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Physiological responses of the leaves of a high-altitude plant *Picrorhiza kurroa* to cold stress

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Abstract

Plants growing at high elevations experience different environmental stresses, such as drought, salt, and cold. Among them, cold stress is the most prevalent one that affects the plants differently. Plants undergo biochemical, metabolic, molecular, and physiological changes under cold stress; hence, they adopt various mechanisms to tolerate it. The antioxidant defence system, osmotic regulators, and photosynthetic pigments in the plant provide them with stress tolerance. The present study is conducted on a high-altitude plant, *Picrorhiza kurroa,* which grows in such environmental conditions, to study the physiological parameters that provide a coping mechanism against cold stress. For this study, the leaves were collected from Pothivasa (2200 m.a.s.l) and Tungnath (3600 m.a.s.l) in Rudraprayag, Uttarakhand, India. The photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids), lipid peroxidation, antioxidant enzymes, namely, superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase, and osmoprotectants (protein, soluble sugar, and proline) present in the leaves were determined to visualize the impact of cold stress. It was revealed that the concentration of photosynthetic pigments increased with elevation. The activity of enzymes was analyzed, and they were observed to decrease with altitude. The malondialdehyde concentration, an indicator of lipid peroxidation, is higher in Pothivasa and lower in Tungnath. There is a significant increase in the osmoprotectants' content along the altitudinal gradient. Therefore, the leaves from both sampling locations revealed the physiological changes that occurred in them to adapt to the cold stress conditions.

Keywords

antioxidant; pigments; lipid peroxidation; osmoprotectants; cold stress; *Picrorhiza kurroa*

Introduction

Stress is defined as "any substance or condition which stops a plant's metabolism, development, or growth" (1). Both biotic and abiotic factors have an impact on plant productivity and growth (2). Abiotic stress factors include water deficit, salinity, light stress, chilling, heat, flooding, and soil compaction, freezing, trace element toxicity, and mineral nutrient deficiencies. To maintain growth and developmental processes, as well as cellular homeostasis, under such challenging circumstances, plants undergo biochemical, metabolic, molecular, and physiological changes when the ideal

ambient temperature varies. When the temperature is below the optimum level, then the plants are said to be under cold stress. If the temperature is between 0 to 15°C, then it is termed chilling stress (3). In most hilly areas, cold stress is a prominent abiotic stress that hampers the normal growth of the plant. Cold stress causes dehydration, growth inhibition, metabolite imbalance, metabolic malfunction, plasma membrane rupture, and solute leakage in plants, all of which can result in plant mortality (4-6).

On the other hand, exposure to a lower temperature can induce cold stress tolerance in plants. This phenomenon expresses various complex biochemical, metabolic, and molecular processes (**7**). On exposure of plants to stress related to temperature, there is a modification of the metabolism of plants in two steps. Initially, they try to alter their cellular metabolism, which is influenced by temperature fluctuations; then, by modulating metabolism through enhanced tolerance mechanisms (8).

Cold stress induces oxidative damage in the plant, causing the over-accumulation of reactive oxygen species (ROS), viz., hydrogen peroxide, hydroxyl ions, singlet oxygen, and superoxide anion. Antioxidant enzymes play a vital role in inhibiting lipid peroxidation caused by ROS. Antioxidant enzymes include ascorbate oxidase (EC 1.10. 3.3), ascorbate peroxidase (APOX; EC 1.11.1.1), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione reductase (EC 1.6.4.2), guaiacol peroxide (EC 1.11.1.7), polyphenol oxi dase (EC 1.10.3.1), superoxide dismutase (SOD; EC 1.15.1.1), and so on.

Lipid peroxidation causes membrane damage in plants, which indicates cold stress, and malondialdehyde (MDA) is the indicator of lipid peroxidation in plants (9). Many metabolites can function as osmoprotectants that induce tolerance during stress conditions and protect the plant against abiotic stresses (10). They regulate the water balance within cells and lessen cellular dehydration. In addition, they can stabilize enzymes, membranes, and other biological components because of the behaviour of their compatible solutes. These osmoprotectants include amino acids, lipids, organic acids, polyamines, and soluble carbohydrates (11).

