Pak. J. Bot., 56(4): 1419-1429, 2024. DOI: http://dx.doi.org/10.30848/PJB2024-4(2)

OSMOREGULATION FACILITATED BY LEAF STRUCTURAL AND FUNCTIONAL TRAITS IN MALABAR NUT (*JUSTICIA ADHATODA* L.) ALONG ELEVATION GRADIENT

HUMERA MATLOOB AND MANSOOR HAMEED

Department of Botany, University of Agriculture Faisalabad, Faisalabad, Pakistan
**Corresponding author's email: rehana_ajku@yahoo.com*

Abstract

Elevations possess a specific set of biotic and abiotic factors that determine the vegetation type of the region. Abiotic factors along with biotic factors in a region can change the morphology, physiology and anatomy of vegetation. *Justicia adhatoda* (L.) was collected from different elevations in the northern areas of Pakistan. Morphology of lower elevations (200 m) showed healthy growth representing plenty of nutrients and water along with suitable abiotic climatic conditions. At moderate levels (800 m and 1250 m) total chlorophyll reduced, however lower (200 m) and higher (1550 m) elevations showed increased concentration of photosynthesis. Proline and glycine betaine concentrations were the lowest at 200 m and 800 m. Anatomical changes like increased cuticle thickness, epidermal thickness and number of cystoliths increased with elevations (1400 m) showing environmental variability. The presence of more adaptive features like trichomes and cystoliths at low and high elevations that were linked to tolerance against environmental adversaries. Such morpho-anatomical and physiological study of *Justicia adhatoda* is helpful to understand the elevational adaptation in different abiotic climatic conditions, such as temperature, wind velocity, UV and soil physio-chemistry.

Key words: Morphology, Cystoliths, Anatomical, Physiological, Climatic conditions.

Introduction

Mountainous ecosystem acts as a perfect platform to study climatic changes (Parmesan 2006). Abiotic factors like nutrient and moisture availability, high wind velocity and radiation alter along the altitudinal gradient at short distances (Pauli *et al*., 2012; Fatima *et al*., 2018). Vegetation of high altitudes faces extreme climatic factors like frost, cold, and low oxygen, which help in explaining adaptive mechanism (Ahmed *et al*., 2016). The mechanism of adaptation of plants in response to elevation variations and climatic change is gaining importance in the current era (Zimmerman *et al*., 2015).

Plants adopt environments either by genetic modifications or by expressing plasticity in structural and functional traits (Stocklin *et al*., 2009; Ahmad *et al*., 2016). Elevation can significantly influence plant growth, internal structure, function and metabolism (Berli *et al*., 2013). Morphological and physiological traits of a plant vary with elevation gradient (Pellissier *et al*., 2013). Plants of high elevations consist specific morphological, anatomical, physiological, and biochemical changes with increasing elevations, such as thicker leaves but a decrease in leaf size (Guo *et al*., 2016), and reduced growth and biomass (Vitasse *et al*., 2014). Plants usually show optimum growth and development at specific elevations, but thereafter negatively affected with increase or decrease in elevation (Zhang *et al*., 2022). Anatomical modifications like dense pubescence on leaf, reduced leaf area, enhanced sclerification on tissues, size of vascular tissues and parenchyma tissues thickness alters with elevation levels (Korner *et al*., 2007). Stomata are considered as main characters of stress tolerance in plants. Stomatal size and density vary with elevation (Soheili *et al*., 2023). Lignin deposition on parenchymatous tissues (Sclerophyll) is adaptive response of stresses that are affiliated with elevations (Psidowa *et al*., 2018). Alpine plants tend to improve water retention capacity with increased cuticle and epidermal thickness during osmotic adjustments (Yang *et al*., 2021). The diameter area of conducting vessels (xylem and phloem) is negatively associated with rise in elevation level (Olson *et al*., 2018).

Physiological processes in plants are also altered along elevation gradient. Photosynthetic rate negatively correlates with the increase in elevation level (Fujimura *et al*., 2010). The effectiveness of enzymatic antioxidants and scavenging enzymes (SOD and POD) increases with the rise in elevation (Agrwal, 2011; Hashim *et al*., 2020). Defensive mechanism in alpine forests became stronger by the increase of protein antioxidant and other metabolites (Li *et al*., 2020). The concentrations of nutrients within plant tissues are negatively associated with elevation, however soil nutrients concentrations remain unchanged (Moser *et al*., 2011). Sodium, calcium, potassium, phosphate, and magnesium ions concentrations dramatically decreased with the rise in elevation (Tenikecier & Ates, 2019).

Malabar nut (*Justicia adhatoda* L.) is one of the most dominant plant species in the mountainous region that may be due to its plasticity to osmotic adjustment according to different elevational environments. It can grow up to 1200 m (Malik & Ghafoor, 1988), but we collected this species from higher elevations (1250, 1400 and 1551 m a.s.l.). Climatic conditions in the Salt Range Pakistan show variation in abiotic factors at smaller distances (Ahmed *et al*., 2013). However, sustainability and stability of *J. adhatoda* in the area is due to strong root system and active absorption of nutrients from different rhizosphere. Deep root system prevents soil from erosion (Mukherjee *et al*., 2013). Such plants can easily survive in the diverse environmental conditions (Chanwala *et al*., 2020).

Plant growth is affected by osmotic inhibition, which may cause water uptake by roots (Mumtaz *et al*., 2019). For osmotic regulation, the most important mechanism is the selective uptake of required ions in addition to ion compartmentalization and ion excretion (Flowers & Colmer, 2008). Soil physiochemical attributes along elevation gradient impose structural and functional alterations in plants, so it was hypothesized that plants must adjust osmotic potential for successful survival and ecological fitness. Plasticity in structural and functional traits is critical for the adaptation of wild population in environmental heterogeneity. The present study was, therefore, conducted

to evaluate adaptive components of *J. adhatoda* along elevational gradient that are related to osmoregulation. The research questions to be addressed are, a) Is there any difference in structural and functional feature of *J. adhatoda* along elevational gradient? b) If yes, then how do these differences take part in osmoregulation? and c) what are the key factors involved in osmoregulation of this species?

Material and Methods

Collection of material: Plants of *Justicia adhatoda* were collected from different elevations with the difference of 150 m (Fig. 1). The distance of 150 m was maintained by rounding the actual distance, which is given in parenthesis with round up figures. The collection sites were 200 (206) m - Lillah, 350 (346) m-Peer Da Khara, 500 (502) m - Matan Kalan, 650 (643) m - Katha Forest, 800 (807) m - Ucchali, 950 (942) m - Chitta, 1100 (1087) m - Sakesar, 1250 (1261) m - Balawra, 1400 (1396) m - Bagh, 1550 (1559) m-Nathia Gali. Six largest plants in the population were collected from each elevation, which were considered as replications. Soil from the rhizosphere of each plant was also collected for physiochemical analysis. Soil samples were taken at a depth of 15-20 cm depth, mixed thoroughly and used for the analysis.

