEN on growth parameters and on some of the physiological and biochemical parameters measured in the leaves and roots of *Crocus sativus* L. (Tables 1 and 2).

Leaves relative water content (RWC) increased significantly under drought stress in the absence of PEN (Fig. 3D). Although PEN did not improve RWC at most drought levels, its effects on RWC were statistically significant according to ANOVA (Table 1). Leaves carotenoids content decreased significantly under drought

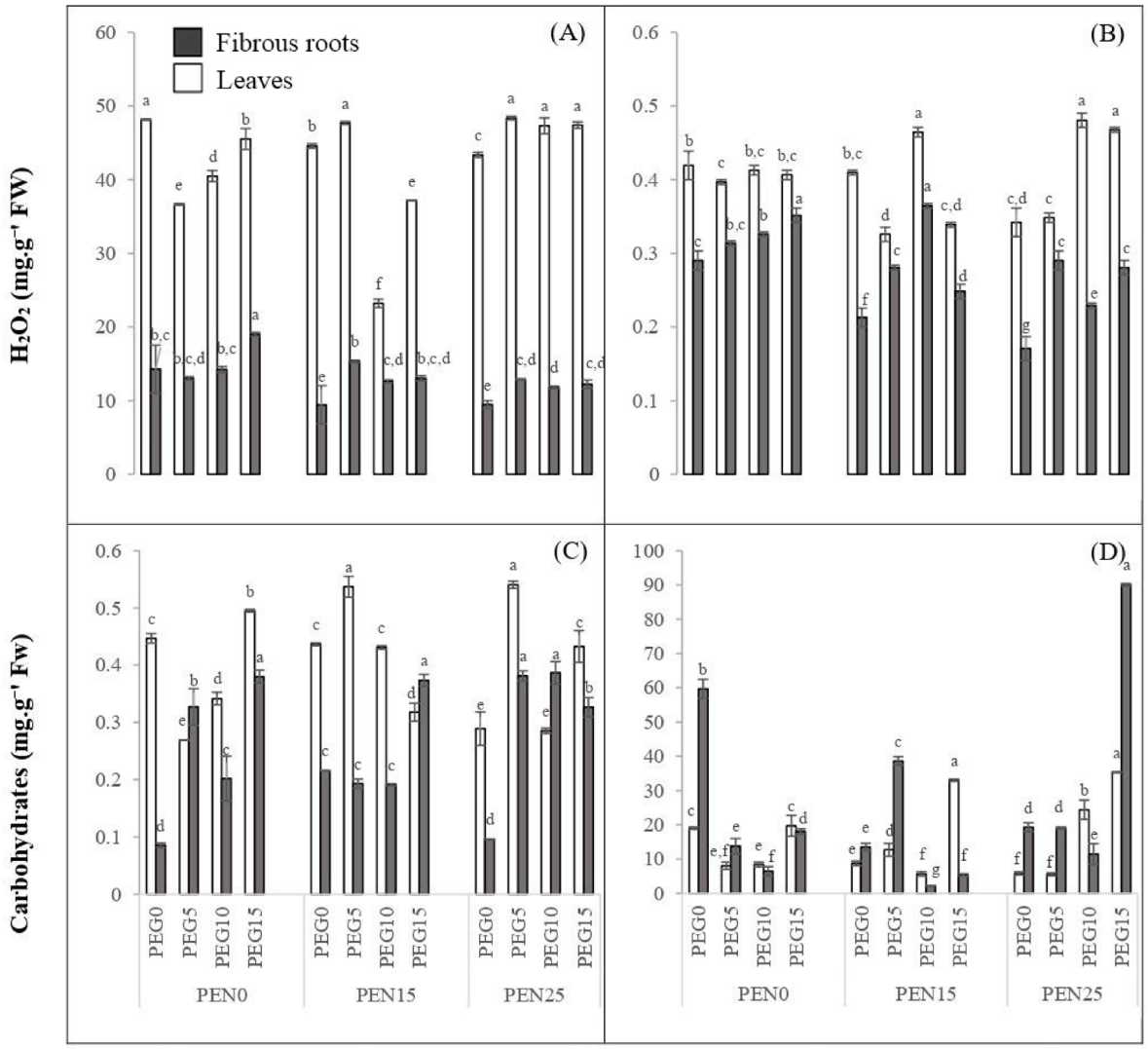

Fig. 3. Effects of PEN (0, 15 and 25 mg/L) pretreatment on H_2O_2 (**A**), MDA (**B**), Carbohydrate (**C**) and Proline (**D**) content in leaves and fibrous roots of *C. sativus* L. under PEG (0, 5, 10 and 15%) induced drought. Vertical bars indicate means ± SE based on three replicates. Different letters above columns indicate a significant difference at $P < 0.05$ using Duncan multiple range test.

stress in the absence of PEN application (Fig. 2E). Both concentrations of PEN increased carotenoid content under unstressed conditions (0% PEG). Moreover, 25 mg/L PEN increased carotenoids content by 100% and 54% under 10 and 15% PEG, respectively (Fig. 2E).

The contents of *Chl a* and *Chl b* in leaves decreased continuously under drought conditions in plants without PEN. *Chl a* and *Chl b* contents decreased by 17% and 50%, respectively, at 15% PEG compared to the control. PEN pretreatment increased *Chl a* content under both 5% and 10% PEG. A positive effect of 15 mg/L PEN on *Chl b* content was observed only under 5% PEG. Moreover, *Chl a* content was also increased by 25 mg/L PEN under 5% PEG (Fig. 2F).

The content of H_2O_2 in fibrous roots was lower than that in leaves under all drought levels, with and without PEN. Remarkably, leaves exhibited approximately twice the H_2O_2 content compared to fibrous roots. Moreover, 25 mg/L PEN led to a relative induction of H_2O_2 content in leaves under 5%, 10%, and 15% PEG (Fig. 3A).

The MDA content, as a lipid peroxidation product and an indicator of oxidative stress in leaves was significantly higher than that in fibrous roots under all the treatment conditions. MDA content in leaves remained relatively constant under all levels of drought without the application of PEN but increased significantly in fibrous root. A significant reductive effect of 25 mg/L PEN on MDA content was observed only in fibrous roots under control and most drought levels. Moreover, a significant ameliorative effect of 15 mg/L PEN on MDA content in fibrous roots was observed under 0% and 15% PEG (Fig. 3B).

The soluble carbohydrates content in the leaves was 400% higher than that in the fibrous roots under control condition. However, under intensified drought stress, the root-to-leaf carbohydrates ratio changed significantly. Under 15% PEG without PEN, the root-to-leaf ratio of carbohydrates increased from 0.25 (control) to 0.76. Change in the root-to-leaf carbohydrates ratio were observed under drought stress with both concentrations of

PEN. Both concentrations of PEN increased the soluble carbohydrates content in leaves by approximately 107% under 5% PEG compared to the control. Moreover, 25 mg/L PEN increased the soluble carbohydrate content in fibrous roots by 12% and 90% under 5% and 10% PEG, respectively (Fig. 3C).