There is also a visible change in the morphology of the leaves, like a reduction in the length and width and an increment in their thickness in response to high elevation (12). The study of the leaves of a plant grown in the North-western Himalayas makes it ideal for studying cold stress tolerance and adaptations. Therefore, to investigate the physiological factors influencing this plant's ability to withstand cold stress, the current study is being conducted on *Picrorhiza kurroa* Royle ex Benth. (Kutki). It belongs to the family Scrophulariaceae and is distributed in the sub-alpine and alpine regions of the North Western Himalayas between 2700-4500 m.a.s.l (13). The Western Himalayan states of Jammu and Kashmir, Himachal Pradesh, and Uttarakhand are home to *P. kurroa*. It is an endangered plant and has a mild, unpleasant aroma and a bitter flavour. The iridoid glycosides secreted by this plant infer implicit medicinal values. These glycosides include active substances such as apocynin, drosin, cucurbitacins, picrosides I, II, III, and V, and kutkosides (14-16). The plant has excellent economic worth due to its ability to treat various illnesses, including leishmaniasis, anaemia, jaundice, inflammation, and gastrointestinal problems (17).

Since *P. kurroa* is an endangered plant, in the present study, samples were collected from nurseries of HAPPRC, HNBGU located at two altitudes, with a difference of approximately 1400 m, and a temperature difference of around 10°C gives the baseline information on the physiological parameters responsible for the cold stress tolerance.

Materials and Methods

Experimental Design and Sample Collection

For the study, the leaves of *Picrorhiza kurroa* were collected from the nurseries of HAPPRC, HNBGU, located in Pothivasa (30°28′N lat; 79°16′E long at an altitude of 2200 m asl) and Tungnath (30°14′N lat; 79°13′E long at an altitude of 3600 m asl) in Rudraprayag district, Uttarakhand, India (Fig. 1 & 2). There is an altitudinal variation of about 1400 m in both sampling locations. Pothivasa has evergreen forests with 99.2-1401.2 mm of rainfall and a humidity of 69.39%. The soil in Pothivasa has a pH of 3.29-4.49, organic carbon content of 3.83-4.08%, available nitrogen of 0.02-0.16%, and very low levels of phosphorus and potassium. Tungnath has alpine meadows with rainfall ranging from 139.81 to 1550.31 mm and humidity of 78.8%. The soil has a pH of 3.29-4.49, organic carbon content of 3.83-4.08%, available nitrogen of 0.02-0.16%, and low levels of phosphorus and potassium.

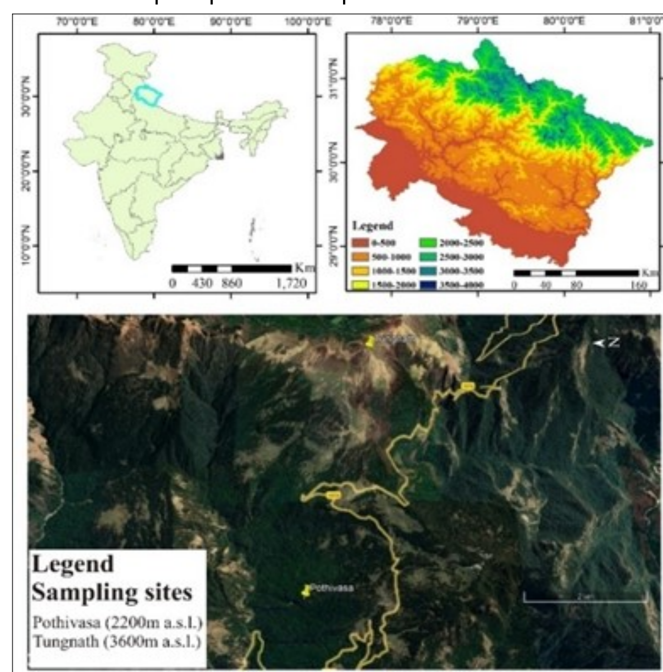

Fig. 1. Location map of the study area Pothivasa (2200m asl) and Tungnath (3600m asl)

To evaluate the physiological attributes responsible for the tolerance of cold stress in the leaves of *P. kurroa,* the biological replicates of the apical leaves of *P. kurroa*

Fig. 2. *Picrorhiza kurroa* cultivated in Pothivasa

were collected for three consecutive years from 2020 to 2022 in July during the monsoon season when the plant was in full bloom. Fifty fully expanded leaves were gathered from different plants at each sampling site. The samples were taken at noon at the same time and on the same day from both locations each time. The temperatures in Pothivasa and Tungnath sampling sites observed during sampling were 13°C and 1°C, respectively. The samples were stored at -20˚C to perform the analysis, and all the assays were performed in triplicate and expressed as mean± standard error. The parameters were also correlated with the altitude.