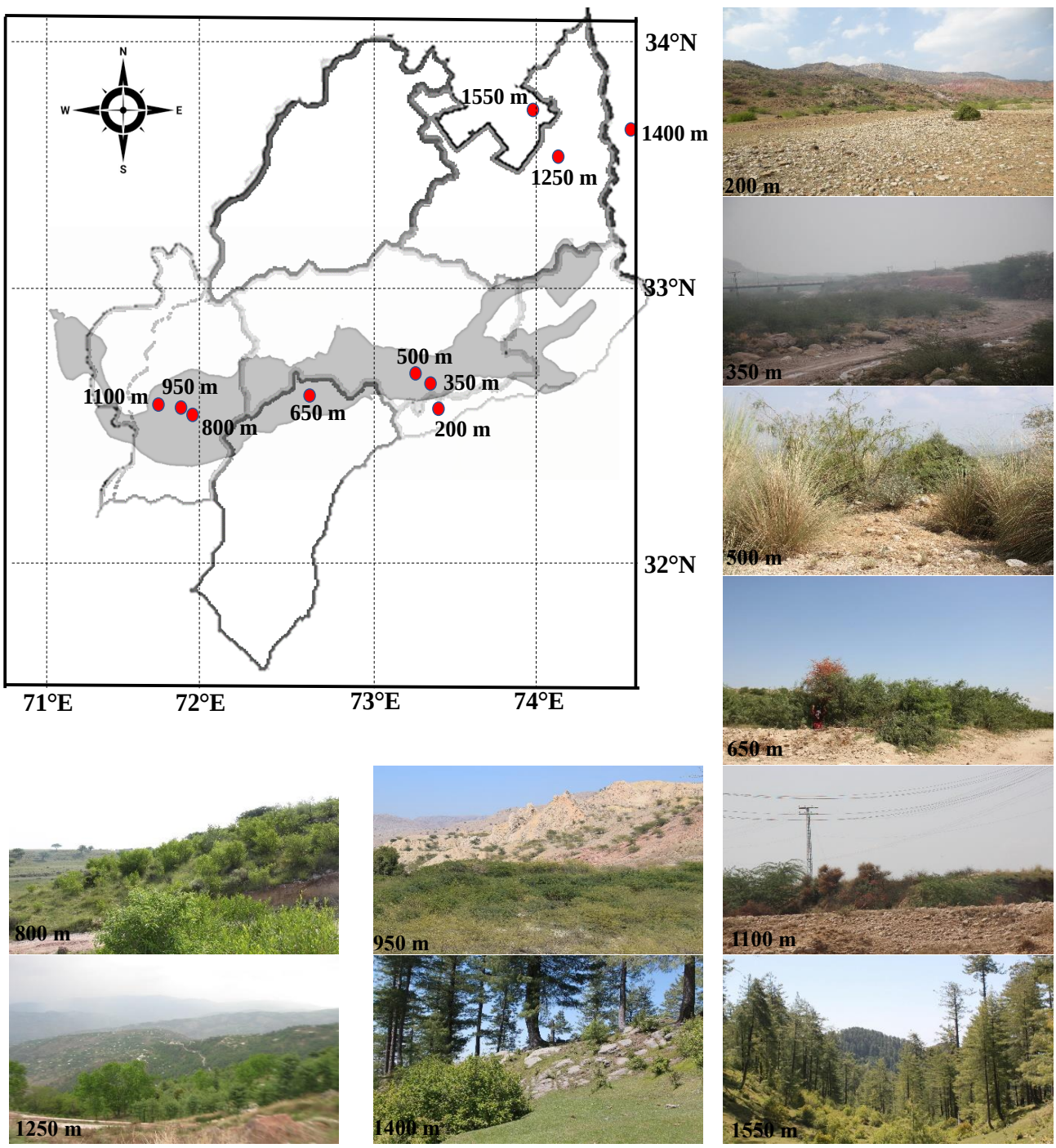

Fig. 1. Geographical presentation of different habitats of *Justicia adhatoda* along elevational gradient 200 m-Lillah, 350m-Peer Da Khara, 500 m-Matan Kalan, 650 m-Katha Forest, 800 m-Ucchali, 950 m-Chitta, 1100 m-Sakesar, 1250 m-Balawra, 1400 m-Bagh, 1550 m-Nathia Gali.

Soil physicochemical analysis: The soil's ECe and pH were estimated using an ECe/pH meter (WTW series Ino LAB pH/Cond 720, USA). Sodium, potassium, and calcium ions in the soil saturation paste extract were measured using a flame photometer (PFP-7, Jenway, UK). According to the Walkley-Black, soil organic matter was quantified using the titration technique (Walkley & Black, 1934). The formula used to calculate organic carbon is as follows:

$$\text{Organic C (\%)} = V_{blank} - V_{sample} \times M_{Fe^{2+}}\, 0.003 \times 100 \times f \times mcf$$

where MFe^{2+} is the concentration of standardized $FeSO_4$ solution (molarity), 0.003 is the amount of carbon that has been oxidized, f is the correction factor (1.3), and mcf is the moisture concentration factor.

The following formula was used to determine organic matter:

$$\text{Organic matter (\%)} = 1.723 \times \text{total organic C}$$

Grewal *et al*., (1990) process was followed for the estimation of saturation percentage (SP). Oven dried soil (200g), which was dried at 70°C for 5 days was used to make the soil paste. Following formula was applied to determine the saturation percentage:

$$\text{SP (\%)} = \frac{\text{Amount of water (g)}}{\text{Oven dried soil (g0}} \text{ x } 100 - Pw$$

where PW is water content used for making saturation paste.

Morphology: The largest branch of each plant was selected for shoot length. Three random branches were selected and then averaged for the calculations. Fresh weight was taken on a portable digital balance immediately after uprooting the plants in the field. The plants were then kept in paper bags and brought back to the laboratory. The material was then dried for one week in an oven at 60°C. Numbers of leaves per shoot were counted.

For leaf area measurements, Zhao *et al*., (2012) method was followed. Leaves were arranged on graph paper with 1 cm^2 square grid boxes, and the boundary was then drawn to determine the leaf area. Following formula was used:

$$\text{Leaf area} = \frac{\text{Number of covered grids} + \text{Number of incompletely covered grids}}{2}$$

Anatomy: Fresh leaves were placed in acetic alcohol (one liter solution was prepared by adding 50 ml formalin, 100 ml acetic acid, 500 ml ethanol and 350 ml distilled water). The material was transferred to acetic alcohol solution after 48 h, one liter solution was prepared by adding 250 ml acetic acid and 750 ml ethanol. Thin sections were cut by a razor blade and dehydrated gradually with alcohol grades. Standard double stained technique was used for staining the sections. Safranin was used for staining lignified tissue, while fast green for parenchymatous tissue. The sections were mounted with Canada balsam on a glass slide. Measurements were taken on a compound microscope (Model MT300H, Meiji Techno Co. Ltd., Japan).

