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RESEARCH ARTICLE

Effects of exogenous proline treatment on antioxidant and biochemical parameters in *Lepidium sativum* L. plants cultivated in a water-stressed condition

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Abstract

Proline, an organic compatible solute that acts as an osmoprotectant, is a crucial part of many plant's responses to water stress. In the current study, the impact of proline on enzymatic, non-enzymatic antioxidant and biochemical parameters in *Lepidium sativum* L. growing under water stress was examined. Plants were raised in controlled environments with a temperature of 25°C 16 h of light and 8 h of darkness. The concentrations of 50 µg/L, 100 µg/L and 250 µg/L proline were standardized and applied to plants grown under different water potential of -0.01Ψ_wMPa, -0.02Ψ_wMPa and -0.03Ψ_wMPa through foliar spray. After 35, 75 and 110 days of plant growth, the enzymatic antioxidants catalase, peroxidase, superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, glutathione-s-transferase activity and non-enzymatic antioxidants like phenols, ascorbic acid, tocopherol, flavonoid content, were measured. The foliar application of proline increased enzymatic and non-enzymatic antioxidant assay activity in stressed growing plants compared to control plants. However, lipid peroxidation was reduced in water stressed plant due to proline application. The present study investigated that proline played a significant role in overcoming water stress in *L. sativum*. Moreover, this study supported that *L. sativum* plant copes with water stress by foliar application of proline.

Keywords

catalase; osmolyte; proline; peroxidase; superoxide dismutase; water stress

Introduction

Plant growth and development are severely hampered by abiotic stress factors such as drought, salinity, cold, freezing, high temperatures, anoxia and intense light (1). The majority of abiotic stressors are intricate and genetically controlled. Depending on the different abiotic stresses, they experience and the inevitable environmental conditions they are subject to, plants undergo a variety of morphological, physiological and biochemical changes that adversely affect their growth and development (2). Water stress is one of the main abiotic

stresses that seriously harm plants. The major causes of the development of drought conditions in field crops include global warming, decreased precipitation, a low groundwater table and a decrease in the level of soil water (3). The major causes of the development of drought conditions in field crops include global warming, decreased precipitation, a low groundwater table and a decrease in the level of soil water (3). A severe water shortage condition may result in a reduction in the stability and transport activity of plant membranes (4). There have also been reports of other significant metabolic changes in plants under water stress, such as proline buildup, a decrease in chlorophyll content, protein content and soluble sugar levels (4). Transpiration, photosynthesis and stomatal conductance are the three physiological functions most significantly impacted by drought stress (3). Water is one of the key physical factors in an ecosystem that affects whether or not plants can establish themselves (5). The main factor restricting crop productivity is water stress, which is becoming a more serious issue in many parts of the world. The ability of a plant to grow and reproduce successfully in the face of water stress is known as drought resistance, but the capacity of a plant to gradually change its structure and function to more effectively withstand drought is known as drought acclimation (6).

Reactive oxygen species (ROS) produced by oxidative stress brought on by a drought can damage membranes and destroy certain enzymes (7). Normal cellular metabolism or unfavourable environmental factors like drought, salt, heavy metals, herbicides, food deprivation and radiation can cause the production of reactive oxygen species in plant cells (8). Numerous enzymatic and non-enzymatic antioxidant defence mechanisms regulate their synthesis. Ascorbate, glutathione, carotenoids, phenolic compounds, sugar and polyamines are examples of non-enzymatic antioxidant defence systems. Enzymatic antioxidant defence systems include catalase, ascorbate peroxidase, peroxidase, superoxide dismutase, monodehydroascorbate reductase and dehydroascorbate reductase (9).

Osmotic adjustment enables organelles and cytoplasmic functions to proceed roughly at a normal rate, enhancing plant growth, photosynthesis and assimilation partitioning to grain fullness (10). Enzymatic antioxidants include peroxidase, superoxide dismutase, catalase, glutathione peroxidase, ascorbate peroxidase, glutathione-s- transferase, glutathione reductase, nicotine amide adenine dinucleotide hydrogen and monodehydroascorbate reductase. While non-enzymatic antioxidants like flavonoids, carotenoids, tocopherols, ascorbic acid, reduced glutathione (GSH) and osmolyte proline. Enzymatic and non-enzymatic defence mechanisms collaborate to remove ROS (10). Signaling or damage depends on the balance between ROS generation and the activation of scavenging enzymes. However, considerations related to climate change have forced us to adopt new technologies in order to understand how stress sensing, signal transduction and plant stress tolerance mechanisms work (11). A frequent response seen in several plant systems is the accumulation of compatible solutes, which has been demonstrated to occur when the osmotic adjustment is altered (12). Although plants can accumulate osmolytes to protect themselves against stress. Enhancement in osmolytes has typically been thought to be a measure of stress tolerance. Proline, polyamines, glycine betaine, sugar and sugar derivatives, glycerol, sorbitol, mannitol and a number of other osmolytes that are frequently measured in cells play important roles in defending cells from cell-damaging stress agents (10). One of these osmolytes, proline, is one of the most significant cytosolutes and higher plants, algae, mammals and bacteria commonly accumulate it freely in response to low water potential (10). Increased biosynthesis and slow oxidation in mitochondria work together to produce its production in leaves with low water potential. Despite some debate, free proline has been implicated in a variety of physiological functions, including the stability of macromolecules, the elimination of excess reductant and the storage of carbon and nitrogen for use after the water deficit has been corrected (13). Not all plant species possess the capability to accumulate suitable solutes for effective stress tolerance. Understanding the enzymatic and non-enzymatic antioxidant responses, as well as the biochemical adaptations of plants under abiotic stress, is crucial for improving stress resilience. *Lepidium sativum*, a medicinal and nutritionally important plant, is known for its moderate drought tolerance; however, its physiological and biochemical responses to water stress remain inadequately explored. Proline, a well-documented osmoprotectant, plays a significant role in mitigating oxidative damage and maintaining cellular homeostasis under stress conditions. Therefore, this study aims to investigate the impact of exogenous proline application on the enzymatic and non-enzymatic antioxidant defense systems, as well as key biochemical attributes of *L. sativum* under water deficit conditions. The findings of this study will contribute to a deeper understanding of the potential of proline in enhancing drought tolerance and may have implications for improving the resilience of similar plant species in water-limited environments.