Photosynthetic Pigments

The fresh leaves of *P. kurroa* from Pothivasa (PVLE) and Tungnath (TNLE) were grounded with ethanol, extracted completely, and then filtered to determine the photosynthetic pigments in the leaves. The absorbance of chlorophyll a (Chl a), chlorophyll b (Chl b), and total carotenoids (Car) were all measured at wavelengths of 665 nm, 649 nm, and 470 nm, respectively. Their concentrations (mg g^{-1} DW) were then determined using the following equations (18):

$$Chl\ a = (13.95A_{665} - 6.88\ A_{649}) \times \text{volume of supernatant/ sample weight/ 1000}$$

$$Chl\ b = (24.96A_{649} - 7.32A_{665}) \times \text{volume of supernatant/ sample weight/ 1000}$$

$$\text{Carotenoids} = (1000A_{470} - 2.05chl\ a - 114.8chl\ b)/245 \times \text{volume of supernatant/ sample weight/ 1000}$$

Antioxidant Enzymes Activity

In ice-cold conditions, 5 gm of leaves were homogenised in 0.15 M potassium phosphate buffer at pH 7.0 before being centrifuged at 15000 rpm for 20 min (19). For the investigation of enzymatic antioxidant activity and protein estimation, the supernatant, or enzyme extract, was collected and stored at -20°C.

Superoxide Dismutase (SOD) (EC 1.15.1.1)

The reaction mixture was prepared using sodium carbonate buffer, NBT, triton X-100, hydroxylamine hydrochloride, and enzyme extract. The amount by which the rate of NBT degradation was inhibited was determined by a rise in absorbance at 540 nm. The percent inhibition of NBT reduction (y%) and sample producing 50% inhibition (z µl) were calculated (20).

Catalase (CAT) (EC 1.11.1.6)

The phosphate buffer, hydrogen peroxide, and enzyme extract were added to formulate the reaction mixture. At 240 nm, the absorbance was measured. The rate of decomposition of H_2O_2 decreased the absorbance. Utilizing the molar Extinction coefficient of $6.93*10^{-3} mM^{-1} cm^{-1}$, the enzyme activity was computed (21).

Guaiacol Peroxidase (POD) (EC 1.11.1.7)

To determine the activity of POD enzymes in leaves, a test cuvette was filled with phosphate buffer, guaiacol solution, enzyme sample, and H_2O_2 solution. At 436 nm, the rate of GDHP production was monitored spectrophotometrically. The extinction coefficient was taken at 25 mM^{-1} cm^{-1} when calculating enzyme activity (22).

Ascorbate Peroxidase (APOX) (EC 1.11.1.11)

Using 3 ml of the reaction mixture, which included phosphate buffer, ascorbate, H_2O_2, and enzyme extract, the decrease in absorbance was measured at 290 nm. The enzyme activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} (23).

Glutathione Reductase (GR) (EC 1.6.4.2)

GR activity was determined by detecting the oxidation of NADPH at 340 nm in a reaction mixture containing phosphate buffer, EDTA, NADPH, and oxidised glutathione (GSSG). The minute decrease in absorbance was followed at 340 nm. The enzyme activity was calculated using the extinction coefficient (6.22 $mM^{-1}cm^{-1}$) (24).

Osmoprotectants

Total Soluble Sugar

The sample was hydrolyzed with 2.5 N HCl for three hours in the water bath at 100°C and then cooled to room temperature. Once the effervescence had stopped, it was neutralized using sodium carbonate. The volume was then increased to 100 ml, centrifuged, and the supernatant was collected and used for analysis. Working standards were also prepared, and anthrone reagent was added to all the test tubes. The mixture was first heated in boiling water and then cooled quickly, and the absorbance was taken at 630 nm (25).