Proline content in control fibrous roots (60 µg g^{-1}FW) was three times higher than in leaves (20 µg g^{-1}FW). Moreover, proline content in fibrous roots under most drought levels and PEN pretreatments was also significantly higher than that in leaves. Although PEN increased proline content in leaves under some drought levels, the induction of proline content in fibrous roots was significantly higher than in leaves. PEN (15 and 25 mg/L) increased proline content of fibrous roots by 160% and 374% under 5% and 15% PEG, respectively (Fig. 3D).

Activities of antioxidant enzymes, including CAT, SOD, PPO, and POX were analyzed in leaves and fibrous roots under PEG-induced drought stress, with and without PEN pretreatment. CAT activity was significantly induced in fibrous roots under drought stress. CAT activity in fibrous roots under 15% PEG was 89% higher than that of the control. Moreover, CAT activity in fibrous roots was remarkably higher than that in leaves. Under 15% PEG, it was 177% higher than that in leaves. PEN (15 mg/L) increased CAT activity in fibrous roots at all levels of drought. In addition, PEN (25 mg/L) induced CAT activity in fibrous roots under 0% and 5% PEG (Fig. 4A). SOD activity, which in fibrous roots was 53% higher than that in leaves under control conditions, was significantly augmented by both concentrations of PEN in fibrous roots. Under PEN pretreatment, SOD activity in fibrous roots remained higher than that in leaves at all levels of drought. Thus, PEN continuously induced SOD activity in fibrous roots under drought stress. Such a huge induction of SOD activity by PEN did not occur in leaves (Fig. 4B). POX activity, which in fibrous roots was approximately 550 times than in leaves, did not change in either organs in a consistent pattern under drought and PEN treatments. The outstanding activity of POX in fibrous roots identifies this organ in *C. sativus* L. as a potential new source for POX (Fig. 4C, D). PPO activity, which was higher in fibrous roots than in leaves under all levels of PEG without PEN pretreatment, also did not change in either organs in a consistent pattern under PEN treatments. However, irregular induction of PPO by PEN was observed in both organs (Fig. 4E).

Leaves exhibited significantly higher inhibition of DPPH radicals under control and drought stress conditions, with and without PEN, in comparison to fibrous roots. However, PEN increased the inhibition of DPPH radicals in fibrous roots more significantly (Fig. 4F).

The content of all the phenolic compounds in leaves was significantly higher than in fibrous roots. Under control conditions, the contents of phenolics, flavonoids, flavonols, and anthocyanins in leaves were 340%, 250%, 150%, and 1,450% higher, respectively, than in fibrous roots. Despite the higher phenolic content in the leaves, the levels of these compounds did not increase under 5% and 15% PEG or with 25 mg/L PEN compared to the control. However, in the presence of 15 mg/L PEN, a consistent increase in the contents of phenolics, flavonols and anthocyanins under drought stress was observed in leaves. This trend except for anthocyanins was also observed in fibrous roots under 15 mg/L PEN during drought stress. Moreover, in contrast to the leaves, both concentrations of PEN increased phenolics contents in fibrous roots under drought conditions, compared to when PEN was absent. Under the influence of 15 mg/L PEN, phenolics content in leaves and fibrous roots increased by 44% and 118%, respectively, under 15% PEG compared to 0% PEG with PEN, (Fig. 5A–D).

All the biochemical and physiological parameters under PEG levels and PEN pretreatments in leaves and fibrous roots were subjected to PCA. In addition to PCA, hierarchical cluster analysis (HCA) was also used to check the correlations among studied parameters. The results obtained from PCA and HCA analyses are presented in Figs. 6, 7, 8 and 9. As displayed in Figs. 6 and 8, the first two principal components in leaves and fibrous roots explained 51.17 and 51.63% of the total variance, respectively.

Discussion

The ameliorative effects of PEN on morphology and growth parameters were observed under specific levels of drought stress (Figs. 1 and 2). Corms, which are used for *C. sativus* propagation, produce two structurally and functionally different roots (Fig. 1C). Fibrous roots play a central role in plant nutrition, while thick contractile roots pull the corm down to its desired depth in the soil[34] (Fig. 1C).

The biocidal effect of PEN was confirmed due to the growth of algae under control conditions and the lack of algal growth and contamination under all the drought and PEN pretreatments (Fig. 1A). According to the literature, no data have been reported concerning the effects of PEN on saffron under drought stress. PEN, as a triazole compound, has plant growth-regulating characteristics and fungicidal effects and can protect plants against different abiotic stresses[20]. Fungicidal properties of triazoles like PEN cause various morphological and biochemical changes such as increased cytokinin synthesis, improved photosynthetic activity, changes in stem length and seedling weight, inhibition of gibberellin synthesis, and induction of chlorophyll and carotenoid content, lipid peroxidation, and changes in membrane permeability[35–37]. Saffron cultivation worldwide is constrained by a range of biotic stresses, including damage from fungi, viruses, and bacteria[38]. It is essential to acknowledge that fungal contamination of saffron corms poses a significant risk both under normal and stress conditions. Given saffron's unique status as both an important spice and a medicinal plant predominantly cultivated in Iran and other regions, and considering the global significance of drought stress, this study investigates the potential of PEN as a remedy.

The decrease in the growth of fibrous roots under drought was continuous and more pronounced than that of the leaves and corms. Under the effects of PEG, the fresh weight (FW) of leaves, fibrous roots, and corms decreased by 12.5%, 37.1%, and 23.9%, respectively, under 15% PEG treatment (Fig. 2A–C). Thus, fibrous roots are the most sensitive organ to drought stress in *C. sativus*. A decline in plant growth is the most typical sign of water shortage, and cell growth is one of the most drought-sensitive processes due to the reduction in turgor pressure[39].