Materials and Methods

The seeds propagated in seed trays comprising soil placed in a polyhouse with regulated temperatures ranging from 20 to 25° C, with a photoperiod of 16 h of light and 8 h of darkness. 10 days later, seedlings were shifted to different earthen pots of 30 cm diameter. After 20 days of transplanting water stress and osmolyte treatments were started. Water stress was imposed using weighing method and tensiometers. Three water potentials (-0.01Ψ_wMPa, -0.02Ψ_wMPa and -0.03Ψ_wMPa) were achieved in one month old seedlings, which were maintained until the end of the experiment and were monitored daily. Proline concentration used for treatments was 50 µg/L, 100 µg/L and 250 µg/L and applied through foliar spray after 20, 40, 60 and 80 days of transplant. For optimal penetration of proline, Tween-20 was added to the foliar solution at 0.1 % by volume. Seedlings were given Hoagland nutrient solution weekly. Three replicate sets of pots were used in a randomized block design to arrange the pots. Each treatment featured a randomized design with three replication sets, each of which had ten pots. After 35, 75 and 110 days, samples of leaves were taken to determine the antioxidant activity of enzymes like catalase, peroxidase, superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, glutathione s-transferase, as well as the content of non-enzymatic antioxidants like phenol, ascorbic acid, tocopherol and flavonoids.

Catalase (CAT)

Plant tissue (leaf and root) was homogenized in a blender with phosphate buffer (0.067 M, pH 7.0) (assay buffer diluted 10 times) at 1-4°C and centrifuged. Cold phosphate buffer was used to stir the sediment before allowing it to stand in the cold while being sometimes shaken and repeating the extraction a couple of times. The assay mixture's final volume was around 3 mL and it was read at 240 nm wavelength against a control cuvette that contained the same enzyme solution as the experimental cuvette but with H_2O_2-free PO_4 buffer. Pipette 3 mL of the H_2O_2-PO_4 buffer into the experimental cuvette. 0.01-0.04 mL of the sample was blended with a glass or plastic rod that had one end flattened. Noted the duration needed for an absorbance drop from 0.45 to 0.4. The 20 cc of one g of homogenized tissue is used for the assay, which is then diluted 1 to 10 times with water. Used the extinction coefficient of 0.036/mole/mL to get the H_2O_2 concentration (14).

Peroxidase (POD)

The plant sample (leaf and root) was homogenized to a 20 % concentration in 0.1 M phosphate buffer (pH 6.5), centrifuged and the supernatant was used for the test. 0.1 mL of the enzyme extract was added to 3.0 mL of pyrogallol solution after the spectrophotometer was set to read zero at 430 nm. 0.5 mL of H_2O_2 was added to the test cuvette and stirred. A spectrophotometer was used to record the change in absorbance every 30 sec for up to 3 min. The change in absorbance per minute at 430 nm is used to measure peroxidase activity (14).

Superoxide dismutase (SOD)

The supernatant from the centrifugation at 2000 rpm for 10 min with the ground-up leaves (0.5g) and 3.0 mL of potassium phosphate buffer were utilized for the experiment. In the test mixture, which had a total volume of 2.8 mL, there were 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of PMS, 0.3 mL of NBT, 0.2 mL of the enzyme preparation and water. The addition of 0.2 mL of NADH started the process. After 90 sec of incubation at 30°C, the mixture was stopped by adding 1.0 cc of glacial acetic acid. After shaking the reaction mixture with 4.0 mL of n-butanol, the mixture was left to stand for 10 min before centrifuging. Using a spectrophotometer, the amount of chromogen in the butanol layer was determined at 560 nm. The amount of enzyme that inhibited NBT decreased by 50% over the course of one minute and is considered one unit of enzyme activity (14).

Ascorbate peroxidase (APX)

In order to homogenize the samples, 5.0 mL of phosphate buffer was used. The supernatants from the centrifugation of the homogenates at 5000 rpm for 10 min was utilized for the test. 50 mM phosphate buffer (pH 6.0), 0.1 mM EDTA, 0.5 mM ascorbate, 1.0 mM H_2O_2 and 50 mL of enzyme extract was all included in the reaction mixture (1.5 mL). H_2O_2 was added to start the process and ascorbate oxidation was detected at 290 nm in 1 min. The molar extinction coefficient for ascorbate (2.8 $mM^{-1}cm^{-1}$) was used to measure enzyme activity and the result was expressed in mol H_2O_2 Min^{-1} g^{-1} FW (1).

Glutathione peroxidase (GPX)

The following reaction is catalyzed by glutathione peroxidase: Se-GPx GSSH + $2H_2O$ 2GSH + H_2O by combining with 5,5'-dithio -bis (2-nitrobenjoic acid) to form a molecule that absorbs at 412 nm, glutathione can be quantified. Two test tubes marked "test" and "control" each contained 0.4 mL of buffer, 0.2 mL of EDTA, 0.1 mL of sodium azide, 0.2 mL of reduced glutathione and 0.1 mL of H_2O_2. 0.2 mL of the sample was introduced to the test and 0.2 mL of water was placed in the control. After 10 min of incubation at 37°C with thorough mixing, the reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid. By centrifuging 1.0 mL of the supernatant, 3.0 mL of buffer and 0.5 mL of Ellman's reagent were added to the supernatant to assess the glutathione concentration. At 412 nm, the colour created was read. Standards weighing between 40 and 200 g were collected and handled similarly. The activity was represented as grammes of glutathione consumed per minute per milligram of protein (1).

Glutathione S-transferase (GST)

The glutathione S-transferase levels in the leaves of the chosen plant were measured. The ability of the enzyme to combine reduced glutathione and 1-chloro-2,4-dinitrobenzene was tested, with the degree of conjugation generating a corresponding change in the absorbance at 340 nm. In order to homogenized the sample (0.5g), phosphate buffer (5.0 mL) was used. The supernatant from the centrifugation of the homogenate at 5000 rpm for 10 min was utilized for the test. In a spectrophotometer, the change in absorbance at 340 nm was tracked to assess the enzyme activity. In a total amount of 2.9 mL, the assay mixture contained phosphate buffer, 0.1 mL of CDNB and 0.1 mL of GSH. The reaction was triggered by adding 0.1 mL of enzyme extract to this combination and measurements were recorded for a minimum of three min against a blank of distilled water. To track non-specific substrate binding, the entire test combination without the enzyme was used as the control. The number of nmoles of CDNB conjugated per minute is used to define one unit of GST activity (1).

Phenol content

A pestle and mortar were used to grind 0.5 g of the sample (leaf and root) in a 10-time volume of ethanol that was made up of 80% alcohol. Saved the supernatant after centrifuging the homogenate at 10,000 rpm for 20 min. After centrifuging and collecting, the supernatant the residue was once again extracted using five times as much 80 % ethanol. The supernatant was dried by evaporation. The residue was dissolved in a known amount of distilled water (5 mL). Pipette various aliquots (ranging from 0.2 to 2 mL) into test tubes. Water was used to bring the volume in each tube to 3 mL. 0.5 mL of Folin-Ciocalteau reagent was added. 2 cc of a 20 % Na_2CO_3 solution was added to each tube and properly mixed after three min. After cooling the tubes in a boiling water bath for a minute, the absorbance at 650 nm was measured in comparison to a blank for the reagent. Created a standard curve using various gallic acid concentrations. The amount of phenols in the test sample was calculated using the standard curve and expressed as mg phenols/100g of material (15).