Total Protein

The reagents and protein standard stock solution containing BSA were prepared and mixed. They underwent a 30-minute incubation period at ambient temperature and in complete darkness. The blue colour was developed. At 550 nm, the readings were recorded. The amount of protein in the samples was determined by plotting an absorbance vs. concentration graph for standard solutions of proteins. Protein content was expressed as mg/g FW (26).

Total Proline

The frozen leaves were mixed with sulphosalicylic acid to homogenise them. The residue was centrifuged at 12000 rpm for 10 minutes to separate it. The process was stopped in an ice bath after dissolving equal amounts of acid-ninhydrin, glacial acetic acid, and homogenised

tissue. 2 ml of toluene was used to extract the reaction mixture, which was then violently agitated and kept at room temperature to allow the two phases to separate. The absorbance of 1 ml of upper phase and toluene (blank) was taken at 520 nm. D-proline was used to prepare a standard curve to calculate the proline concentration (27).

Determination of Lipid Peroxidation

The leaves were ground in trichloroacetic acid (TCA), centrifuged, and then the supernatant was collected and mixed with thiobarbituric acid (TBA). It was heated and then cooled in an ice bath. It was centrifuged again, and the absorbance of the supernatant was determined at wavelengths of 450 nm, 532 nm, and 600 nm (28). The malondialdehyde (MDA) content was computed using the following equation and represented as µmolg^{-1} dry weight:

$$MDA = 6.45(A_{532} - A_{600}) - 0.56A_{450}$$

Results

Photosynthetic Pigments

The effect of altitudinal variation was studied on chlorophyll a, chlorophyll b, and carotenoids present in the leaves of *Picrorhiza kurroa*, collected from two locations, i.e., Pothivasa and Tungnath, at different elevations. It was observed that the concentration of chlorophyll a, chlorophyll b, and carotenoids in PVLE were 0.073±0.002 mg g^{-1} DW, 0.287±0.003 mg g^{-1} DW, and 0.066±0.002 mg g^{1} DW, respectively, and that in TNLE were 0.074±0.001 mg g^{-1} DW, 0.303±0.003 mg g^{-1} DW, and 0.072±0.0002 mg g^{-1} DW, respectively (Fig. 3). Hence, these pigments positively correlated with altitude, i.e., TNLE contained higher photosynthetic pigments than PVLE (Table 1).

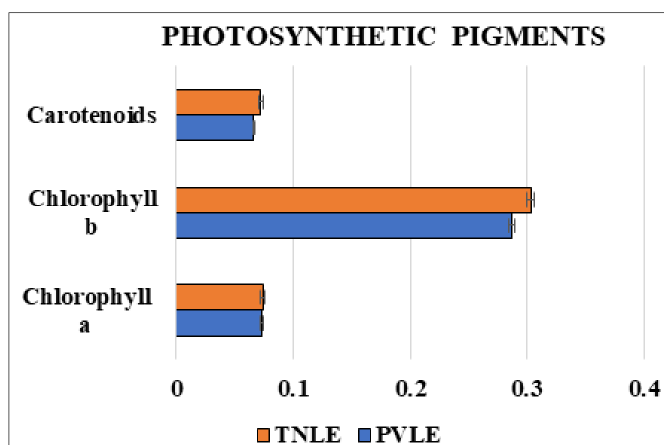

Fig. 3. Photosynthetic pigments (Chl a, Chl b, and Car) present in the leaves extract

Enzymatic Antioxidants

The activity of antioxidant enzymes is affected at high altitudes. Superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase were studied for their change in activity with respect to altitude. SOD results revealed that the sample required for 50% inhibition of NBT reduction was very high in the case of PVLE (286± 13.93 µl) in comparison to TNLE (4.82±0.61 µl) (Table 2). Other enzymes, viz., CAT, POD, APOX, and GR, also showed higher specific activity in PVLE than TNLE (Fig. 4). The specific activity of CAT, POD, APOX, and GR in PVLE was calculated to be 16.64± 0.47 U/mg protein/min, 11.75± 0.65 U/mg protein/min. 3.34± 0.25 U/mg protein/min and 0.17 ± 0.01 U/mg protein/min, respectively, and that in TNLE were 4.76 ± 0.83 U/mg protein/min, 2.24 ± 0.92 U/mg protein/min, 1.63± 0.21 U/mg protein/min, and 0.77± 0.01 U/mg protein/min, respectively. Hence, all these enzymes showed a negative correlation with respect to altitude, as shown in Table 1.