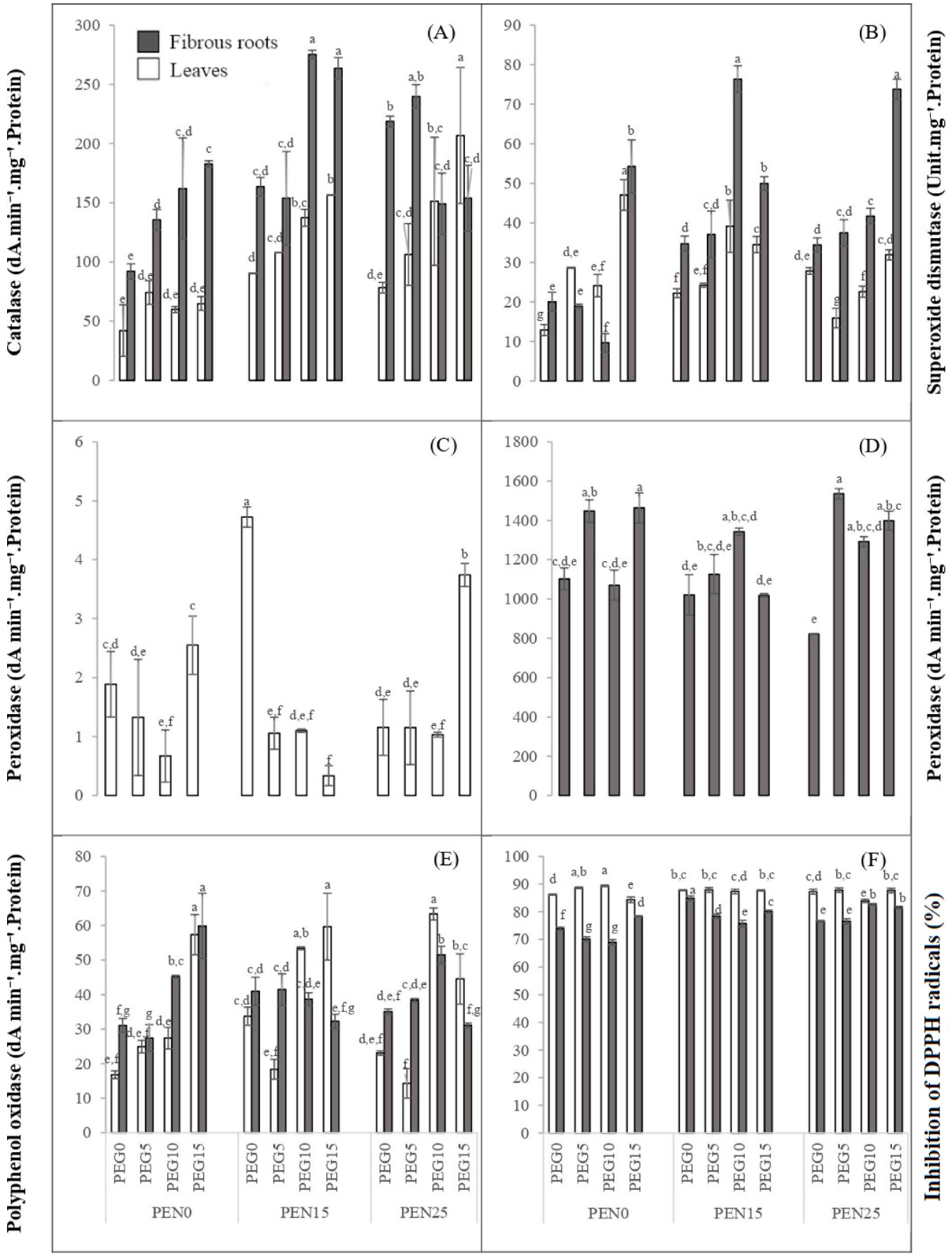

Fig. 4. Effects of PEN (0, 15 and 25 mg/L) pretreatment on Catalase (**A**), Superoxide dismutase (**B**), Peroxidase (**C**), (**D**), Polyphenol oxidase (**E**) activity, and inhibition of DPPH (*1*, 1-diphenyl- 2- picrylhydrazyl) radicals (**F**) in leaves and fibrous roots of *C. sativus* L. under PEG (0, 5, 10 and 15%) induced drought. Vertical bars indicate means ± SE based on three replicates. Different letters above columns indicate a significant difference at $P < 0.05$ using Duncan multiple range test.

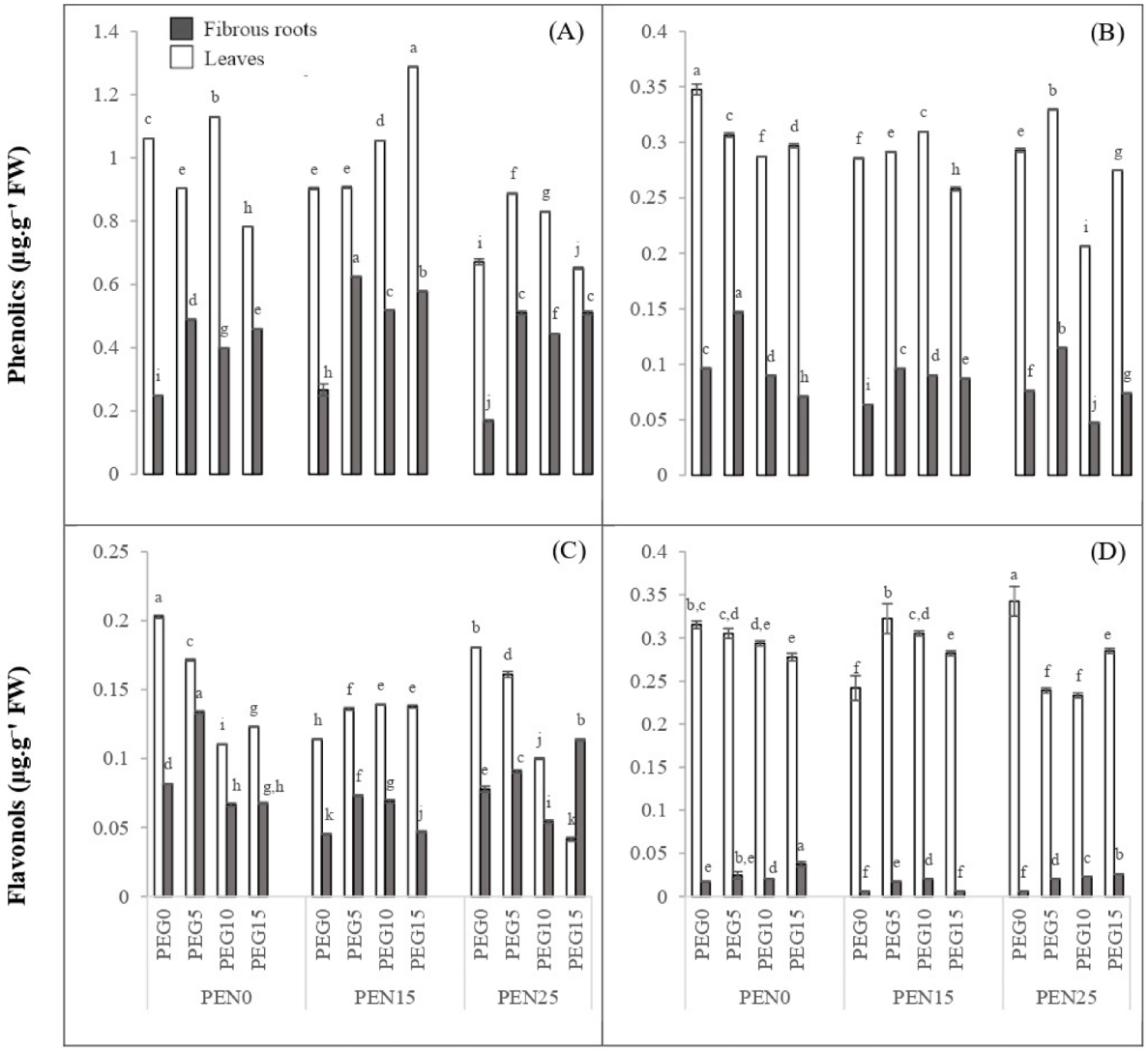

Fig. 5. Effects of PEN (0, 15 and 25 mg/L) pretreatment on total phenolics (**A**), flavonoids (**B**), flavonols (**C**) and anthocyanins (**D**) content in leaves and fibrous roots of *C. sativus* L. under PEG (0, 5, 10 and 15%) induced drought. Vertical bars indicate means ± SE based on three replicates. Different letters above columns indicate a significant difference at $P < 0.05$ using Duncan multiple range test.