Ascorbic acid content

Using 4 % TCA, ascorbate was extracted from 1g of the plant material and the volume was then increased to 10 mL using the same method. After 10 min of centrifugation at 2000 rpm, the resulting supernatant was treated with a small amount of activated charcoal, violently mixed with a cyclomixer and then left for 5 min. By using centrifugation, the charcoal particles were separated and aliquots were employed for the estimate. A sample of the supernatant and standard ascorbate between 0.5 and 1.0 mL each were taken. 2.0 mL of the volume containing 4 % TCA was added. To each tube, 0.5 mL of the DNPH reagent was added, followed by 2 drops of the 10 % thiourea solution. Osazone crystals were created after the mixture was incubated for three hours at 37°C. In 2.5 mL of cool, 85 % sulphuric acid, the crystals were dissolved. After adding sulphuric acid, the DNPH reagent and thiourea were applied to the blank alone. The tubes were chilled using ice and a spectrophotometer was used to measure the absorbance at 540 nm. Using an electronic calculator configured to the linear regression mode, a typical graph was created. Calculated and represented in terms of mg/g of sample, ascorbate content in the samples was determined (16).

Tocopherol content

After being homogenized in 50 mL of 0.1N sulfuric acid with the plant sample (2.5g), the sample was left to stand overnight. The flask's contents were rapidly stirred before being filtered with Whatman No. 1 filter paper. For the estimate, aliquots of the filtrate were employed. 1.5 mL of plant extract, 1.5 mL of the standard and 1.5 mL of water was pipetted out separately into 3 centrifuge tubes. 1.5 mL of ethanol and 1.5 mL of xylene was added to each tube, well mixed and centrifuged. A 1.0 mL layer of xylene was transferred to a new stoppered tube. 1.0 mL of dipyridyl reagent was added to each tube and thoroughly mixed. Pipetting 1.5 mL of the mixture into a cuvette allowed the extinction to be measured at 460 nm. All of the tubes received the 0.33 mL of ferric chloride solution after being thoroughly mixed. After 15 min, a spectrophotometer reading at 520 nm revealed the red colour that had grown (16).

Flavonoids

The samples were homogenized in 80 % methanol at a rate of 0.1 g per mL. A 5 % $NaNO_2$ solution and 1.25 mL of distilled water was added to 250 µL of methanolic extract. 150 µL of 10% $AlCl_3$. After adding 500 µL of 1M NaOH and 275 µL of distilled water to the mixture and thoroughly shaking it, the amount of pink colour intensity was measured at 510 nm. Quercetin (QU) was used as a reference to calculate the concentration of total flavonoids (15, 49). The findings were presented as mg of quercetin/g of freshly weighed plant material.

Lipid peroxidation

200 mg of leaf and root plant tissue were homogenized with 2 mL of 0.1% trichloroacetic acid. The supernatant was obtained after the homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant 2 mL was combined with 4 mL of 20% TCA that contained 0.5% thiobarbituric acid to initiate the reaction (TBA). The mixture was then cooked for 45 min at 95°C. For five minutes, it was quickly cooled in an ice bath. At 532 nm, absorbance was measured. By deducting the absorbance at 600 nm, measurements were adjusted for general turbidity (16).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism® 5.2. Data were expressed as mean ± standard deviation (SD) from three independent replicates (n = 3). One-way analysis of variance (ANOVA) was conducted to determine significant differences among treatments, followed by Tukey's post hoc test at a significance level of $P \leq 0.05$.

Results

The impact of proline on catalase activity in plants grown under varied water potential has been presented in Table 1. It is evident that catalase activity increased with plant growth irrespective of treatment. The proline treated plants had higher catalase activity compared to untreated plants. It increased by 38.8% in 50 µg/L proline treated plants, by 52.5% in 100µg/L proline treated plants and by 55.7% in 250 µg/L proline treated plants of 35 days. In 75 days old plants the catalase increased by 33.7% in 50 µg/L, by 37% in 100 µg/L and by 38.2% in 250 µg/L proline treated plants. Similarly, as the plant matures by 110 days, the catalase activity increased by 20%, 28.2% and 35.6%, in 50 µg/L, 100 µg/L and 250 µg/L proline treated plants, respectively (Table 1). These increases were significant at $P< 0.05$. The impact of proline in plants grown under different water stress conditions showed that 250µg/L proline was more effective compared to 50µg/L and 100µg/L in increasing the catalase activity in 35 days old

Table 1. Effect of proline and water stress on peroxidase activity (U (µmol/min)

Treatments	35 Days	75 Days	110 Days
Control	0.219±0.073^{a}	0.356±0.012^{a}	0.443±0.015^{a}
Proline 50µg/L	0.304±0.037^{b}	0.476±0.023^{b}	0.532±0.033^{b}
Proline 50µg/L and -0.01Ψ_wMPa	0.290±0.029^{a}	0.466±0.022^{c}	0.522±0.026^{c}
Proline 50µg/L and -0.02Ψ_wMPa	0.284±0.027^{a}	0.457±0.008^{d}	0.515±0.024^{d}
Proline 50µg/L and -0.03Ψ_wMPa	0.275±0.028^{a}	0.436±0.016^{e}	0.507±0.026^{e}
Proline 100µg/L	0.334±0.011^{c}	0.488±0.020^{f}	0.568±0.028^{f}
Proline100µg/L and -0.01Ψ_wMPa	0.320±0.003^{d}	0.440±0.018^{g}	0.556±0.007^{g}
Proline 100µg/L and -0.02Ψ_wMPa	0.314±0.009^{e}	0.434±0.003^{h}	0.547±0.019^{h}
Proline 100µg/L and -0.03Ψ_wMPa	0.311±0.011^{f}	0.420±0.004^{i}	0.536±0.019^{i}
Proline 250µg/L	0.341±0.008^{g}	0.492±0.003^{j}	0.601±0.005^{j}
Proline 250µg/L and -0.01Ψ_wMPa	0.332±0.029^{h}	0.450±0.023^{k}	0.592±0.030^{k}
Proline 250µg/L and -0.02Ψ_wMPa	0.318±0.049^{i}	0.438±0.010^{l}	0.565±0.005^{l}
Proline 250µg/L and -0.03Ψ_wMPa	0.314±0.049^{j}	0.422±0.008^{m}	0.554±0.022^{m}

Data are mean ± SD, of three replicates (n=3) and analyzed using graph pad prism 5.2 by one -way Anova followed by Tukey post-test at P<0.05 significance level. Different lower-case letters in a column indicate significant difference between control and treatments.