Physiological attributes: For the estimation of ionic content, selected samples (0.1 g) of plant material were oven dried before being ground. According to Wolf's method) (Wolf, 1982), plant material was digested in concentrated H_2SO_4, and the amount of Na^+, K^+, and Ca^{2+} was measured using a portable flame photometer (Jenway, PFP-7, UK).

A technique developed by Mukherjee and Choudhuri (1983) was used to determine the concentration of ascorbic acid. The leaf part was homogenized with trichloroacetic acid (TCAA) and 2mm of it was mixed with 2,4-dinitrophenylhydrazine (2, 4 DNP) and a drop of CH_4N_2S. After boiling the mixture for 15 min., it was cooled and 5 ml of H_2SO_4 was added. The measurements were taken at 530 nm on spectrophotometer (Hitachi 220, Japan).

Julkenen-Titto (Rita, 1985) method was used for the measurement of phenolics. Fresh plant parts (0.1 g) were ground in acetone, centrifuged for 15 min. and then took 100 µl from the mixture and added 1ml of water. Folinphenolic acid was added to it, shook and then added bicarbonate. The material was placed on vortex for 5 min. Absorbance was calculated at 720nm on spectrophotometer (Hitachi 220, Japan).

Hydrogen peroxide was detected by the procedure proposed by Velikova *et al*., (2000). The plant part (fresh leaf) was crushed by 5 ml of trichloric acid (0.1 percent). Extraction was centrifuged at 12000 rpm for 900 sec. 0.5 ml of mixture + 0.5 ml of phosphate (pH: 7.78) + 0.1 ml of KI. The material was then vortexed. Absorbance was measured at 390 nm with blank on spectrophotometer (Hitachi 220, Japan).

For the determination of malondialdehyde, a mixture prepared from fresh leaf following Carmark & Horst (1991). Absorbance was calculated at 390 nm on spectrophotometer (Hitachi 220, Japan). Mixture prepared from fresh material was analyzed for peroxidase by spectrophotometer (Hitachi 220, Japan) with the interval of 30 seconds at 470 nm.

Arnon method (1949) used a technique to detect chlorophyll content. Fresh leaf was homogenized with acetone and filtrate was used to record calculations under 480, 663 and 645 absorbance. The content was calculated by the following formulae:

$$\text{Chl } a \left(\frac{mg}{g} f.w.\right) = [12.7(OD\ 663 - 2.69(OD\ 645) \times V/1000 \times W]$$

$$\text{Chl } b \left(\frac{\text{mg}}{\text{g}} f.w.\right) = [22.9(OD\ 645) - 4.68(OD\ 663) \times V/1000 \times W$$

$$\text{Carotenoids } \left(\frac{\text{mg}}{\text{g}} f.w.\right) = A^{car}/Em \times 100$$

where V= volume of the sample, W= weight of fresh tissue, A^{car}= OD 480+0.114 (OD 663) –0.638 (OD 645), Em= 2500.

Free proline was detected by the method of Bates *et al*., (1973). Absorbance was taken at 520nm on spectrophotometer (Hitachi 220, Japan). In this technique fresh leaf tissues (0.5g) grinded in 10 ml of sulfo-salicylic acid (3%). The homogenized mixture was filtered. Two ml. of the filtrate was mixed with the acid ninhydrin (1.25g of ninhydrin + glacial acetic acid). The mixture was then placed in an ice tube. Four ml of toluene was added to this mixture. It was mixed vigorously by passing a stream of air (1-2 min). The chromophore containing toluene was aspirated from the aqueous phase. Reading was calculated as follows:

µmol proline g-1 fresh weight = [(µg proline/ml × ml of toluene)/115.5 ×µg/µmol]/[(g of sample)/5]

Hamilton and Van – Slyke (1943) defined the procedure for the calculations of free amino acids. Fresh leaf (g) grinded in the phosphate buffer and ninhydrin and pyridine was added. The mixture was heated in water bath and absorbance was calculated at 535nm on spectrophotometer (Hitachi 220, Japan).

For the estimation of flavonoids, fresh leaf portion mashed up in the acetone. Filtered extract was taken in flask and mixed with water. After 5 min NaOH was added in ($NaNO_2$ + $AlCl_3$) mixture. Absorbance was calculated at 510 nm by spectrophotometer (Hitachi 220, Japan).

Statistical analysis

One way ANOVA (Steel & Torrie, 1980; Fernands, 1992). was used to determine the significance of the results. Principal component analysis was applied to check the association among different variables using XLSTAT (ver. 2014).

Results

Environmental and soil traits: Environmental and soil physicochemical traits such as minimum average temperature (12°C), soil ECe (3.43 dS m^{-1}) and Na^+ (269.2 mg kg^{-1}) were the greatest at 350 m elevation, while soil organic matter (0.76%) and K^+ (96.4 mg kg^{-1}) were the lowest (Table 1). The minimum of soil organic matter (0.76%), Na^+ (104.9 mg kg^{-1}) was recorded at 650 m, annual rainfall (244 mm) at 800 m, soil ECe (0.49 dS m^{-1}) at 950 m, and soil Ca^{2+} (89.3 mg kg^{-1}) at 1400 m. The maximum average annual temperature (48°C), soil Ca^{2+} (171.7 mg kg^{-1}) and K^+ (177.8 mg kg^{-1}) and the minimum saturation percentage (29%) was observed at 1100 m. Annual rainfall (1610 mm), soil organic matter (1.63%) and pH (8.3) were the highest at 1550 m elevation. Average minimum temperature (-6°C) and soil PO_4^{3-} (0.131 mg kg^{-1}) were the lowest at this elevation.

Morphological traits: Plants collected from 200 m elevation showed the maximum shoot length (136 cm), number of branches (35), number of leaves (525), fresh weight (59.8 g) and dry weight (21.4 g). The minimum number of branches (8) at 1100 m, shoot length (36 cm) at 1250 m, fresh weight (30 g) and dry weight (7.5g) at 1400 m and number of branches (6) at 1500 m were noted (Table 2).

Table 1. Environmental and soil physiochemical attributes of *Justicia adhatoda* collected from different elevations.