Improvement in the growth of all organs under drought stress was achieved by PEN (Fig. 2A–C). A positive correlation was observed between leaf fresh weight (FW) and dry weight (DW) under 10% PEG and 15 mg/L PEN (Fig. 6). The FW and DW of fibrous roots under 5% PEG also positively correlated with 15 mg/L PEN (Fig. 8). The effects of PEN on leaf RWC were also statistically significant, based on ANOVA results (Table 1). The improvement in leaf DW can be attributed to increased proline content and elevated activities of polyphenol oxidase (PPO) and superoxide dismutase (SOD) (Fig. 7), while the improvement in fibrous root DW was associated with increased levels of phenolic compounds such as anthocyanins, flavonoids, and flavonols (Fig. 9). The positive effects of PEN on plant growth have been reported previously[28–33] and may be due to the enhancement of phytohormone levels and subsequent cell division[40,41].

Positive effects of PEN on carotenoid content were observed under drought, and the carotenoid content under 15% PEG was augmented by 25 mg/L PEN (Fig. 2E). Carotenoids stabilize biological membranes and inhibit lipid peroxidation[42]. Moreover, carotenoids are accessory pigments that, in addition to absorbing light for photosynthesis, exhibit antioxidant activity against oxidative stress induced by abiotic factors. As non-enzymatic antioxidants, these pigments protect plants against reactive oxygen species (ROS) and prevent the photo-oxidation of chlorophyll under high light intensity and abiotic stresses such as drought.

Chlorophyll content in leaves decreased continuously under drought stress. The decreasing trend under drought was altered by PEN pretreatment. Positive effects of PEN on *Chl a* and *Chl b* contents under 5% PEG were observed at 25 and 15 mg/L PEN, respectively (Fig. 2F). A decrease in chlorophyll content can be regarded as a typical symptom of oxidative stress under drought. The positive effects of PEN on chlorophyll content under water stress could be due to an increase in chlorophyll biosynthesis or a decrease in its degradation and oxidation,

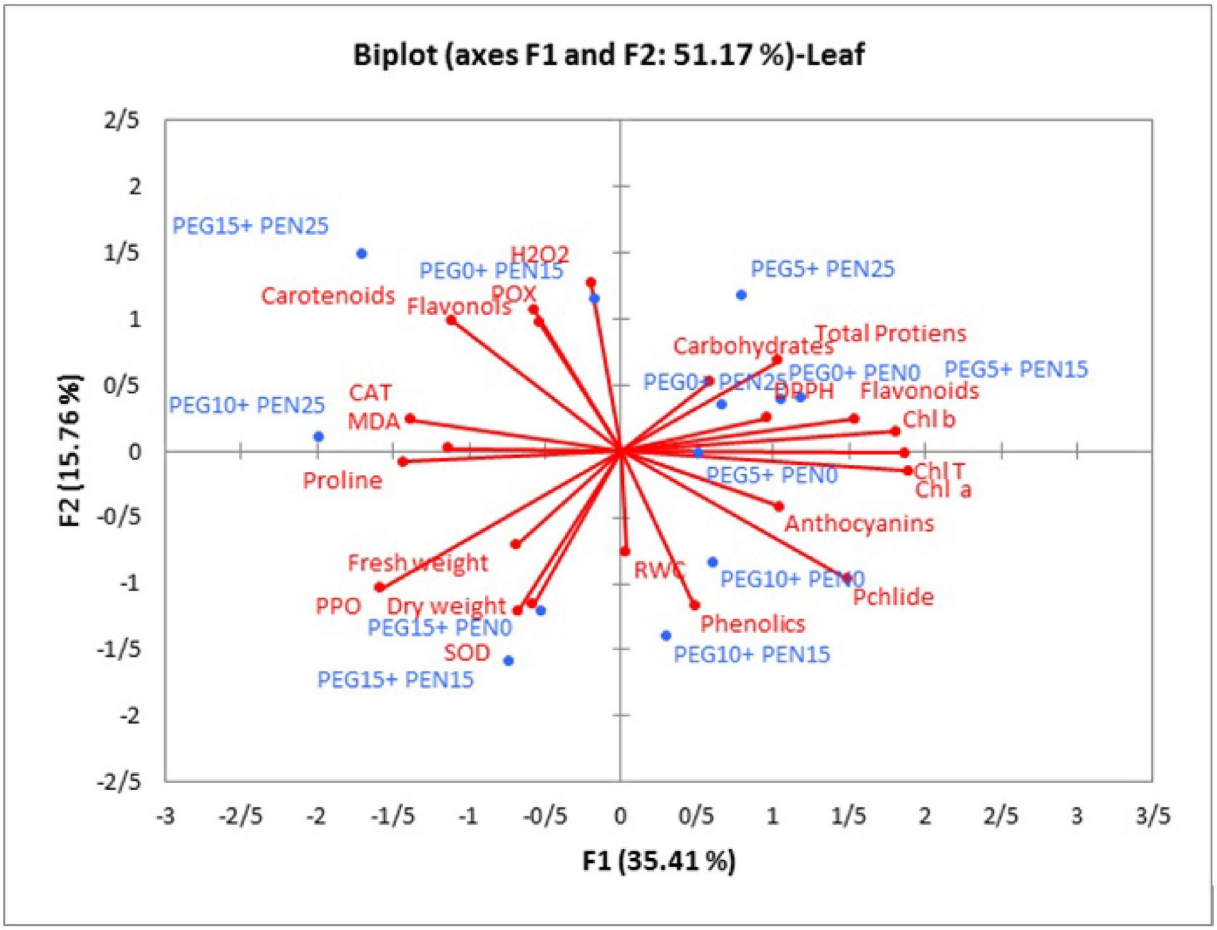

Fig. 6. Loading plots of principle components 1 and 2 of the PCA of physiological and biochemical changes in leaves of *C. sativus* L. under PEN pretreatment and PEG induced drought. CAT *Catalase*, POX, *Peroxidase*, SOD *Superoxide dismutase*, PPO, *Polyphenol oxidase*, H_2O_2*Hydrogen peroxide*, MDA *Molondyaldehyde*, RWC *relative water content*, Chl a, *Chlorophyll a*, Chl b *Chlorophyll b*, Chl T *Total Chlorophyll*, Pchlide *Protochlorophyllide*, DPPH *1, 1-diphenyl- 2- picrylhydrazyl*.

and are consistent with the reports of Kishorekumar et al.[43] and Aly and Latif[44] under triazole compound treatment. The effects of triazoles in preventing chlorophyll degradation can be attributed to an enhancement in cytokinin content. This hypothesis is consistent with the findings of Fletcher et al.[45]. Moreover, according to our results, the ameliorative effect of PEN in preventing chlorophyll degradation can also be attributed to the induction of enzymatic and non-enzymatic antioxidants. According to HCA analysis, positive correlations were observed between chlorophyll content and non-enzymatic antioxidants (anthocyanins and flavonoids) in leaves (Fig. 7).