plants. The catalase activity increased between 25.5 to 51.5 % in water stressed plants. However, the application of 250µg/L proline to the plants grown in -0.01, -0.02 and -0.03 Ψ_wMPa showed enhanced catalase activity in 35 days plants. In 75- and 110-days old plants the trends were almost similar to that seen in 35 days plant. The treatment of proline, including the lowest concentration applied, was seen to be less effective compared to higher concentration to nullify the impact of water stress in 35, 75 and 110 days old plants in catalase activity as it was found to be more in proline treated plants compared to untreated plants (Table 1). Peroxidase activity also enhanced in plants treated with different concentrations of proline and showed significant variation ($P<0.05$) as presented in Table 2. It was observed that the peroxidase activity increased by 58.9% in 50µg/L proline treated plants, by 67.2% in 100µg/L proline treated plants and by 80% in 250µg/L proline treated plants in 35 days old plants (Table 2). A similar trend was found in peroxidase activity between proline treated and untreated plants in 75 and 110 days old plants. It was also found that proline was effective in increasing the peroxidase activity in plants grown under varied water potential. This increase was proline concentration dependent but as the growth of plants increased, it was less effective in 75 and 110 days old plants compared to that of 35 days old plants treated with proline (Table 2). Superoxide dismutase activity exhibited a rise in response to water stress compared to untreated plants with foliar application of proline (Table 3). The foliar spray of proline on an unstressed plant significantly increased superoxide dismutase activity the most effective concentration was 250µg/L proline. In association with water stress, it raised the activity of superoxide dismutase assay by 9.9 %, 9.5 % and 9.2 % in 50µg/L proline treated plants, by 12.8 %, 12.4 % and 11 % in 100µg/L proline treated plants and 13.9 %, 13.5 % and 12.6 % in 250µg/L proline treated plants growing under varied water potential of -0.01ΨwMPa -0.02ΨwMPa and -0.03ΨwMPa, respectively, in 35 days old plants. The superoxide dismutase activity in untreated 75 days old plants was enhanced by 7.6 %, 7.9 % and 8.2 % in 50µg/L, 100µg/L and 250µg/L proline treated plants, respectively. In contrast, the SOD activity in these plants was 4.7%, 5.9% and 7.3% in 50µg/L, 100µg/L and 250µg/L proline treated 110 days old plants, respectively (Table 3). In water stressed plants also the trend with respect to the increase in superoxide dismutase was comparatively more in young plants compared to old plants and was dependent upon the concentration of proline applied. The results presented in Table 4 illustrate the APX assay activity in leaves of *L. sativum* under foliar application of proline. It is evident that the proline application in varied concentrations significantly ($P<0.05$) alleviated the impacts of water stress. Ascorbate peroxidase activity increased as the concentration of applied proline increased in water stressed plants. Ascorbate peroxidase increase was 56.8% in 50 µg/L, 63.2% in 100 µg/L and 74.9% in 250 µg/L glycine betaine treated 35 days old plants. In 75 and 110 days, plants had similar trend as it was seen in 35 days old plants. The increase in ascorbate peroxidase activity was observed more in 35 days plants

Table 2. Effect of proline and water stress on peroxidase activity (U (µmol/min)

Treatments	35 Days	75 Days	110 Days
Control	0.241±0.071^a	0.314±0.078^a	0.527±0.055^a
Proline 50µg/L	0.383±0.108^a	0.448±0.047^a	0.662±0.016^b
Proline 50µg/L and -0.01Ψ_wMPa	0.366±0.104^a	0.414±0.025^a	0.621±0.007^c
Proline 50µg/L and -0.02Ψ_wMPa	0.362±0.104^a	0.395±0.028^a	0.588±0.030^a
Proline 50µg/L and -0.03Ψ_wMPa	0.349±0.086^a	0.379±0.038^a	0.581±0.030^a
Proline 100µg/L	0.403±0.089^a	0.469±0.029^b	0.697±0.026^d
Proline100µg/L and -0.01Ψ_wMPa	0.394±0.083^a	0.432±0.010^a	0.666±0.027^e
Proline 100µg/L and -0.02Ψ_wMPa	0.389±0.080^a	0.414±0.078^a	0.649±0.019^f
Proline 100µg/L and -0.03Ψ_wMPa	0.384±0.077^a	0.372±0.110^a	0.635±0.046^g
Proline 250µg/L	0.434±0.059^a	0.484±0.060^c	0.708±0.008^h
Proline 250µg/L and -0.01Ψ_wMPa	0.392±0.067^a	0.448±0.058^a	0.682±0.015^i
Proline 250µg/L and -0.02Ψ_wMPa	0.388±0.070^a	0.444±0.059^a	0.659±0.036^j
Proline 250µg/L and -0.03Ψ_wMPa	0.365±0.073^a	0.440±0.060^a	0.649±0.027^k

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at $P<0.05$ significance level. Different lower case letters in a column indicate significant difference between control and treatments.

Table 3. Superoxide dismutase activity (U(µmol/min/mg protein)) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	90.493±0.576^a	98.911±0.352^a	105.033±0.445^a
Proline 50µg/L	101.792±0.754^b	106.491±0.231^b	110.046±0.267^b
Proline 50µg/L and -0.01Ψ_wMPa	99.503±0.628^c	106.215±0.056^c	109.936±0.301^c
Proline 50µg/L and -0.02Ψ_wMPa	99.170±0.432^d	105.686±0.215^d	109.790±0.393^d
Proline 50µg/L and -0.03Ψ_wMPa	98.836±0.333^e	104.533±0.230^e	109.640±0.190^e
Proline 100µg/L	102.792±0.304^f	106.738±0.211^f	110.270±0.326^f
Proline 100µg/L and -0.01Ψ_wMPa	102.125±0.944^g	106.325±0.121^g	110.053±0.266^g
Proline 100µg/L and -0.02Ψ_wMPa	101.792±0.881^h	106.286±0.129^h	109.726±0.462^h
Proline 100µg/L and -0.03Ψ_wMPa	100.458±0.586^i	105.636±0.181^i	109.223±0.171^i
Proline 250µg/L	103.458±0.737^j	107.071±0.171^j	112.723±0.132^j
Proline 250µg/L and -0.01Ψ_wMPa	103.125±0.454^k	106.956±0.259^k	110.610±0.196^k
Proline 250µg/L and -0.02Ψ_wMPa	102.792±0.304^l	106.824±0.346^l	110.256±0.199^l
Proline 250µg/L and -0.03Ψ_wMPa	101.958±0.322^m	106.638±0.265^m	109.873±0.179^m

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at $P<0.05$ significance level. Different lower case letters in a column indicate significant difference between control and treatments.