	Elevations (m a.s.l.)									
	200	350	500	650	800	950	1100	1250	1400	1550
Coordinates	32°33'22"N 72°45'39"E	32°39'09"N 72°47'19"E	32°39'12"N 72°43'39"E	32°33'28"N 72°23'07"E	32°31'55"N 72°01'45"E	32°33'22"N 71°58'25"E	32°31'28"N 71°54'25"E	33°43'43"N 73°28'35"E	33°51'21"N 73°26'48"E	34°09'25"N 73°17'50"E
Annual rainfall (mm)	346c	271d	247e	250e	244e	323d	144f	1123b	1057c	1610a
Minimum temperature (°C)	11b	12b	8c	6d	9c	8c	15a	-2e	-3e	-6f
Maximum temperature (°C)	48a	46b	42cd	41d	43c	41d	49a	33e	32e	28f
Saturation percentage	31b	32b	34a	32b	32b	34a	29c	34a	32b	35a
Organic matter	0.83e	0.76f	0.83e	0.76f	0.96d	0.97d	0.97d	1.33c	1.41b	1.63a
pH	7.4d	7.7bc	7.3d	7.6c	7.1e	7.6c	7.9b	7.8b	7.8b	8.3a
Electrical conductivity	1.54d	3.43a	2.16c	1.52d	0.98f	0.49g	2.21c	2.65b	3.17a	1.25e
Soil Na^+ (mg kg^{-1})	112.6e	269.2a	213.8c	104.9e	178.3e	144.6d	233.2b	225.6b	267.1a	110e
Soil Ca^{2+} (mg kg^{-1})	124.7d	116.4e	125d	99.4f	134.4c	167.9b	171.7a	118.8e	89.3f	93f
Soil K^+ (mg kg^{-1})	124.7c	96.4c	118.9d	120.7cd	149.14b	152.7b	177.8a	110.7e	99.1f	98.8f
Soil PO_4^{3-}(mg kg^{-1})	0.142cd	0.143c	0.141d	0.145b	0.141d	0.134e	0.146b	0.148a	0.146b	0.131f

Table 2. Morphological attributes of *Justicia adhatoda* collected from different elevations.

A.S.L (m)	Elevations (m a.s.l.)									
	200	350	500	650	800	950	1100	1250	1400	1550
Shoot length (cm)	136.0a	99.0b	87.0c	68.0d	67.0de	65.0e	48.0f	36.0i	43.7g	39.0h
Number of branches per plant	35.0a	21.7b	13.0c	14.0c	11.0c	12.0c	8.0d	7.0d	7.0d	6.0d
Number of leaves per branch	525.0a	366.0b	291.0c	230.0e	242.0d	230.0e	212.0f	216.7f	184.0g	126.6h
Leaf area (cm^2)	4851a	3896b	2628c	2231e	2236e	2396d	2015g	2182f	1076i	1631h
Shoot fresh weight (g $branch^{-1}$)	59.8a	45.6b	44.5b	37.3c	36.1c	35.6c	36.2c	30.2d	30.0d	30.3d
Shoot dry weight (g $branch^{-1}$)	21.4a	15.1b	14.8b	15.1b	10.2d	11.6c	10.2d	9.6d	7.5e	7.7e

Leaf anatomical traits: Among leaf anatomical traits, midrib thickness (1590.3 µm), metaxylem area (243.3 µm) and parenchymatous cell area (542.6 µm) were the greatest at 200 m elevation (Table 3, Fig. 2). Adaxial epidermal thickness (18.6 µm) was the minimum at 350 m. At 500 m, phloem area (112.6 µm) was the maximum, whereas density of cystoliths (1) and trichomes (2) were the lowest. Spongy thickness (243.2 µm) was the highest at 650 m, but lamina thickness (243.2 µm), abaxial epidermal thickness (16.7 µm), palisade thickness (112.6 µm) and trichome length (72.4 µm) were the lowest. The thickest abaxial epidermis (74.8 µm) was recorded at 800 m, while the thickest palisade (299.3 µm) and trichome density (3) were at 950 and the thickest lamina (505.1 µm) was at 1100 m. Density of cystoliths (5) and trichomes (8) were the highest at 1250 m (Fig. 3), whereas phloem thickness (37.4 µm) and spongy mesophyll thickness (93.6 µm) were the minimum. The metaxylem were the narrowest (74.8 µm) along with lowest stomatal density (15.9) at 1400 m. Adaxial epidermal thickness (83.1 µm) was the maximum at the highest elevation (1550 m). The thinnest midrib (710.8 µm) and the smallest parenchyma cells (74.8 µm) were noted at this elevation.

Physiological traits: Ascorbic acid (1.3 mg g^{-1} dw), glycine betaine (0.5 mg g^{-1} dw), proline (20.5 mg g^{-1} dw), and peroxidase (0.1 mg g^{-1} dw) were the lowest at 200 m. The highest K^+ (19.8 mg g^{-1} dw) and the lowest phenolics (3.2 mg g^{-1} fw) were found at 350 m. Shoot PO_4^{3-} (50.5 mg g^{-1} dw) and MDA (2.5 mg g^{-1} dw) were the minimum at 500 m. The carotenoids (0.331 mg g^{-1} fw), chlorophyll *a* (1.74 mg g^{-1} fw), chlorophyll *b* (0.419 mg g^{-1} fw), total chlorophyll (2.16 mg g^{-1} fw), Ca^{2+} (344.9 mg g^{-1} dw), proline (112.8 mg g^{-1} dw), flavonoids (7.6 mg g^{-1} dw) and peroxidase (1.2 mg g^{-1} dw) were the greatest at 800 m. The lowest flavonoids (3.2 mg g^{-1} dw) were recorded at 950 m. The lowest chlorophyll *b* (0.026 mg g^{-1} fw) was found at 1400 m. Plant growing at 1400 m elevation showed the maximum ascorbic acid (1.9 mg g^{-1} dw), glycine betaine (1.7 µmol g^{-1} fw), phenolics (7.0 µmol g^{-1} fw) and MDA (15.7 µmol g^{-1} fw). The maximum Na^+ (39.6 mg g^{-1} dw), Ca^{2+} (53.4 mg g^{-1} dw) and K^+ (11.3 mg g^{-1} dw) (Table 4).

Correlation studies: Annual rainfall was positively correlated with leaf trichome length and shoot phosphate however negatively related with shoot Na^+ and Ca^{2+} (p<0.05). The minimum temperature was positively associated with leaf midrib thickness, while a negative association was observed with shoot PO_4^{3-} (p<0.01). The maximum temperature was positively related with shoot length, number of leaves, leaf area, shoot fresh and dry weights, leaf midrib thickness, metaxylem thickness and parenchyma thickness (p<0.05) and positively related with leaf midrib thickness (p<0.01). Saturation percentage was negatively related with the proline and stomatal density (p<0.05). Organic matter was negatively correlated with shoot length, number of leaves, leaf area, and shoot fresh and dry weights (p<0.01). Organic matter showed a positive relationship with MDA (p<0.05). Soil pH was positively correlated with PO_4^{3-} (p<0.05). Soil ECe was positively related to K^+ (p<0.05). Soil Na^+ was positively linked soot K^+ (p<0.01). Soil Ca^{2+} showed a strong positive association with lamina thickness (p<0.01). Soil K^+ showed a positive correlation with leaf lamina thickness, abaxial epidermal thickness and palisade tissues (p<0.05), while negatively related with chlorophyll *b*. Soil PO_4^{3-} showed a negative relationship with epidermal thickness (p<0.01).

