PLANT SCIENCE TODAY
Vol x(x): xx–xx
https://doi.org/10.14719/pst.12768

eISSN 2348-1900

RESEARCH ARTICLE

Anatomical variation in Walnut (*Juglans regia* L.) populations from Dir and Swat Districts, Pakistan: Implications for plant taxonomy and adaptation to climate change

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Received: 16 November 2025; Accepted: 16 March 2026; Available online: Version 1.0: 22 April 2026

Cite this article: Mohammad K, Ikramullah K, Hamad UR, Rafi U, Fazal U, Muhammad I, Mursaleen, Rimsha Z, Mohammad R. Anatomical variation in Walnut (*Juglans regia* L.) populations from Dir and Swat Districts, Pakistan: Implications for plant taxonomy and adaptation to climate change. Plant Science Today (Early Access). https://doi.org/10.14719/pst.12768

Abstract

In the fight against climate change, every ring in anatomy tells a story and every leaf holds a secret. Similarly, walnut populations in Pakistan's Dir and Swat districts face varying climatic conditions that affect their anatomy. However, the current study aims to explore the anatomical variations in walnut leaves and nuts to understand climate-driven adaptations. In the assessed study, the 44 sites examined were analyzed using scanning electron microscopy (SEM) to analyse epidermal cells, pith cells and vascular tissues. Walnut populations showed clear anatomical variations between districts. In the results, the Swat-selected specimens had larger epidermal cells (31.55–56.12 µm), phloem and pith cells, whereas the Dir specimens had larger xylem and palisade cells. Although high-altitude walnuts produced thinner shells and bigger cotyledons, the low-altitude trees had thicker shells. In short, these traits decreased with rising temperature and increased with altitude and light intensity. These findings have implications for plant taxonomy and conservation, suggesting anatomical variations can inform adaptation strategies and breeding programs in mountainous areas.

Keywords: anatomical variation; climate change; Dir and Swat districts; Pakistan; plant taxonomy; walnut

Introduction

Juglans regia L., commonly known as walnut, is a deciduous perennial plant that belongs to Juglandaceae. Walnuts are known for their nutritional values and high market prices. The walnut leaves are about 25–40 cm long and are odd-pinnate, with 5–9 leaflets arranged alternately. The walnut root system is characterized by a deep taproot that develops early (1). It typically grows in pure stands or as individual mature trees rather than in mixed woodlands. The species also requires a long growing season and usually thrives in warm, sheltered locations (1). Walnuts thrive in deep, rich soil with a pH between 6 and 7.5 (2). Walnut is a widely distributed medicinal plant that has significant medical properties for the treatment of many diseases, such as skin disorders, stomach pain, diarrhoea, arthritis, asthma, fever and some endocrine diseases like thyroid dysfunctions, diabetes mellitus, cancer and other diseases (3). Nuts, especially walnuts, are a nutritional powerhouse and offer numerous health benefits (4). World-wide, walnut is valuable crop due to rich in nutrients (5). The food and Agriculture Organisation (FAO) included walnut in the priority plant group (6). In recent years, the by-products of walnuts, such as husk and shell, contain 70 % of the weight of the fruit (7), used for bioethanol production (8, 9), pyroligneous acid extraction(10), activated carbon and charcoal (11) and nutty carbon used for battery anodes (12). Besides, skin (13), bark (14), root (15) and leaves (16) can be used in various industries as low-cost material (17). Previously, (6) reported that walnut diversity has increased over the years in their native regions, particularly their kernel colour, shell colour, nut shape, size and thickness. Walnuts are indeed cultivated commercially in various regions around the world, including East Asia, North Africa, Southern Europe, South America and the USA. The global production of walnuts has increased significantly in recent years, with Asian countries leading the growth.