The lower content of MDA in fibrous roots compared to that in leaves demonstrated higher levels of protective systems in fibrous roots. The significant and continuous increase in MDA content in fibrous roots under drought, compared to that in leaves, confirmed the higher sensitivity of fibrous roots. The significant attenuative effect of 25 mg/L PEN on MDA content in fibrous roots under control and most drought levels, and the similar effect of 15 mg/L PEN on MDA content under 0% and 15% PEG (Fig. 3B), could be attributed to the prominent induction of antioxidative systems in fibrous roots by PEN. Thus, the ameliorative effects of PEN on drought stress in fibrous roots were confirmed by the decrease in MDA and the increase in DPPH radical inhibition (Fig. 4F). Flavonols, as non-enzymatic antioxidants, can be regarded as regulatory compounds in controlling MDA content in fibrous roots (Fig. 8). MDA content, as a product of biological membrane peroxidation, is considered an indicator of oxidative stress. The ameliorative effects of PEN on membrane stability under drought stress may be due to changes in phytosterol composition in the plasma membrane. The effects of drought stress on ROS production and induction of oxidative stress can be explained by perturbations in photosynthetic electron transport and by an imbalance between reductive power production and its consumption for CO_2 assimilation, as also mentioned by others[46]. MDA content induction under some abiotic stresses has been demonstrated[47], and the effect of PEN in reducing MDA content in other plants, such as *Brassica napus* L. under drought, has been reported in another study[29]. According to Zhang et al.[37], a parallel increase in MDA content and electrical conductivity under drought stress, as well as the attenuative effect of uniconazole on both parameters, was reported in the leaves of soybean.

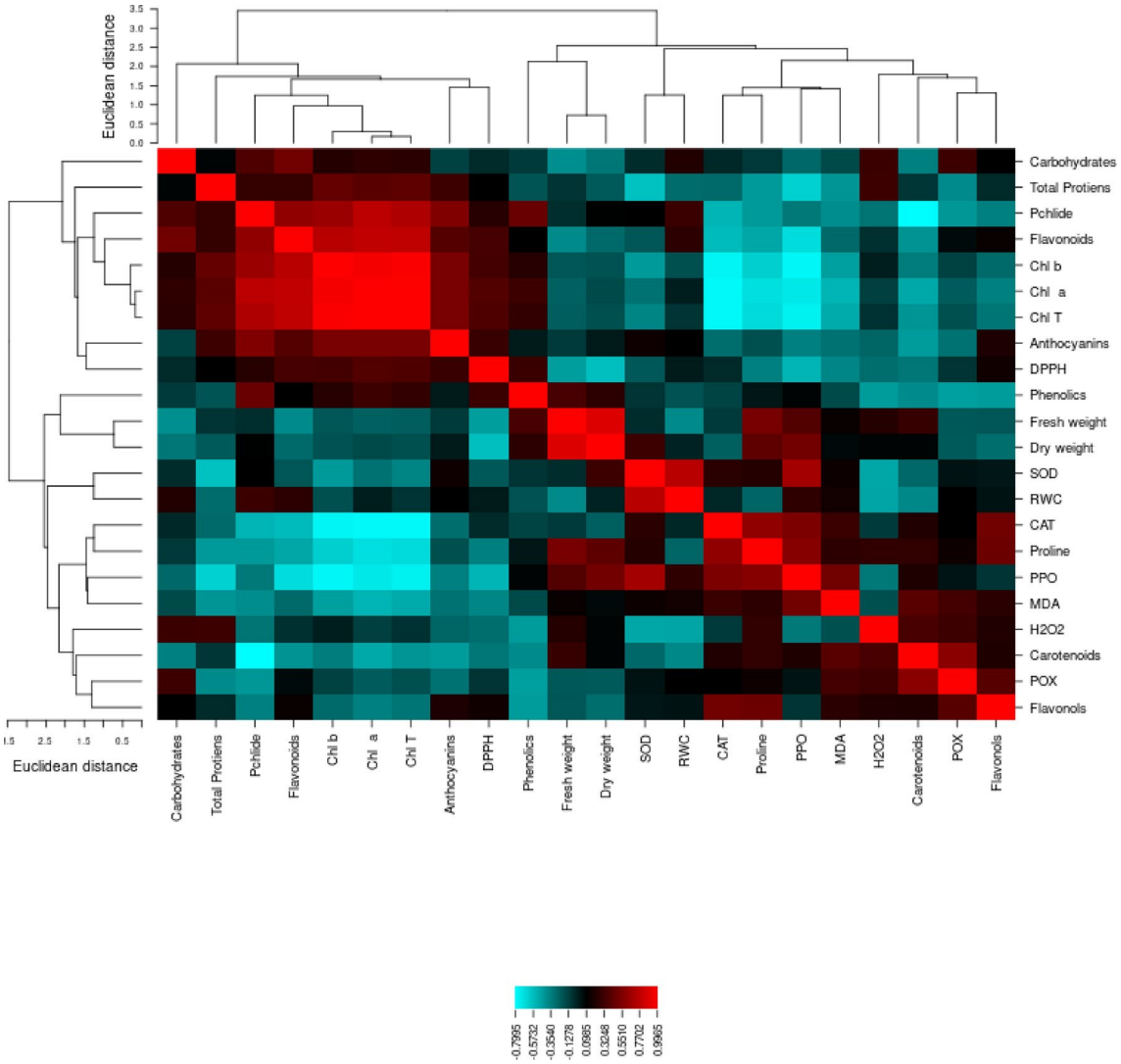

Fig. 7. Heatmap of physiological and biochemical parameters changes in leaves of *C. sativus* L. under PEN pretreatment and PEG induced drought. CAT *Catalase*, POX, *Peroxidase*, SOD *Superoxide dismutase*, PPO, *Polyphenol oxidase*, H_2O_2*Hydrogen peroxide*, MDA *Molondyaldehyde*, RWC *relative water content*, Chl a, *Chlorophyll a*, Chl b *Chlorophyll b*, Chl T *Total Chlorophyll*, Pchlide *Protochlorophyllide*, DPPH *1, 1-diphenyl- 2-picrylhydrazyl*.

During stress conditions such as drought, cells experience an overproduction of reactive oxygen species (ROS). ROS are highly reactive molecules, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH). These ROS can damage lipids, proteins, and nucleic acids, leading to oxidative stress and cellular injury. Plants contain enzymatic and non-enzymatic antioxidants to detoxify ROS[48,49]. Higher activities of all antioxidative enzymes, including CAT, SOD, PPO, and POX, were observed in fibrous roots under both control and drought stress conditions compared to those in leaves (Fig. 4A–E). The inductive effects of PEN on CAT and SOD activities in fibrous roots under certain levels of drought could be regarded as one of the contributing factors to the lower MDA content in fibrous roots. The unresponsiveness of PPO and POX activities to PEN suggests differential roles for antioxidative enzymes under alleviator treatment.

PEN enhances plant antioxidant defenses by modulating redox signaling and upregulating genes encoding key antioxidant enzymes such as SOD, CAT, and ascorbate peroxidase (APX). It promotes the accumulation of non-enzymatic antioxidants like ascorbate, glutathione and proline, thereby reducing oxidative damage under abiotic stress. PEN's effects can be linked to elevated levels of signaling molecules such as salicylic acid and abscisic acid, which further stimulate the antioxidant machinery. This coordinated regulation ultimately maintains cellular redox homeostasis and enhances stress tolerance[23,25,29,32,33,37].