Principal component analysis: Leaf phloem thickness was associated with the calcium-ion at 500 m. Similarly leaf epidermal thickness and electrical conductance at 1550 m showed association, however at 200m metaxylem thickness and temperature were linked closely (Fig. 4). Plants glycine betaine, plants trichomes, plants ascorbic acid and pH at 650 m were closely associated. The electrical conductivity and sodium-ion concentration were linked with plant per oxidase at 1250 m while annual rainfall, electrical conductivity, plant midrib thickness and plant proline concentrations are associated at 1400 m. Soil Ca^{2+}, organic matter and maximum temperature were grouped together at 800 m, while number of leaves, shoot length, number of branches and saturation percentage were closely clustered at 200 m.

Discussion

Plant biomass is an important feature that is directly linked to overall growth and development of a plant. Fresh and dry weights at 200 m show better environment for the growth. Soil was fertile with nigh nutrient content; rainfall was high and temperature is not too harsh. All these were favourable for the growth and development of *J. adhatoda* plants colonizing the lowest elevation. Plant productivity and growth is affected by abiotic factors of the environment (Shrivastava & Kumar, 2015). Growth trends of plants are directly linked to abiotic attributes of environments. Leaf morphology alters significantly by level of altitudes. Climatic conditions are responsible for the biochemical and growth changes (Vitasse *et al*., 2009) Plants at lower elevations (200 m) in the present study showed more growth. Another important feature at 200 m was the high rate of photosynthesis, which was linked to photosynthetic pigments as seen at lower elevation. Energy is produced in the plant by photosynthesis, and this is closely associated with growth and development (Parihar *et al*., 2015). At high elevations plants must face various environmental stress factors such as drought, atmospheric factor, CO_2 concentration, UV radiation, temperature and light intensity. These stressful conditions result in production of ROS, leading to redox imbalance (Rana *et al*., 2019). That might be the reason for more concentrations of glycine betaine and proline at higher elevations. Biochemical alterations such as high antioxidant activities, increased chlorophyll ratio and carotenoids, peroxidation, phenolics and proline contents are the major factors for survival of plants at high elevations. Magnesium is structural component of the chlorophyll, while Shoot Ca^{2+} ions concentrations linked with the epidermal thickness (Cui *et al*., 2019).