Table 4. Impact of proline and water stress on ascorbate peroxidase activity (U (µmol/min)) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	1.671±0.657[a]	2.409±0.026[a]	2.791±0.208[a]
Proline 50µg/L	2.321±0.614[a]	3.285±0.227[b]	3.787±0.223[b]
Proline50µg/L and -0.01Ψ_wMPa	2.295±0.617[a]	3.207±0.037[c]	3.686±0.058[c]
Proline50µg/L and -0.02Ψ_wMPa	2.228±0.469[a]	3.201±0.046[d]	3.632±0.051[d]
Proline50µg/L and -0.03Ψ_wMPa	2.138±0.414[a]	3.137±0.068[e]	3.581±0.041[e]
Proline 100µg/L	2.378±0.380[a]	3.825±0.201[f]	4.174±0.207[f]
Proline100µg/L and -0.01Ψ_wMPa	2.100±0.604[a]	3.738±0.059[g]	3.977±0.049[g]
Proline100µg/L and -0.02Ψ_wMPa	2.085±0.488[a]	3.626±0.082[h]	3.857±0.098[h]
Proline100µg/L and -0.03Ψ_wMPa	1.985±0.541[a]	3.531±0.078[i]	3.743±0.069[i]
Proline 250µg/L	2.638±0.428[a]	3.967±0.164[j]	4.371±0.321[j]
Proline250µg/L and -0.01Ψ_wMPa	2.404±0.628[a]	3.919±0.129[k]	3.994±0.222[k]
Proline250µg/L and -0.02Ψ_wMPa	2.326±0.344[a]	3.811±0.095[l]	3.931±0.213[l]
Proline250µg/L and -0.03Ψ_wMPa	2.311±0.386[a]	3.758±0.023[m]	3.814±0.502[m]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level. Different lower case letters in a column indicate significant difference between control and treatments.

compared to the other two growth stages (Table 4). The glutathione peroxidase assay activity was increased by the exogenous application of proline in *L. sativum* in response to water stress. Plants treated with proline it was found that glutathione peroxidase increased by 38.9% in 50 µg/L proline treated plants, by 42.3% in 100 µg/L proline treated plants and by 57.8% in 250µg/L proline treated plants in 35 days old plants (Table 2). A similar significant variation was found in glutathione peroxidase assay was observed between proline treated and untreated plants in 75 and 110 days old plants. It was also observed that proline was effective in enhancing glutathione peroxidase in plants grown under varied water potential. The increase in glutathione peroxidase activity was in proline dose dependent manner and it is significant at P<0.05 (Table 5). Proline was effective in enhancing glutathione-s-transferase assay activity in water stressed growing plants compared to control as shown in (Table 6). In the initial stage of plant growth (35 and 75 days old plants), the percentage increase was higher compared to 110 days old plants. The maximum enhancement in glutathione-s-transferase was observed in 75 days plants treated with proline compared to control. In 35 days old plants the

Table 5. Influence of proline and water stress on glutathione peroxidase activity (µg/min/mg protein) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	1.913±0.613[a]	3.071±0.287[a]	4.111±0.338[a]
Proline 50µg/L	3.022±0.438[b]	4.112±0.654[a]	4.984±0.176[b]
Proline 50µg/L and -0.01Ψ_wMPa	2.880±0.373[c]	4.013±0.556[a]	4.689±0.481[a]
Proline 50µg/L and -0.02Ψ_wMPa	2.842±0.398[d]	3.937±0.481[a]	4.671±0.473[a]
Proline 50µg/L and -0.03Ψ_wMPa	2.563±0.411[a]	3.857±0.401[a]	4.611±0.418[a]
Proline 100µg/L	3.122±0.438[e]	4.312±0.568[a]	5.076±0.057[c]
Proline 100µg/L and -0.01Ψ_wMPa	3.114±0.347[f]	4.160±0.422[a]	5.003±0.128[d]
Proline 100µg/L and -0.02Ψ_wMPa	3.018±0.118[g]	4.109±0.374[a]	4.886±0.094[e]
Proline 100µg/L and -0.03Ψ_wMPa	2.985±0.240[h]	3.886±0.352[a]	4.775±0.070[a]
Proline 250µg/L	3.347±0.118[i]	4.463±0.603[b]	5.397±0.285[f]
Proline 250µg/L and -0.01Ψ_wMPa	3.175±0.109[j]	4.351±0.638[a]	5.039±0.098[g]
Proline 250µg/L and -0.02Ψ_wMPa	3.010±0.302[k]	4.206±0.657[a]	5.012±0.041[h]
Proline 250µg/L and -0.03Ψ_wMPa	3.000±0.265[l]	4.138±0.690[a]	4.873±0.274[i]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level. Different lower case letters in a column indicate significant difference between control and treatments.

Table 6. Application of proline and water stress on glutathione -s-transferase activity (U (nmoles/min)) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	1.133±0.028[a]	1.932±0.050[a]	2.902±0.146[a]
Proline 50µg/L	1.689±0.165[b]	3.256±0.029[b]	4.148±0.092[b]
Proline 50µg/L and -0.01Ψ_wMPa	1.658±0.098[c]	3.025±0.252[c]	4.101±0.073[c]
Proline 50µg/L and -0.02Ψ_wMPa	1.630±0.212[d]	2.992±0.236[d]	4.044±0.196[d]
Proline 50µg/L and -0.03Ψ_wMPa	1.524±0.191[a]	2.852±0.176[e]	4.076±0.094[e]
Proline 100µg/L	1.737±0.194[e]	3.441±0.015[f]	4.311±0.071[f]
Proline 100µg/L and -0.01Ψ_wMPa	1.531±0.236[a]	3.356±0.186[g]	4.201±0.041[g]
Proline 100µg/L and -0.02Ψ_wMPa	1.529±0.238[a]	3.249±0.053[h]	4.160±0.081[h]
Proline 100µg/L and -0.03Ψ_wMPa	1.491±0.182[a]	3.131±0.117[i]	4.069±0.052[i]
Proline 250µg/L	1.891±0.036[f]	3.487±0.020[j]	4.557±0.206[j]
Proline 250µg/L and -0.01Ψ_wMPa	1.706±0.207[g]	3.373±0.028[k]	4.478±0.239[k]
Proline 250µg/L and -0.02Ψ_wMPa	1.606±0.304[a]	3.257±0.119[l]	4.407±0.284[l]
Proline 250µg/L and -0.03Ψ_wMPa	1.503±0.249[a]	3.191±0.143[m]	4.279±0.096[m]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level. Different lower case letters in a column indicate significant difference between control and treatments.