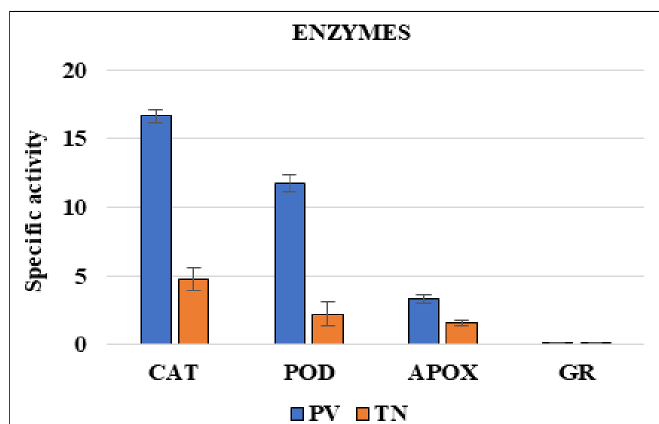

Fig. 4. Specific activity of catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APOX) and glutathione reductase (GR)

Osmoprotectants

Soluble protein, soluble carbohydrates, and proline are the osmotic regulators that are accumulated or induced directly or indirectly by the abiotic stress in the environment (29). In the present study, it was observed that the concentration of these osmoprotectants increases with the increase in altitude, as shown in Table 1. The total protein content was found to be 4.337±0.01 mg/ml in PVLE and 7.069±0.1 mg/ml in TNLE. Total soluble sugar was 0.35± 0.005 mg/ml in PVLE and 0.55±0.001 mg/ml in TNLE. Total proline content was 4.53± 0.2 µmoles/gm and 6.73± 0.09 µmoles/gm in PVLE and TNLE, respectively (Fig. 5).

Table 1: Correlation among photosynthetic pigments, MDA content, and enzymatic antioxidant with altitude

	Altitude (m.a.s.l)	*chl a*	*chl b*	*Car*	*MDA*	*SOD*	*CAT*	*POD*	*APOX*	*GR*
Altitude (m.a.s.l)	1									
chl a	1	1								
chl b	1	1	1							
Car	1	1	1	1						

MDA	-1	-1	-1	-1	1					
SOD	-1	-1	-1	-1	1	1				
CAT	-1	-1	-1	-1	1	1	1			
POD	-1	-1	-1	-1	1	1	1	1		
APOX	-1	-1	-1	-1	1	1	1	1	1	
GR	1	1	1	1	-1	-1	-1	-1	-1	1

Table 2. Activity of superoxide dismutase (SOD); y: % inhibition of NBT and z: µl of sample for 50% inhibition

SOD	y (%)	z (µl)
PVLE	12.26±0.58	286± 13.93
TNLE	733.61± 86.08	4.82±0.61

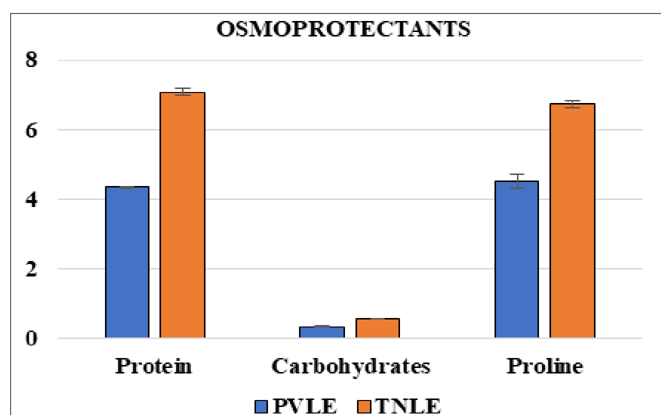

Fig. 5. Concentration of soluble protein (µg protein/100µl), soluble carbohydrates (mg sugar/ml), and proline (µmoles/gm) in PVLE and TNLE

Lipid Peroxidation

The oxidative damage was estimated by evaluating the malondialdehyde (MDA) content, which acts as an indicator for lipid peroxidation. The result showed increased levels of MDA in PVLE (2.214±0.09 µmol l^{-1}) in comparison to TNLE (1.697± 0.15 µmol l^{-1}), representing a negative correlation between the altitude and MDA concentration (Fig. 6).