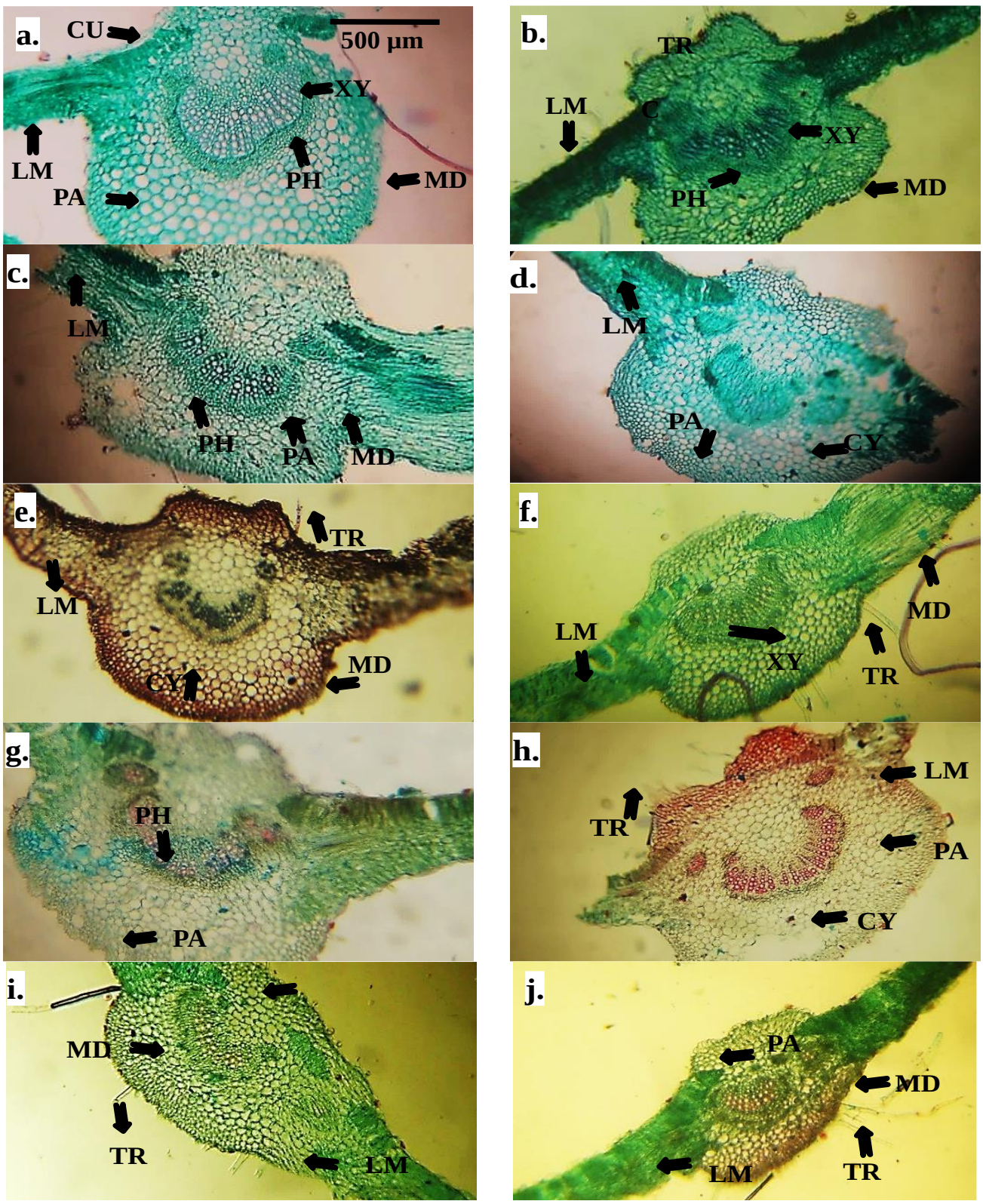

Fig. 2. Leaf anatomical studies of *Justicia adhatoda* collected along elevational gradient.
a. 200 m-Cuticle (CU): Increased cell size; Xylem (XY): length increased; Phloem (PH): reduced; Parenchyma (PA): enhanced thickness; Mid rib (MD): mid rib thickness increased prominent, b. 350 m-Trichome (TR): more number of trichomes; Cuticle (CU) increased; Phloem (PH) thickness enhanced; Cytoliths (CY); large number of cytoliths, c. 500 m-Parenchyma (PA) thickness reduced; Phloem (PH) area increased; Lamina thickness (LM) increasd, d. 650 m-Parenchyma (PA) cell thickness increased; Visible numbers of cytoliths, e. 800 m- Trichome (TR) length and number increased; Lamina (LM) decreased; Cytoloiths (CY) number increased, f. 950 m- Trichomes number (TR) and length inctreased; Xylem (XY) thickness decreased; Lamina thickness (LM) increased, g. 1000 m- Phloem (PH) thickness increased; Midrib thickenss (MD) and Parenchyma (PA) thickness increased, h. 1250 m- Cyyoliths (CY) and number of trichomes (TR) increased, Parenchyma (PA) thickness decreased, i. 1400 m- Midrib thickness (MD) reduced; Lamina thickness (LM) increased; Trichomes (TR) increased, j. 1550 m- Parenchyma (PA) and Mid rib (MD) thicknesses decreased.

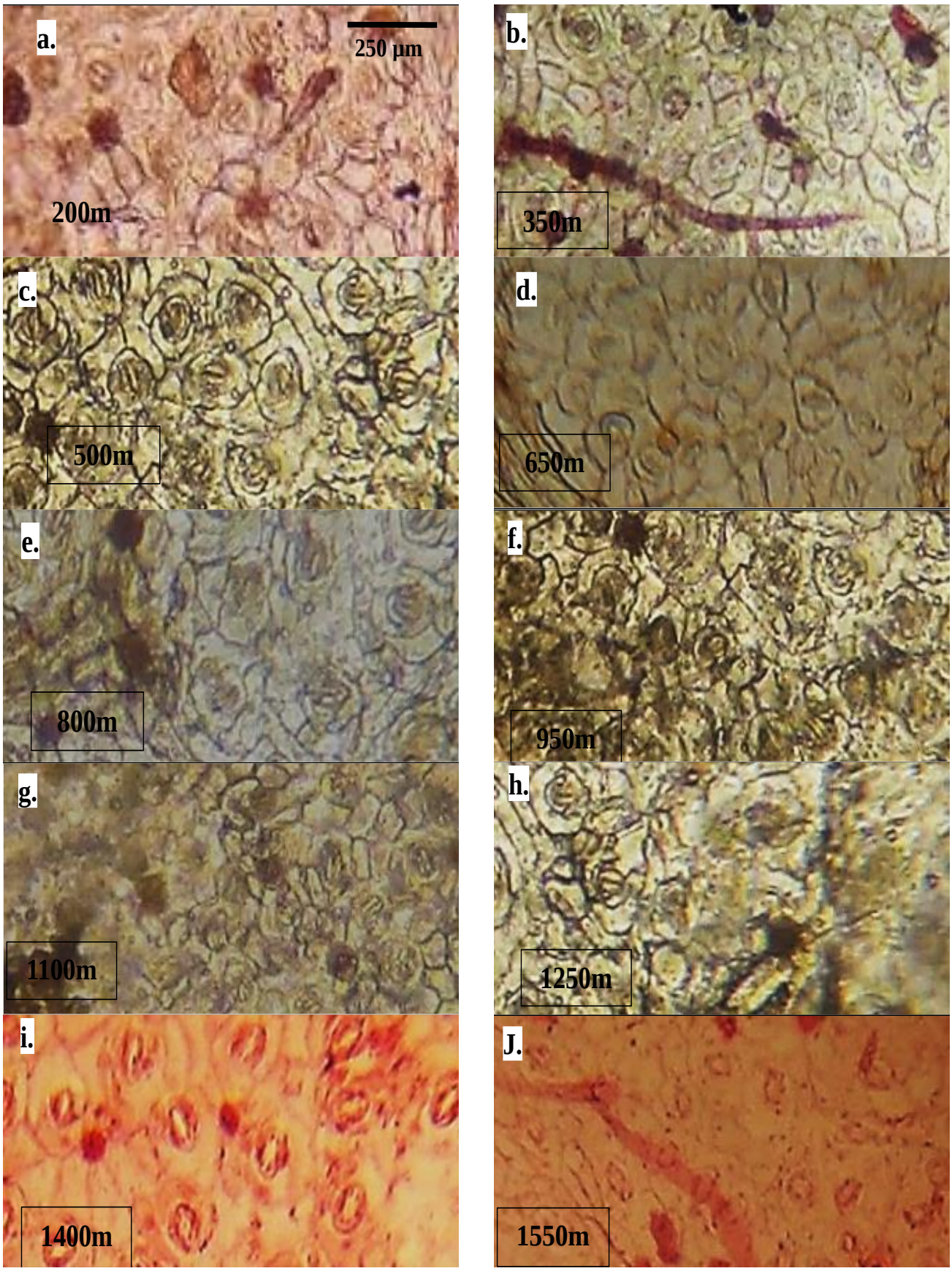

Fig. 3. Leaf anatomical studies of Justicia adhatoda collected along elevational gradient.
a. 200 m- stomata large, more cytoliths , b. 350 m-Trichomes length increased; Cytoliths number enhanced, c. 500 m- Stomatal area increased, d. 650 m-Stomatal size decreased, increased number of cytoliths, e. 800 m- increased size of cytolithss; trichome number decreased, f. 950 m- Stomatal size decreased, g. 1100 m- stomatal size reduced, h. 1250 m- Cytoliths and number of trichomes reduced, i. 1400 m- Trichome number reduced, size increased, j. 1550 m- trichome length enhanced with the decreased stomatal size.

Table 3. Anatomical attributes of *Justicia adhatoda* collected from different elevations.