Table 7. Effect of proline and water stress on phenol content (mg/g) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	1.856±0.427[a]	2.455±0.273[a]	3.915±0.261[a]
Proline 50 µg/L	2.297±0.119[a]	3.253±0.309[b]	4.484±0.115[b]
Proline 50 µg/L and -0.01Ψ_wMPa	2.258±0.151[a]	3.216±0.292[c]	4.438±0.159[a]
Proline 50 µg/L and -0.02Ψ_wMPa	2.227±0.161[a]	3.176±0.278[a]	4.396±0.201[a]
Proline 50 µg/L and -0.03Ψ_wMPa	2.193±0.191[a]	3.090±0.347[a]	4.354±0.164[a]
Proline 100 µg/L	2.446±0.097[a]	3.329±0.233[d]	4.718±0.128[c]
Proline 100 µg/L and -0.01Ψ_wMPa	2.412±0.130[a]	3.183±0.204[e]	4.627±0.203[d]
Proline 100 µg/L and -0.02Ψ_wMPa	2.362±0.181[a]	3.141±0.214[a]	4.619±0.284[e]
Proline 100 µg/L and -0.03Ψ_wMPa	2.326±0.216[a]	3.112±0.218[a]	4.523±0.395[f]
Proline 250 µg/L	2.488±0.127[a]	3.384±0.391[f]	4.914±0.318[g]
Proline 250 µg/L and -0.01Ψ_wMPa	2.432±0.089[a]	3.283±0.309[g]	4.854±0.238[h]
Proline 250 µg/L and -0.02Ψ_wMPa	2.397±0.078[a]	3.237±0.270[h]	4.794±0.175[i]
Proline 250 µg/L and -0.03Ψ_wMPa	2.332±0.277[a]	3.202±0.444[i]	4.733±0.149[j]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level. Different lower case letters in a column indicate significant difference between control and treatments.

glutathione-s-transferase was 49%, 53.3% and 66.9% in proline treated plants. Whereas in plants grown in various water potential of -0.01 Ψ_wMPa, -0.02Ψ_wMPa and -0.03Ψ_w MPa and sprayed with 250µg/L, it was increased by 50.5%, 41.7% and 32.6%, respectively (Table 6).

Proline was found effective in increasing glutathione-s-transferase enzyme activity in water stressed growing plants at three growth stages studied. Phenols are secondary plant products with a vast array of possible functions, including anti-oxidative activity. Foliar application of proline significantly increased phenol content in water stressed plants as compared to control plants. Proline was effective in increasing the phenol content in the leaves of *L. sativum* under varied water potential growing plants. In 35 days old plants, the percentage increase was 23.8%, 31.7% and 34% in 50µg/L, 100µg/L and 250µg/L proline, respectively. Phenol content increase was higher in 75 days plants compared to 35 and 110 days old plants. In 75 days old plants the phenols content was 32.5%, 35.6% and 37.8% in proline treated plants while in water stressed plants grown in -0.03Ψ wMPa and sprayed with 250µg/L it was 30.4% (Table 7). In the present study, the results revealed that the ascorbic acid content showed an increase in water stress growing plants due to foliar application of proline in dose-dependent manner in three growth stages studied (Table 8). It is also evident that ascorbic acid content increased with plant growth irrespective of treatment. In the early growth stage plants found more enhancement in ascorbic acid content as compared to mature plants with foliar application of proline. Proline treated plants had higher ascorbic acid content compared to control plants. In proline treated plants, ascorbic acid content increased by 24.3% in 50 µg/L proline, by 27.4% in 100 µg/L proline and 32.6% in 250 µg/L proline in 35 days plants. Likewise, as the plant matures by 75 and 110 days, a similar trend was observed for ascorbic acid content enhancement as it was seen in 35 days old plants. All these observations of ascorbic acid content were significant at P<0.05 (Table 8). The foliar application of proline increased tocopherol content in the leaves of water stressed plants signifying that osmolyte may increase water stress tolerance in *L. sativum*. In proline treated 35 days old plants tocopherol content increased by 31.3%, 37.7% and 44.1% in 50 µg/L, 100 µg/L and 250 µg/L proline, respectively. In contrast, the tocopherol content in these plants was 19.3%, 21.9% and 26.5% higher in 50 µg/L, 100 µg/L and 250 µg/L proline treated 110 days old plants, respectively. The increase in tocopherol content was observed in 35 days plants as compared to 75 and 110 days old plants (Table 9). Table 10 evident that flavonoid content significantly increased in proline treated water stressed plants. Flavonoid content was more increased in 35 days plants as compared to 75- and 110-days old plants. In 35 days proline treated plants flavonoid content increased by 41.8%, 48.4% and 51.6% in 50 µg/L, 100 µg/L and 250 µg/L proline applied, respectively. Interestingly, in water stressed plants treated with 250µg/L proline flavonoid content

Table 8. Impact of proline and water stress on ascorbic acid content (mg/g) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	1.588±0.445[a]	2.891±0.118[a]	3.871±0.284[a]
Proline 50µg/L	1.975±0.318[a]	3.444±0.220[b]	4.440±0.053[b]
Proline 50µg/L and -0.01Ψ_wMPa	1.958±0.375[a]	3.346±0.133[a]	4.380±0.074[a]
Proline 50µg/L and -0.02Ψ_wMPa	1.937±0.266[a]	3.309±0.105[a]	4.276±0.086[a]
Proline 50µg/L and -0.03Ψ_wMPa	1.918±0.273[a]	3.215±0.082[a]	4.196±0.077[a]
Proline 100µg/L	2.024±0.249[a]	3.577±0.143[c]	4.594±0.132[c]
Proline 100µg/L and -0.01Ψ_wMPa	1.981±0.159[a]	3.500±0.071[d]	4.435±0.094[d]
Proline 100µg/L and -0.02Ψ_wMPa	1.952±0.387[a]	3.472±0.050[e]	4.338±0.046[a]
Proline 100µg/L and -0.03Ψ_wMPa	1.925±0.121[a]	3.429±0.037[f]	4.276±0.087[a]
Proline 250µg/L	2.106±0.181[a]	3.677±0.152[g]	4.670±0.158[e]
Proline 250µg/L and -0.01Ψ_wMPa	1.992±0.376[a]	3.632±0.130[h]	4.446±0.044[f]
Proline 250µg/L and -0.02Ψ_wMPa	1.973±0.210[a]	3.573±0.124[i]	4.378±0.196[a]
Proline 250µg/L and -0.03Ψ_wMPa	1.946±0.305[a]	3.501±0.150[j]	4.316±0.158[a]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level. Different lower case letters in a column indicate significant difference between control and treatments.

Table 9. Application of proline and water stress on tocopherol content (µg/g) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	6.693±0.547[a]	9.795±0.393[a]	10.946±0.218[a]
Proline 50µg/L	8.793±0.899[b]	11.151±0.445[a]	12.262±0.373[b]
Proline 50µg/L and -0.01Ψ_wMPa	8.653±0.358[c]	11.051±0.409[a]	12.196±0.301[c]
Proline 50µg/L and -0.02Ψ_wMPa	8.582±0.796[d]	10.918±0.393[a]	12.149±0.293[d]
Proline 50µg/L and -0.03Ψ_wMPa	8.482±0.655[e]	10.841±0.655[a]	12.102±0.374[e]
Proline 100µg/L	9.218±0.745[f]	11.282±0.315[a]	12.673±0.424[f]
Proline 100µg/L and -0.01Ψ_wMPa	8.962±0.393[g]	11.182±0.284[a]	12.564±0.533[g]
Proline 100µg/L and -0.02Ψ_wMPa	8.826±0.450[h]	11.105±0.491$_a$	12.399±0.648[h]
Proline 100µg/L and -0.03Ψ_wMPa	8.712±0.582[i]	11.048±0.198[a]	12.271±0.726[i]
Proline 250µg/L	9.650±0.600[j]	11.551±0.445[a]	12.877±0.491[j]
Proline 250µg/L and -0.01Ψ_wMPa	9.316±0.691[k]	11.418±0.424[a]	12.631±0.473[k]
Proline 250µg/L and -0.02Ψ_wMPa	9.250±0.311[l]	11.318±0.498[a]	12.497±0.475[l]
Proline 250µg/L and -0.03Ψ_wMPa	9.060±0.273[m]	11.213±0.399[a]	12.352±0.482[m]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level. Different lower case letters in a column indicate significant difference between control and treatments.