The content of phenolics and all individual phenolic compounds in leaves was significantly higher than that in fibrous roots. However, the content of phenolics in fibrous roots increased significantly under drought stress with PEN, compared to the control (Fig. 4). The tolerance of saffron plant fibrous roots to salinity is also attributed to the induction of polyphenols[50]. In agreement with the DPPH analysis (Fig. 4F), an increase

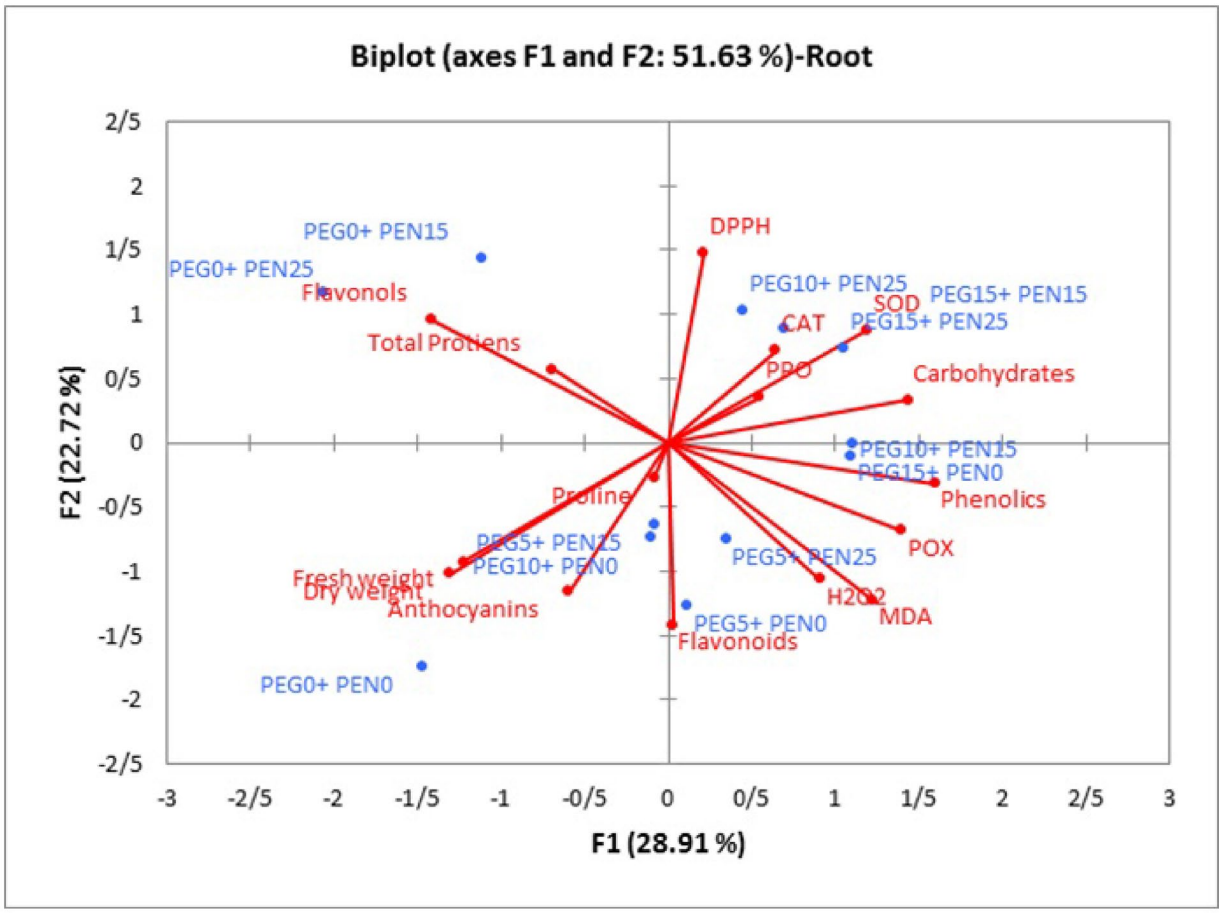

Fig. 8. Loading plots of principle components 1 and 2 of the PCA of physiological and biochemical changes in fibrous roots of *C. sativus* L. under PEN pretreatment and PEG induced drought. CAT *Catalase*, POX, *Peroxidase*, SOD *Superoxide dismutase*, PPO, *Polyphenol oxidase*, H_2O_2*Hydrogen peroxide*, MDA *Molondyaldehyde*, RWC *Relative water content*, Chl a, *Chlorophyll a*, Chl b *Chlorophyll b*, Chl T *Total Chlorophyll*, Pchlide *Protochlorophyllide*, DPPH *1*, *1-diphenyl- 2- picrylhydrazyl*.

in content of antioxidants such as phenolics was expected in fibrous roots under drought stress and PEN application. However, according to HCA analysis of fibrous roots, DPPH radical inhibition showed positive correlations with CAT and SOD activities as well as soluble carbohydrates content (Fig. 9).

In spite of the higher content of soluble carbohydrates in control leaves compared to that of the fibrous roots, a change in the root-to-leaf carbohydrates ratio was observed under drought, even without PEN (Fig. 3C). Some physiological processes, such as distribution of carbohydrates, are well known to influence root growth under abiotic stress[51,52]. A Change in the root-to-leaf carbohydrate ratio was observed with both concentrations of PEN under drought. The increase in root-to-leaf carbohydrate ratio can be regarded as an adaptive response to increased drought in saffron and may be due to an increase in the transport of soluble carbohydrates from leaves to fibrous root. Under drought stress, carbohydrates can act as compatible osmolytes for osmotic adjustment and as a source of energy to cope with stress conditions. In the present study, the increased proportion of soluble sugars in roots under drought indicates that carbohydrates were distributed more to fibrous roots than to leaves.

Proline content in fibrous roots under control conditions, and most drought levels, and PEN treatment was significantly higher than that in leaves. Moreover, despite an increase in proline content of leaves due to PEN under some drought levels, the induction in proline content (as a compatible osmolyte and an osmotic protectant) in fibrous roots was significantly greater than that in leaves (Fig. 3D). Therefore, PEN by increasing proline content in fibrous roots, can enhance plant survival under drought conditions and lead to amelioration.

Based on correlation analyses using Pearson's coefficient in leaves, FW and DW showed positive correlations with SOD and PPO activity. MDA content in leaves displayed negative correlations with chlorophylls, flavonoids and anthocyanins contents. Moreover, PEG15 + PEN15 exhibited positive correlations with leaf DW and FW. Inhibition of DPPH radicals showed positive correlations with chlorophylls, flavonoids, soluble carbohydrates and anthocyanins content (Fig. 7).