	Elevations (m a.s.l)									
	200	**350**	**500**	**650**	**800**	**950**	**1100**	**1250**	**1400**	**1550**
Leaf anatomy										
Lamina thickness (µm)	486.4b	318.2f	355.4d	243.2g	374.2c	486.4b	505.1a	316.0f	336.7e	318.1f
Midrib thickness (µm)	1590.3a	1552.9b	1253.5d	1103.f	1178.7e	898.8h	1328.1c	1029.5g	729.6i	710.8j
Adaxial epidermis thickness (µm)	56.3b	18.6e	37.2d	52.1b	36.4d	59.4b	61.2b	74.4a	37.2d	83.1a
Abaxial epidermal thickness (µm)	72.4a	35.2e	18.7f	16.7f	74.8a	74.2a	93.6a	37.2e	56.1d	37.2e
Parenchyma thickness (µm),	542.6a	336.7d	299.3f	318.7e	355.4c	130.9h	392.9b	336.7d	205.80g	74.8i
Spongy thickness (µm)	112.2f	168.9b	130.9e	243.2a	149.8d	130.7e	145.6d	93.6g	130.9e	143.2d
Palisade thickness. (µm)	132.4g	149.6f	168.3e	112.6h	243.2c	299.3a	261.4b	112.6h	168.9e	205.8d
Phloem thickness (µm)	93.2b	54.3e	112.6a	56.1e	74.4c	74.8d	92.5b	37.4f	93.5b	71.8d
Metaxylem thickness (µm)	243.3a	205.1b	149.1d	149.7d	112.6e	168.4c	205.8b	112.2f	74.8g	168.9c
Cystoliths density per mm^2	2.0d	4.0b	1.0e	3.0c	3.0c	2.0 b	2.0d	5.0a	2.0d	3.0c
Hair density per mm^2	3.0c	7.0a	2.0c	4.0b	2.0c	6.0b	3.0c	8.0a	5.0b	4.0b
Epidermis										
Stomatal density per mm^2	25.7b	16.1c	16.4c	15.9c	16.3c	35.2a	23.8b	16.8c	16.5c	33.2a
Stomatal area (μm^2)	84.4c	224.2b	148.3d	72.4g	130.9f	149.6d	74.8g	131.9e	163.3c	299.3a
Trichome density per mm^2	26.0c	21.0d	21.1d	29.0b	19.0f	13.0h	34.0a	17.0h	13.0h	19.0f
Trichome length (μm^2)	329.9h	404.1g	432.2e	411.6f	449.4d	561.3b	291.9i	432.2e	785.8a	471.4c

Table 4. Physiological attributes of *Justicia adhatoda* plants collected from different elevations.

	Elevations (m a.s.l.)									
	200	**350**	**500**	**650**	**800**	**950**	**1100**	**1250**	**1400**	**1550**
Shoot Na^+ (mg g^{-1} d.w.)	73.1g	317.3c	291.7e	299.6d	331.1a	325.2b	283.9f	291.7e	45.5h	39.6i
Shoot Ca^{2+} (mg g^{-1} d.w.)	86.9g	331.1c	305.5e	313.4d	344.9a	339.0b	297.6f	305.5g	59.3h	53.4i
Shoot K^+ (mg g^{-1} d.w.)	12.5f	19.8a	19.1b	15.2e	14.1be	16.2d	16.8c	20.0a	17.1c	11.3g
Shoot PO_4^{3-} (mg g^{-1} d.w.)	115.0h	51.8i	50.5i	235.6c	127.0g	185.1e	178.5f	230.8d	315.8a	301.7b
Ascorbic acid (µg mol^{-1} f.w.)	1.3c	1.5bc	1.5bc	1.5bc	2.3a	1.7bc	1.4bc	1.4bc	1.9ab	1.4bc
Glycine betaine (µmol g^{-1} f.w.)	0.5e	1.0cde	0.9cde	1.1bcd	0.6ab	1.3abc	0.8cde	0.7de	1.7a	0.7bde
Proline (µmol g^{-1} f.w.)	20.5b	42.3b	33.7b	41.4b	112.8a	55.4b	32.8b	31.7b	62.8b	41.7b
Phenolics (mg g^{-1} f.w.)	4.5e	3.2j	3.5i	3.9f	5.4b	3.7h	5.2c	3.8c	7.0a	4.6d
Flavonoids (mg g^{-1} f.w.)	4.8e	4.1h	3.2i	4.5g	7.6a	3.0j	6.4c	4.7f	7.0b	5.5d
Hydrogen peroxide (µmol g^{-1} f.w.)	32.0a	13.0g	14.0ef	18.3c	15.1d	14.4e	13.8f	17.9c	24.5b	12.2h
Malondialdehyde (µmol g^{-1} f.w.)	5.3i	12.3c	2.5j	11.7d	11.3f	9.2g	8.9h	11.5e	15.7a	15.2a
Peroxidase (Units μg^{-1} Protein)	0.1d	0.3cd	0.4bcd	0.7abc	1.2a	0.7abc	0.9ab	0.8abc	0.8abc	0.8abc
Chlorophyll *a* (mg g^{-1} f.w.)	1.69a	1.30d	1.56b	1.72a	1.74a	1.18e	1.49b	1.33d	1.40c	1.21e
Chlorophyll *b* (mg g^{-1} f.w.)	0.369b	0.123f	0.301d	0.413a	0.419a	0.354d	0.300d	0.164e	0.168e	0.126f
Total chlorophyll (mg g^{-1} f.w.)	2.06b	1.42f	1.86c	2.13a	2.16a	1.53de	1.52e	1.49d	1.57d	1.34g
Chlorophyll *a*:*b* ratio	4.58f	10.57a	5.18e	4.16g	4.15g	3.33	4.97e	8.11d	8.33c	9.60b
Carotenoids (mg g^{-1} f.w.)	0.019b	0.012de	0.034a	0.033a	0.331a	0.021b	0.014cd	0.017bc	0.014cd	0.009e

Leaf anatomy alters by environmental abiotic factors. An increase in epidermal and cuticle thickness at higher elevations shows more adaptability to stressful environments (Paradiso *et al*., 2017). Enhanced metaxylem thickness is responsible for the better conduction of water and nutrients (Naseer *et al*., 2017). Similarly large phloem area at low elevations facilitates assimilation of food that contributes a step towards better growth of plant at moderate elevations (Akhtar *et al*., 2016). Stomatal area and density at high elevations are important features to demonstrate the osmoregulation of a plant. Smaller stomata at relatively hotter sites are responsible for the control of excessive transpiration under stressed conditions (Naseer *et al*., 2017).

Anatomical adaptations like trichome density and length, leaf cystoliths density, leaf mid rib thickness associated with the soil saturation percentage, electric conductivity, soil sodium and annual rainfall at higher elevations (Griffiths *et al*., 2009). The presence of adaptive characters like trichomes number, trichome density reduces the wind velocity, light effects and subsequent changes in osmoregulation. Cystoliths are salt storage cavities that increase the defensive mechanism by denaturing of membranes. Abiotic factors like temperature of plane areas may also be considered responsible for conducting tissues thickness, stomatal density and physiological changes such as hydrogen per oxidase and carotenoids (Mark and Jacqueline 2006). High average temperature increases the transpiration rate. The long thin vascular system facilitates the conduction of water at higher elevation. The availability of ions in the soil controls the phloem thickness, abaxial epidermal thickness, chlorophyll ratio, ascorbic acid, flavonoids and proline concentrations (Jim *et al*., 2017).

Overall, almost all growth parameters decreased with rise in elevation resulting in a bushy habit. Such reduction in size favoured survival at high elevations by combating low temperatures. Leaf anatomical adaptations such as midrib, lamina and mesophyll thicknesses significantly contributed to the survival at high elevations. Another critical factor for cold tolerance is the long trichomes, which protect leaves from environmental adversaries. High elevations adversely affected ionic content (Na^+, Ca^{2+} and K^+) but PO_4^{3-} increased. Flavonoids, Phenolics and enzyme MDA positively correlated with elevation.