Table 10. Effect of proline and water stress on flavonoid content (mg/g) in *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	2.535±0.051[a]	3.473±0.175[a]	4.743±0.233[a]
Proline 50µg/L	3.595±0.208[b]	4.580±0.243[b]	5.263±0.186[a]
Proline 50µg/L and -0.01Ψ_wMPa	3.354±0.140[c]	4.533±0.240[c]	5.219±0.289[a]
Proline 50µg/L and -0.02Ψ_wMPa	3.213±0.115[d]	4.496±0.244[d]	5.179±0.289[a]
Proline 50µg/L and -0.03Ψ_wMPa	3.163±0.027[e]	4.460±0.253[e]	5.141±0.196[a]
Proline 100µg/L	3.763±0.089[f]	4.660±0.171[f]	5.363±0.302[b]
Proline 100µg/L and -0.01Ψ_wMPa	3.585±0.249[g]	4.627±0.143[g]	5.322±0.037[c]
Proline 100µg/L and -0.02Ψ_wMPa	3.523±0.155[h]	4.580±0.109[h]	5.311±0.046[a]
Proline 100µg/L and -0.03Ψ_wMPa	3.415±0.128[i]	4.529±0.088[i]	5.267±0.111[a]
Proline 250µg/L	3.843±0.104[j]	4.727±0.117[j]	5.470±0.172[d]
Proline 250µg/L and -0.01Ψ_wMPa	3.643±0.181[k]	4.682±0.075[k]	5.282±0.359[a]
Proline 250µg/L and -0.02Ψ_wMPa	3.581±0.236[l]	4.649±0.046[l]	5.212±0.041[a]
Proline 250µg/L and -0.03Ψ_wMPa	3.566±0.235[m]	4.616±0.029[m]	5.164±0.415[a]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level. Different lower case letters in a column indicate significant difference between control and treatments.

increased by 43.7%, 41.3% and 40.7% in different water potential of -0.01ΨwMPa, -0.02ΨwMPa and -0.03ΨwMPa, respectively, in 35 days old plants. The concentration of 250 µg/L proline was more precise in all three growth stages studied in *L. sativum*. Proline plays an important role in plant response to water stress. In this study, we examined the effectiveness of exogenous proline in alleviating water stress tolerance in *L. sativum* plants exposed to three water stress levels (Table 11). The impact of proline on lipid peroxidation was studied in this study and it was observed that it overcame the lipid peroxidation in stressed plants. In 35 days old plants the amount of malondialdehyde content decreased by 32.3% in 50µg/L, by 66.8% in 100µg/L and 90.8% in 250µg/L proline treated plants in 35 days of plants. In the same way, the amount of MDA content in 75 and 110 days plants also decreased with foliar application of proline. The amount of MDA content decreased more in young plants as compared to mature plants in all three-growth stages studied. Generally, it seems that exogenous proline supplementation alleviated the deleterious effects in water stressed growing plants. The results of the present study also revealed that the amount of MDA content reduction in this plant depends on the severity of water potential and concentration of proline applied (Table 11).

Table 11. Impact of proline and water stress on lipid peroxidation (MDA) content (µmol/l) in leaves of *Lepidium sativum* L.

Treatments	35 Days	75 Days	110 Days
Control	1.893±0.270[a]	2.307±0.582[a]	3.272±0.256[a]
Proline 50µg/L	1.431±0.217[a]	1.910±0.467[a]	2.687±0.256[a]
Proline 50µg/L and -0.01Ψ_wMPa	1.491±0.246[a]	1.927±0.299[a]	2.713±0.549[a]
Proline 50µg/L and -0.02Ψ_wMPa	1.546±0.301[a]	1.944±0.033[a]	2.750±0.764[a]
Proline 50µg/L and -0.03Ψ_wMPa	1.613±0.307[a]	1.980±0.322[a]	2.789±0.774[a]
Proline 100µg/L	1.135±1.081[a]	1.677±0.355[a]	2.420±0.582[a]
Proline 100µg/L and -0.01Ψ_wMPa	1.191±0.272[a]	1.694±0.573[a]	2.527±0.182[a]
Proline 100µg/L and -0.02Ψ_wMPa	1.259±0.412[a]	1.710±0.313[a]	2.567±0.780[a]
Proline 100µg/L and -0.03Ψ_wMPa	1.382±0.382[a]	1.748±0.347[a]	2.593±0.760[a]
Proline 250µg/L	0.992±0.273[a]	1.410±0.573[a]	2.213±0.462[a]
Proline 250µg/L and -0.01Ψ_wMPa	1.062±0.406[a]	1.465±0.306[a]	2.240±0.226[a]
Proline 250µg/L and -0.02Ψ_wMPa	1.114±0.318[a]	1.497±0.337[a]	2.282±0.332[a]
Proline 250µg/L and -0.03Ψ_wMPa	1.141±0.012[a]	1.517±0.400[a]	2.298±0.117[a]

Data are mean ± SD, of three replicates (n=3) and analyzed by one-way Anova followed by Tukey post-test at P<0.05 significance level.