Based on the correlation analyses in fibrous roots, FW and DW showed positive correlation with anthocyanins content. MDA content in fibrous roots displayed negative correlations with flavonol and proteins content. Moreover, PEG15 + PEN15 showed synergistic effect and exhibited positive correlations with leaves DW and

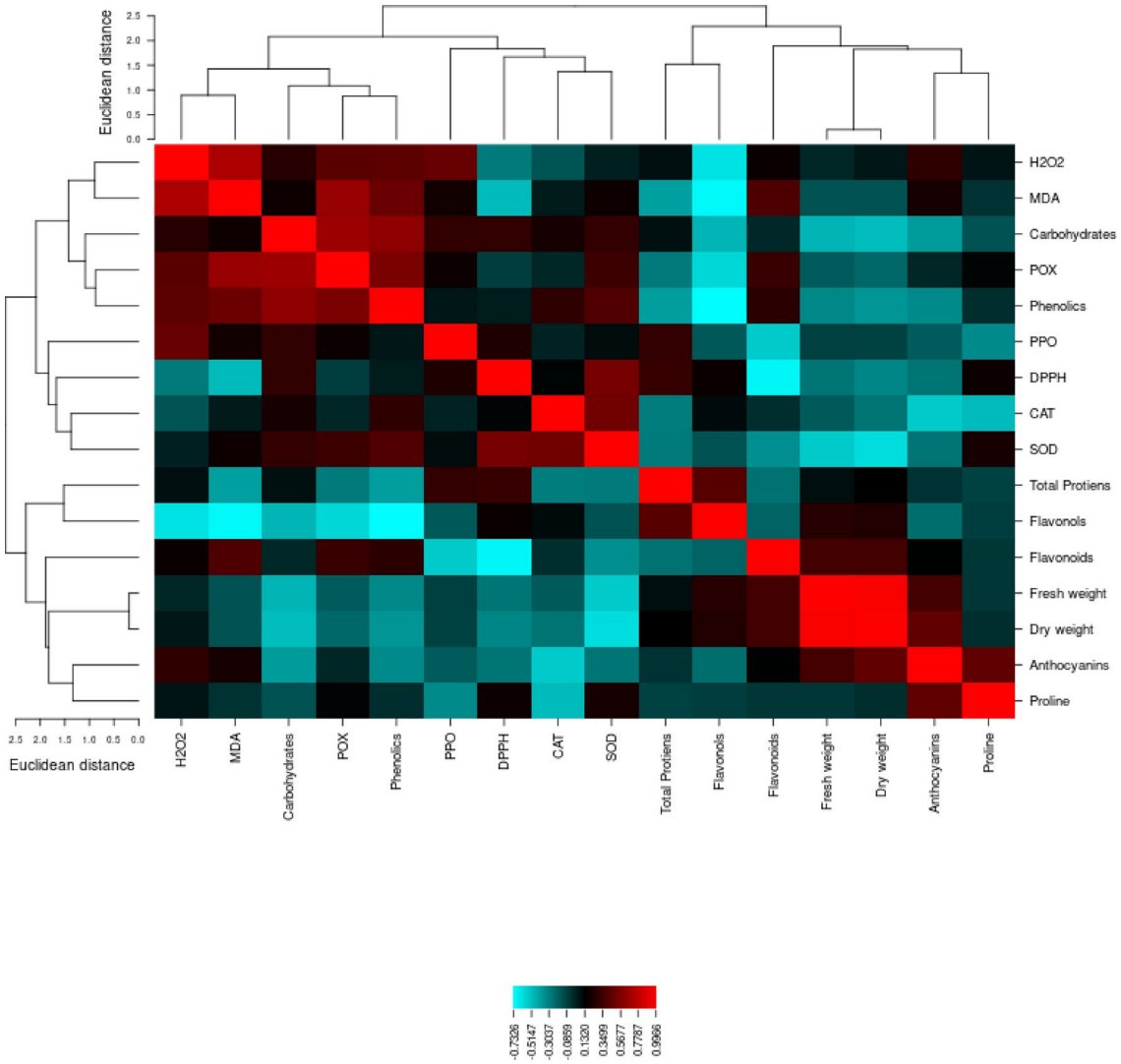

Fig. 9. Heatmap of physiological and biochemical parameters changes in fibrous roots of *C. sativus* L. under PEN pretreatment and PEG induced drought. CAT *Catalase*, POX, *Peroxidase*, SOD *Superoxide dismutase*, PPO, *Polyphenol oxidase*, H_2O_2*Hydrogen peroxide*, MDA *Molondyaldehyde*, RWC *relative water content*, Chl a, *Chlorophyll a*, Chl b *Chlorophyll b*, Chl T *Total Chlorophyll*, Pchlide *Protochlorophyllide*, DPPH *1, 1-diphenyl- 2-picrylhydrazyl*.

FW. Negative correlations were also observed between MDA content and the PEG0 + PEN15 and PEG0 + PEN25 treatments. Inhibition of DPPH radicals showed positive correlations with CAT, SOD, PPO activity and soluble carbohydrate content (Fig. 9).

Despite the higher sensitivity of fibrous roots to drought stress, the MDA content of this organ is much lower than in leaves and is significantly reduced under the influence of PEN. Furthermore, inhibition of DPPH radical in fibrous roots showed positive correlations with SOD, PPO, and CAT activity, as well as with protein and soluble carbohydrate content. Moreover, proline was identified as a key metabolite involved in regulating MDA content in fibrous roots.

In conclusion, given the inherently lower MDA content in fibrous roots, the significant attenuation of lipid peroxidation under drought stress with PEN, and the higher activities of all antioxidative enzymes compared to those in leaves, it was deduced that fibrous roots play a pivotal role in the drought tolerance of saffron. Moreover, the application of the fungicide PEN not only protects saffron against drought stress but also reduces the risk of fungal infection.

Materials and methods

Plant materials and treatments

Saffron corms, sourced from Mashhad, underwent a thorough disinfection and pre-treatment process[11]. Based on preliminary experiments, 15 and 25 mg/L of PEN were selected as the optimum concentrations for further studies. For pre-treatment, the corms were soaked in PEN solutions at concentrations of 0, 15, and 25 mg/L for

24 h prior to cultivation. Following the pre-treatment, the corms were planted in plastic pots (14 × 12 cm) filled with perlite and watered with 100 mL of half-strength Hoagland's solution for three weeks. Drought stress was then imposed using PEG 6000, with concentrations of 0% (− 0.01 MPa), 5% (− 0.05 MPa), 10% (− 0.15 MPa), and 15% (− 0.3 MPa) (w/v) in the Hoagland solution[53]. The pots were placed in a growth chamber at 16–18 °C with a relative humidity of 49–53%, under white LED light (40–50 µmol photons m^{-2} s^{-1}) and a 16-h light/8-h dark photoperiod. Three weeks after PEG treatment, the organs from six-week-old plants were collected and immediately frozen in liquid nitrogen before being stored at -70 °C for future analyses. The fresh weight of the organs was measured, and the dry weight was obtained by drying the samples in an oven at 60 °C for 24 h until a constant weight was achieved.

Measurement of relative water content (RWC)

To determine RWC[54], three leaves were randomly chosen and immediately weighed to obtain their fresh weight (FW), and then were immersed in distilled water in a dark environment at room temperature for 24 h. The excess water was gently removed by blotting, and the leaves were weighed again to determine the turgid weight (TW). To determine dry weight (DW), the leaves were placed in an oven at 60 °C for 24 h. The RWC was then calculated using the following formula:

$$\mathrm{RWC}\ (\%) = (\mathrm{FW} - \mathrm{DW}) / (\mathrm{TW} - \mathrm{DW}) \times 100.$$

Measurement of leaf chlorophylls and carotenoids content

For extraction and quantification of *Chl a*, *Chl b*, and carotenoids[55], fresh leaves (0.2 g) were ground in a mortar with 5 mL of 80% (v/v) acetone. The homogenate was filtered, and the absorbances of the filtrate were recorded at 663.6 nm, 646.6 nm, and 440.5 nm. The photosynthetic pigment content was expressed as µg g^{-1} FW.