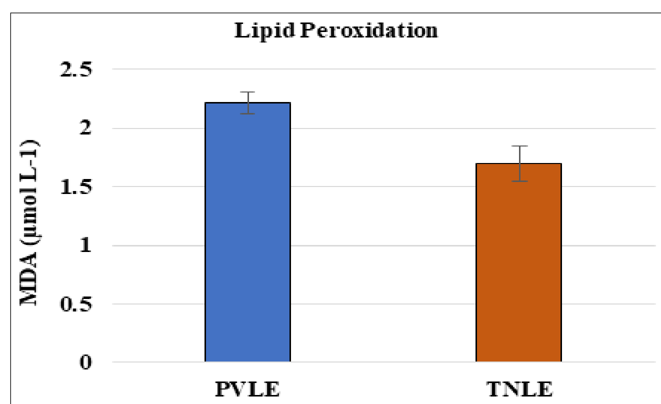

Fig. 6. Malondialdehyde concentration in PVLE and TNLE

Correlation among photosynthetic pigments, MDA content, and enzymatic antioxidant with altitude

The correlation analysis was done among the Chl a, Chl b, Car, MDA content, SOD, CAT, POD, APOX, and GR with respect to the altitude, as shown in Table 1. The findings indicated a significant positive correlation (r = 1) between altitude and the levels of these photosynthetic pigments. Whereas, the trend observed in enzymatic antioxidants and MDA content showed a negative correlation with respect to altitude. This means that as the altitude increased, the activity of these antioxidant enzymes and lipid peroxidation decreased.

Discussion

The plant of the study, *Picrorhiza kurroa*, is a high-value medicinal herb that grows at high altitudes and faces cold stress conditions. In high altitudes, various abiotic factors, including light intensity, oxygen pressure, precipitation, photoperiod, temperature, and UV intensity, act as stress and affect the plant's growth (30). Different plant species generally cope with environmental stresses by employing different adaptation mechanisms, such as altering their morphology, metabolism, and physiology. Under stress, the plant's life cycle is maintained by the antioxidant defence system, osmotic regulators, and photosynthetic pigments (31, 32). Plants may use all or some of these methods to combat stress conditions.

In the current investigation, the concentration of chlorophyll present in the leaves of *P. kurroa* was seen to increase with altitude as chlorophyll a pigment increased from 0.072 mg g^{-1} DW to 0.075 mg g^{-1} DW and chlorophyll b increased from 0.29 mg g^{-1} DW to 0.304 mg g^{-1} DW. Plant photosynthetic capacity is directly impacted by photosynthetic pigments, which are also vulnerable to environmental stresses and are responsible for assimilating and transforming light. However, chlorophyll content can be decreased by water stress, intense light, and high temperatures by slowing synthesis, increasing decomposition, or harming chloroplast structures (33). It is fascinating to note that certain herbaceous plant species have evolved a remarkable adaptation strategy in the face of cold stress. Species such as *Malva neglecta*, winter cereals, and spinach exhibit a surprising upregulation of their photosynthetic processes when confronted with low temperatures (34). Similar patterns of increased chlorophyll content with altitude have been observed in other plant species, such as *Nepeta septemcrenata*, *Origanum syriacum*, *Phlomis aurea*, *Rosa arabica*, and *Silene schimperiana*, and this was associated with the accumulation of total soluble sugar, HCO_3^-, and Mg^{+2} in soil at higher altitudes (35). This acclamatory elevation of photosynthetic capacity implies an investment to increase the overall capacity of photosynthesis and the size of the photosynthetic apparatus to compensate for the decrease in enzyme activity at lower temperatures (36). Carotenoids

are complexes that participate in photosynthesis and have the potential to remove the ROS, as the ROS can accumulate and cause photooxidation, further damaging the chloroplast of the leaves (37, 38). In this study, carotenoids were observed to increase with altitude, i.e., from 0.065 mg g^{-1} DW to 0.072 mg g^{-1} DW, which may be due to the pigments' protective role in dissipating the additional energy and ROS scavenging (39). A similar pattern was observed in the skin tissues of peaches (40).