Discussion

Proline is a compatible solute that plants make under stress and is thought to act as an osmoprotectant. This low molecular weight, neutrally charged substance is crucial for stress-related protein and cell membrane stability and protection (17). One of the most researched plant osmoprotectants, proline is involved in the control of ion homeostasis and redox balance (1). The five-carbon amino acid proline is crucial for stabilising macromolecules, scavenging free radicals and signalling pathways. It also plays a role in osmotic balance under stress (16). Proline is the primary amino acid, as previously mentioned, that raises osmotic pressure and controls water potential under a variety of abiotic stress situations (18). In general, reactive oxygen species cause harm to a wide range of organic molecules, including proteins, lipids and carbohydrates. They can interfere with the activities of cell membranes by altering the enzyme system and they can finally lead to death by methylating or harming DNA (19). CAT, POD, SOD, APX, GPX, GST and non-enzymatic antioxidant molecules like phenol, ascorbic acid, tocopherol and flavonoids, among other enzymes and non-enzymatic antioxidant molecules, are some of (20). Although proline buildup has been thought to be a sign of stress response, it may also help mitochondria produce ROS, which causes plants to have a hypersensitive response (21). Proline accumulation is seen to be advantageous by many plant scientists. Stress tolerance is typically considered to be correlated with the high accumulation of proline under stress, which is generally correlated with enhanced production (22). Exogenously provided osmolytes such as proline facilitate the growth of plants in stressed environments (18). The exogenous use of proline gained considerable attention for its role as a compatible solute as an osmotic balancing agent and helping the plant to overcome environmental stresses (22). As all the plant species respond in a different way to environmental stresses, they also react differently to osmolyte treatments. Numerous enzymes and non-enzymatic substances, such as CAT, POD, SOD, APX, GPX, GST and non-enzymatic phenolics, ascorbic acid, tocopherol and flavonoids, aid in reducing the ROS that is created during stress (23). According to research on grape plants, water stress generated an improvement in the behaviour of enzyme antioxidants such as CAT, POD, APX and SOD (7). In the current research, the exogenous application of proline in response to water stressed in *L. sativum* confirms the importance of proline as a compatible solute. According to the study's findings, proline applied topically to water-stressed plants considerably increased their catalase activity when compared to control plants (Table 1). By applying proline, similar outcomes were observed in bean plants under stress, where catalase activity dramatically increased in comparison to untreated plants (24). Peroxidase serves as the second line of defence for plants, enabling them to deal with excess H_2O_2 (25). In response to varied stresses, the peroxidase scavenges the antioxidant activity of different enzymes. Under stress, it was seen that *Lemna valdiviana* superoxide dismutase and catalase activity were decreased, while peroxidase activity remained unaffected, suggesting that some antioxidant enzymes are still active (26). Proline increases the activity of peroxidase enzymes in plants, which reduces reactive oxygen species (27). Exogenous proline treatment was found to dramatically boost peroxidase activity in stressed plants' leaves as compared to untreated plants in the current investigation (Table 2). Previous research on cucumber is used to support the findings of the current study (28). Because it increases the activity of superoxide dismutase in plants, proline can reduce reactive oxygen species (27). Exogenous injection of 10 mM proline was thought to stimulate the development of tobacco suspension cells under abiotic stress because proline acts as an antioxidant for enzymes and membranes (29). In soybean cell cultures maintained under stress, superoxide dismutase activity rose, which is often associated with higher stress tolerance (29). Similar to the previous work, the current investigation on *L. sativum* showed that foliar proline administration dramatically boosted superoxide dismutase activity in stressed plants as compared to untreated plants (Table 3). Similar outcomes were seen in *Solanum nigrum*, in sugarcane (7) and in *Saccharum spontaneum* (23) where proline increased the activity of superoxide dismutase in stressed plants, helping to detoxify O_2 radicals. Exogenously applied proline increased the activity of antioxidative enzyme ascorbate peroxidase that confers oxidative stress protection. The present study proved that exogenous application of proline had beneficial role in water stress tolerance in *L. sativum*. Proline application enhanced the antioxidative enzyme activity such as ascorbate peroxidase in stressed plants compared to control (Table 4). Similar results in faba bean were obtained where ascorbate peroxidase activity enhanced under drought stress by using exogenous application of proline (30). Exogenous osmolyte was used to increase plant performance because it is a suitable osmolyte and has the antioxidant ability to quench ROS, which makes it a powerful protector of plants from harmful abiotic stresses like water stress (31). Proline increased the activity of antioxidant defence system enzymes including glutathione peroxides, which helped tobacco plants tolerate stress better (28). The present study also demonstrated that exogenous application of proline enhanced glutathione peroxidase activity in plants under water stress. Similar findings were reported in chickpea (32), melon (33), lentil (34) and sugarcane (35). The results of the present study showed an enhancement in glutathione-s-transferase enzyme activity in water stressed plants as compared to untreated plants by exogenous application of proline (Table 6). The findings of this study corroborate those of (25) who found that exogenous application of the osmolyte proline increased glutathione-s-transferase activity in *Vicia faba* under drought stress conditions. Additionally, plant metabolic activities are regulated by cellular metabolites such as leaf phenolics, which also play a part in lowering ROS-induced oxidative damage (36). The current investigation on *L. sativum* found that exogenous proline treatment dramatically boosted the amount of phenolics in plants under water stress (Table 7). Similar outcomes were reported in the *Zea mays* cases Agaiti-2002 and EV-1098 (33), in *V. faba* (25) and in thyme and Cowpea (*Vigna unguiculata*) (37) under drought stress (38). Additionally, metabolites such as ascorbic acid aid in the regulation of a variety of plants' metabolic activities and play a part in reducing ROS-induced oxidative damage (39). When exposed to water stress in the current study, exogenously

applied proline dramatically raised the ascorbic acid concentration in *L. sativum* in comparison to control plants. This improvement might be the result of the foliar application of osmolytes, which were used in the leaves and may have served as a source of carbon as has previously been documented in many research (36). Similar results were in *V. faba* where ascorbic acid content was enhanced via foliar proline application during drought stress (25). Additionally, proline applied topically increased the level of tocopherol under water deficiency stress (33). The current study also demonstrated that exogenous proline administration increased the content of tocopherol in water-stressed plants in a concentration-dependent way (Table 9). Proline applied topically to leaves greatly boosted plant growth, development and flavonoid content. Plants are protected from oxidative damage by proline application, which increases the activities of flavonoid and phenolic content (33). According to the current study, exogenous proline treatment increased the flavonoid content in stressed plant leaves as compared to untreated plants at all growth stages (Table 10). Similar results were obtained in *Zea mays* (40), in *Arabidopsis* (41), in the leaves and roots of barley (42), in wild soybean (43), in poplar plants (44) and in the leaves of *Jerusalem artichokes,* the flavonoids content increased under drought stress (45). In addition to other biochemical characteristics, malonaldehyde accumulation was used to quantify lipid peroxidation. Under drought stress conditions enhanced accumulation of lipid peroxidation in *Medicago sativa* was reported (46), in maize (47), mung bean (48) and *Saccharum spontaneum* (23). The results of the present study evident that malonaldehyde content decreased due to proline application in water stressed plants (Table 11) and similar results were obtained in *Vicia faba* (25).

Conclusion

In stressed plants, proline served adaptive roles in controlling osmotic adjustment and safeguarding subcellular structures. Proline applied topically to plants helps them develop under stress by increasing their tolerance. In the current study, exogenous proline was administered to *L. sativum* plants growing under both stress-free and water-stressed circumstances and it was discovered to be successful. In comparison to control plants, exogenous proline treatment improved enzymatic and non-enzymatic antioxidants and reduced lipid peroxidation in plants under water stress.

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Authors' contributions

SS, AT and KD conceptualized the study. SS, NS, MDC and TA handled data, tables and figures preparation. SS, AT, KD, AJS and NS wrote the original draft. NS, TA and SS reviewed and edited. All the authors reviewed, commented and edited the paper for submission.

Compliance with ethical standards

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