Assessment of H_2O_2 and malondialdehyde (MDA) content

For H_2O_2 determination[56], plant material (0.5 g) was homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) on ice and centrifuged at 11,289 × g for 15 min. One mL potassium phosphate buffer and 1 mL potassium iodide were added to 0.5 mL of the supernatant, then the absorbance of the supernatant was recorded at 390 nm and H_2O_2 content was calculated using a standard calibration curve. H_2O_2 content was reported as mg g^{-1} FW.

Lipid peroxidation was determined by measuring MDA content. A plant sample (0.2 g) was homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 7840 × g for 20 min. To 1 mL of the supernatant, 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA was added, then was heated at 95 °C for 30 min and quickly cooled on ice. After centrifugation for 15 min, the absorbance of the supernatant was measured at 532 and 600 nm. The value for nonspecific absorbance at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}[57]. MDA content was expressed as µmol g^{-1} FW.

Determination of antioxidant enzymes activity

Plant material (0.5 g) was homogenized at 4 °C with 1 M Tris–HCl (pH 6.8) to determine protein content and activities of different antioxidant enzymes. The homogenate was centrifuged at 13,250 × g for 20 min at 4 °C, and the supernatant was kept at − 70 °C until further assays were performed. The protein content was determined according to Bradford, using bovine serum albumin as a standard[58].

Catalase (CAT) activity was determined by measuring the capability to decompose H_2O_2 in a potassium phosphate substrate[59]. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 3% H_2O_2 and 10 µL enzyme extract. The decrease in absorbance at 240 nm was followed for 180s and CAT activity was expressed as dA min^{-1} mg^{-1} protein.

Peroxidase (POX) activity was evaluated by measuring the enzyme's ability to oxidize benzidine in the presence of H_2O_2 substrate[60]. The assay mixture consisted of 2 mL of 200 mM sodium acetate buffer (pH 4.8), 200 µL H_2O_2 (3%), 100 µL of 20 mM benzidine, and 50 µL enzyme extract. The increase of absorbance was recorded at 530 nm and POX activity was expressed as dA min^{-1} mg^{-1} protein.

Polyphenol oxidase (PPO) activity was determined by measuring the oxidation of pyrogallol, a polyphenol[61]. The reaction mixture contained 2.5 mL of 200 mM potassium phosphate buffer (pH 7), 200 µL of 20 mM pyrogallol, and 20 µL enzyme extract. The increase in absorbance was recorded at 430 nm and PPO activity was expressed as dA min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the photochemical reduction of the nitroblue tetrazolium (NBT) to formazan[62]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM ethylenediamine tetraacetic acid (EDTA), 75 µM NBT, 13 mM methionine, 2 µM riboflavin, and 100 µL of protein extract. Reactions were carried out for 16 min at a light intensity of 300 μmol^{-1} $m^{-2}s^{-1}$. The non-irradiated reaction mixture was used as a control and its absorbance was subtracted from that at 560 nm. One unit of SOD was defined as the amount of enzyme that caused 50% inhibition of NBT reduction, and the activity was expressed as U mg^{-1} protein.

Determination of total phenolics, flavonoids, flavonols, anthocyanins content and inhibition of DPPH radicals

Plant tissue (0.5 g) was homogenized in 5 mL of 80% methanol. The mixture was then sonicated in a water bath for 20 min. After centrifugation at 3500 rpm for 20 min, the supernatant was collected for phenolics determination. For total phenolics content measurement 50 µL of the methanolic extract was mixed with 1.25 mL of 10% Folin-Denis reagent. After 5 min, 1 mL of 7% sodium carbonate solution was added to the mixture and then absorbance was recorded at 765 nm[63]. Total phenolics content was calculated as µg g^{-1} FW.

For determination of flavonoids content[64], methanolic extract (0.5 ml) was mixed with 1.5 mL of methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of potassium acetate (1 M), and 2.8 mL of distilled water and the absorbance was measured at 415 nm after 30 min. Gallic acid was used as a standard for the calibration curve. Flavonoids content was calculated as μg g^{-1} FW.

For determination of flavonols content[65], a methanolic extract (0.5 mL), 3 ml of 5% sodium acetate, and 0.5 mL of 2% aluminum chloride solution were mixed. Absorbance was measured at 445 nm after 2.5 h and rutin was used as the standard. Anthocyanin content was determined in 0.3% HCl in methanol at 25 °C using an extinction coefficient of 33,000 cm^2 mol^{-1}at 550 nm[66]. Flavonols content was calculated as μg g^{-1} FW.

To estimate DPPH (1, 1-diphenyl- 2- picrylhydrazyl) radical scavenging activity[67], leaf tissue (0.1 g) was homogenized in 1 mL of 96% ethanol and then insoluble materials were removed by centrifugation at 3500 × g for 5 min. Twenty microliters of extracting solution were mixed with 800 μL of DPPH (0.5 mM in ethanol). The absorbance of the resulting solution was measured at 517 nm after 30 min in darkness. Using the following equation, the free radical scavenging activity was calculated:

$$\text{Inhibition of DPPH radicals } (\%) = [(\text{Absorbance of control} - \text{Absorbance of sample}) / (\text{Absorbance of control})] \cdot 100.$$

Determination of free proline and soluble carbohydrates content

For the proline content measurement[68], 0.5 g of plant tissue was homogenized in 10 mL of 3% sulfosalicylic acid and then was centrifuged at 13,000 rpm for 20 min at 4 °C. Two ml of the supernatant were combined with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid, and then was boiled at 100 °C for 1 h. The reaction mixture was extracted with 4 ml of toluene, and the absorbance was measured at 520 nm using L-proline as a standard. Proline content was calculated as μg g^{-1} FW.

For total soluble carbohydrates content determination[69], plant material (0.1 g) was extracted using 3 mL of de-ionized water. To determine carbohydrate content, 50 μL of extract was mixed with 450 μL of distilled water and 500 μL of a 5% phenol solution and then 2.5 mL of concentrated sulfuric acid was immediately added. The mixture was allowed to stand at room temperature for 30 min. The absorbance of the samples was measured at 485 nm. Total soluble carbohydrates content was calculated as mg g^{-1} FW.

Statistical analysis

The experiments were laid out in a completely randomized design. Each experiment was repeated three times, and the data were analyzed by using either one- or two-way analysis of variance (ANOVA) with SPSS (version 22). Means were compared using Duncan's test at the 0.05 level of confidence. Principal component analysis (PCA) was conducted to obtain the correlation matrix and Pearson's correlation coefficients between each pair of biochemical or physiological variables, using XLSTAT software (2016). The hierarchical cluster analysis (HCA) was performed to evaluate the correlations between each pair of variables, using the online CIMminner software.

Data availability

All datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

M.N. carried out the experiments, also performed statistical analysis and drafted the manuscript. V.N. and H.R. contributed to design of experiment. S.A.S. took part in preparation of methods for the experiments. M.R. critically revised the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

The authors confirm that the present study complies with international, national and/or institutional guidelines.

Additional information

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