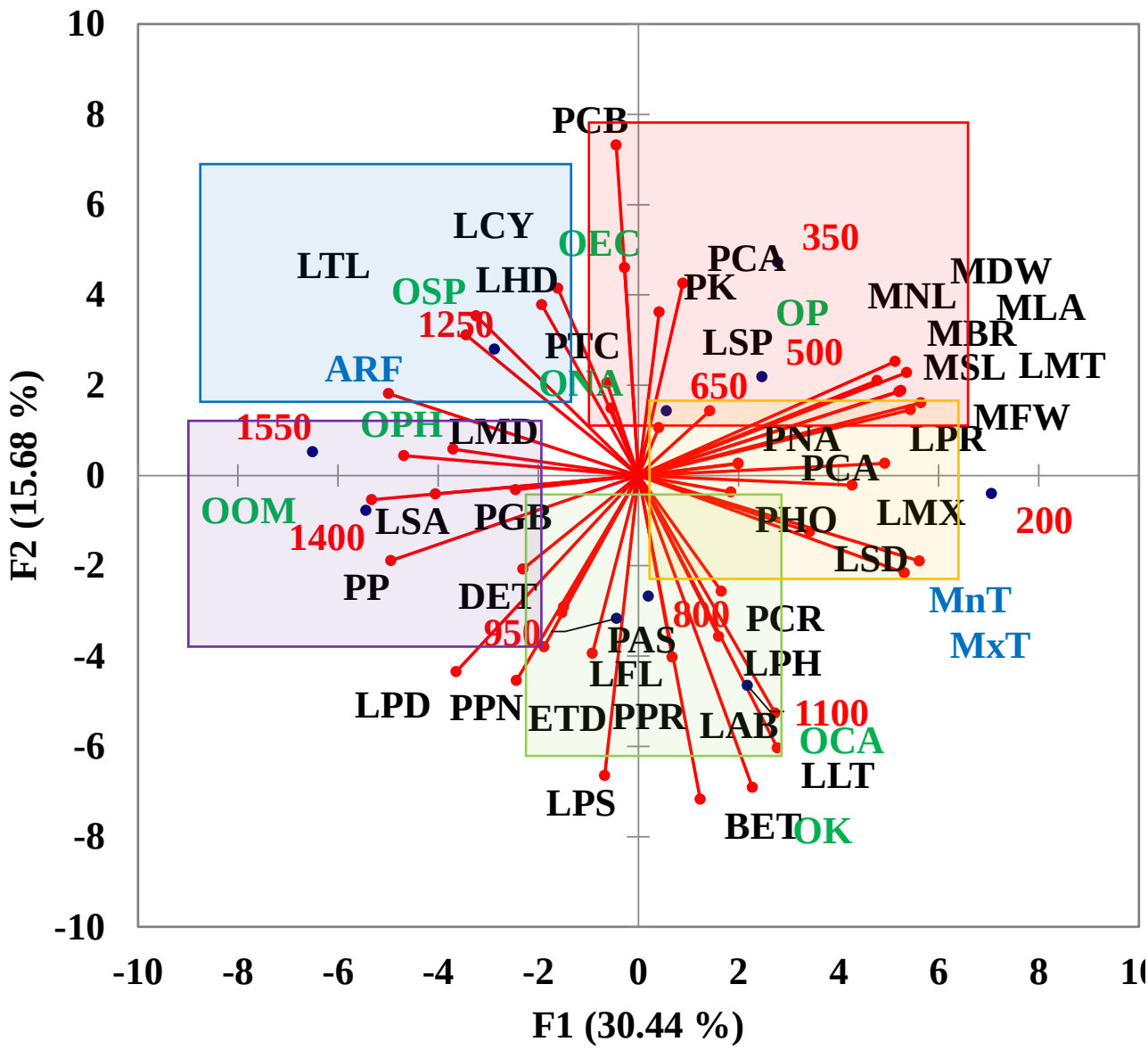

Fig. 4. PCA analysis among morphological, anatomical and soil attributes of *Justicia adhatoda* collected along elevational gradient.

Environmental traits: ARF= Annual rainfall, MnT= Minimum temperature, MxT= Maximum temperature.
Soil physicochemical: OSP= Saturation percentage, OOM= Organic matter, OEC= Electrical conductivity, OPH= Soil pH, ONA= Soil Na^+, OCA= Soil Ca^{2+}, OK= Soil K^+, OP= Soil PO_4^{3-}.
Morphology: MSL-Shoot length, MBR-Number of branches, MNL-Number of leaves, MLA-Leaf area, MFW-Shoot fresh weight, MDW-Shoot dry weight.
Leaf anatomy: LLT-Lamina thickness, LMT-Midrib thickness, LPH-Phloem thickness, LMX-Metaxylem thickness, LCY-Cystoliths density, LHD-Hair density, LPR-Parenchyma thickness, DET-Adaxial epidermis thickness, BET-Abaxial epidermal thickness, LSP-Spongy thickness, LPS-Palisade thickness.
Epidermis: LSD-Stomatal density, LSA-Stomatal area, ETD-Trichome density, LTL-Trichome length.
Physiology: PNA= Sodium ion, PCA= Calcium ion, PK= Potassium ion, PP= Phosphate ion, PAS= Ascorbic acid, PGB= Glycine betaine, PPR= Proline, PPN= Phenolics, LFL= Flavonoids, PHO= Hydrogen peroxidase, PMD= Malondialdehyde, PPD= Peroxidase, PCA= Chlorophyll *a*, PCB= Chlorophyll *b*, PTC= Total chlorophyll, LAB= Chlorophyll *a*:*b* ratio, PCR= Carotenoids.

Conclusion

Plants are not arbitrarily arranged in each elevational site; rather, how they are distributed depends on the conditions under which they develop and to which they are able to adapt functionally. Physiological, morphological and anatomical adaptations in the plants are responses to abiotic changes somehow. Reduction in the plant biomass with increasing elevation levels are the consequences of physiological and anatomical alterations representing the unfavorable environmental conditions. The presence of trichomes, increased epidermal thickness, midrib thickness, metaxylem area, cortical area, chlorophyll contents and presence of osmolytes all regulate the distributional pattern of *J. adhatoda*. These changes represent the plants escape from the severe environments. Different elevation levels possess different climates, i.e., temperature decreases significantly at high elevations. Phytochemical diversity aids plants in coping with unfavourable environments. By controlling its phytochemicals, *J. adhatoda* chemically modified itself

along the elevational range. The ability of plants to adapt to changing environmental conditions is demonstrated by their defense mechanisms, which activate the manufacture of secondary metabolites in high-altitude plants.

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(Received for publication 22 August 2023)