The effect of altitudinal variation on the antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APOX), and glutathione reductase (GR) present in *P. kurroa* resulted in a decrease in their activity with the increase in altitude (Table 2 and Fig. 4). At higher altitudes and under stress conditions, a significant level of ROS causes oxidative damage to plants (35). Plants have evolved an antioxidant defence system that includes antioxidant enzymes to prevent oxidative damage under various difficult environmental conditions. The study of these enzymes on *Coleus forskohlii* along the altitudinal gradient showed the best enzymatic activity at the highest altitude to combat oxidative stress (41). However, the results of this investigation, suggesting the decrease in enzymatic activity, reveal that they were adequate to change O_2 and H_2O_2 levels (42), and plants at higher altitudes may have adapted to higher oxidative stress by regulating the acti-vity of antioxidant defence systems (43).

Plants regulate the osmotic stress developed due to low temperatures with the help of osmoprotectants. The cold stress causes a change in intracellular and metabolic pathways by synthesizing proteins, carbohydrates, and proline (44). In this study, *P. kurroa* leaves also showed changes in response to the cold stress by increasing the synthesis of these osmoprotectants, as shown in Fig. 5. These regulatory proteins control and/or regulate the expression and activity of genes related to stress, hence contributing to cold stress tolerance (45). The increase in carbohydrates helps protect the structure of cell membranes, which plays an essential role in plant stress tolerance (46). It seems increased photosynthetic pigments resulted in increased production of carbohydrates, which are acting as osmoprotectants. The overproduction of proline also acts as a cryoprotectant. Research reported the increase in sugar content in *Aloe vera, Bryophyllum pinnatum, Crassula lacteal, Echinocactus grusonii, Euphorbia resinifer*a, *Sansevieria trifasciata*, and *Yucca aloifolia* after cold stress (47). A research report stated an accumulation of sucrose under cold stress (48). Bano and Fatima (2009) also observed a higher sugar concentration at high elevations. Researchers observed that the proline concentration significantly increases during cold hardening (49).

Reactive oxygen species (ROS) may accumulate due to stress-related cellular damage, resulting in membrane lipid peroxidation (50). Malondialdehyde (MDA), a by-product of lipid peroxidation, has been widely employed as a marker of membrane damage brought on by free radicals under different abiotic conditions (51). Our research revealed that the MDA levels in *P. kurroa* leaves decreased from 2.214 µmol l^{-1} in PVLE to 1.697 µmol l^{-1} in TNLE along the altitude gradient. In the high-altitude region, less damage in the plasma membrane and better ROS scavenging capabilities may be responsible for the decline in MDA levels. The leaves of *L. secalinus* yielded similar outcomes where in Minhe County (1872 to 2185 m.a.s.l), MDA content in the leaves initially elevated and then declined, and in Huangzhong County (2163 to 2935 m.a.s.l) linearly declined along the altitudinal gradient (52).

Therefore, the increase in the photosynthetic pigments and osmoprotectants, along with the decrease in the MDA content in *P. kurroa* leaves, enables the plant to withstand low temperatures at high altitudes.

Conclusion

The current study on the leaves of *Picrorhiza kurroa* showed the adaptation mechanism of this plant against cold stress along the altitude. The increase in the concentration of chlorophyll pigments and carotenoids in the leaves showed their vital role in increasing photosynthesis and scavenging free radicals, respectively. The soluble sugar, protein, and proline may be essential for osmotic regulation at high altitudes, according to the increase in their levels with elevation that has been seen in these plants. The decrease in the MDA content reveals higher ROS scavenging and less damage to the cell membrane. The enzymatic antioxidant activity in the leaves is observed to be decreasing in TNLE. It may be assumed that the plant is not using the enzymatic antioxidant mechanism to combat cold stress, and it reveals the adjustability of the leaves at high altitudes. The study indicated the physiological changes in these leaves to tolerate the stress. Further studies can be done to learn about the molecular changes occurring in these leaves to survive the stress conditions.

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Authors contributions

SA and PS conceptualised the study. SA carried out the experiments. PS and SA wrote and finalized the manuscript.

Compliance with ethical standards

Conflict of interest: There is no conflict of interest with anyone either within the institute or outside the institute.

Ethical issues: None.

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