In this modern time, the practice of scanning electron microscopic study delivers the chance to explore consistent structures to determine plant taxa and information about the anatomical, ecological and geographical significance of diverse groups of plants (18, 19). The nut's surface, exine and shape are valuable characteristics for identifying and classifying plant species within a family. The variation between species has been revealed using scanning electron microscopy (SEM), which provides evidence to support and strengthen the taxonomy of the genus (20). Certain differences in cell structures cannot be detected by SEM (21). Various cell studies depend on the methodology used in the preparation of slides and differences in the individual methods (22).

Different climate conditions, like drought, can significantly impact plant anatomy, reducing tree diameter in regions like mid and Southern Europe (23). Mild winters boost walnut germination, showing that warmer winters can impact their adaptability (24). Low air humidity alone can reduce the productivity and biomass of European beech trees (25). In-plant species distribution environment plays an important role in the entire globe (26). Mean annual precipitation also had an excessive influence on the woody fruit plant species distribution but mean annual temperature, on the other hand, was recognised as a great climatic driver for all herb species distribution and growth (27). It is also described that an increase in the humidity of the air results in a reduced growing level, as well as accumulation of biomass of the sapling of *Betula pendula* (28). Walnut yield is closely linked to its lateral bearing habit. Environmental factors like light, temperature, drought and altitude affect leaf structure and trends show plants in low-temperature zones have thicker mesophyll tissue, epidermal thickness, smaller leaf area and thicker leaves (29). Plants in water-scarce regions often have small cell volume, increased palisade layers, decreased spongy coatings and smaller intercellular spaces (30). Anatomical studies for species from this family are still incipient (31–34) evidencing the need to expand knowledge about anatomy, searching for characters that contribute to the identification and separation of species of this family. Plant morphology plays a key role in understanding early angiosperm evolution and classification (35).

The anatomical features of plant parts, like leaves and stems, provide important extra information about their external appearance, especially when there are only vegetative specimens for analysis (36, 37). Climate change affecting tree species distributions is a good example of the wide magnitude of global changes (38, 39). Therefore, the study aimed to examine how different climatic factors influence the anatomical structure of walnut leaves and nuts in northern Pakistan.

Methodology

Study sites

The plant specimens (leaves and nuts) were collected for anatomical variation from forty-four locations of Dir and Swat districts (Table 1). Dir is situated at 71°50' E and 35°12' N whereas Swat district is

Table 1. Sampling site and their Latitude (Northern) and Longitude (Eastern) of Dir and Swat

S. No	District Dir sites	Latitude (Northern)	Longitude (Eastern)	S.No	District Swat sites	Latitude (Northern)	Longitude (Eastern)
1	Buth Qillah	34° 80'12''	72° 15'75''	1	Milaga	34°41' 47''	72° 47'34''
2	Laram	34° 47' 39''	71° 56'40''	2	Yakhtangy	34°42' 47''	72° 33'43''
3	Khanpur	34° 36' 24''	72° 4'32''	3	Rangila	34°80' 28''	71° 79'33''
4	Siawargar	34° 47' 12''	72° 57'47''	4	Ghaki Banda	34°78' 47''	71° 28'47''
5	Rabat Dara	34° 43' 47''	72° 67'32''	5	Murghozar	34° 66'78''	72° 34'31''
6	New Kaly	34° 51' 47''	71°87'47''	6	Totanu Banda	34°59' 37''	72° 28'74''
7	Bombolai	34° 38' 24''	71° 34'74''	7	Tegako	34°50' 43''	72° 26'73''
8	Abi Shah	34° 40' 32''	71° 35'47''	8	Qalagy	34°87' 30''	72° 17'42''
9	Barimky	34° 43' 32''	72° 67'47''	9	Sar bala	34°89' 43''	72° 36'72''
10	Kasky Toormang	34° 54'55''	72° 1'3.2''	10	Malam Jaba	34°48' 35''	72° 23'58''
11	Balo Rabat	34° 52' 53''	71° 58'26''	11	Kalam	35°28' 41''	72° 35'18''
12	Asilo Rabat	34° 52' 88''	71° 58'45''	12	Lalkoo	35° 6'44''	72° 23'50''
13	Sheringal	35° 15' 12''	72° 3'21''	13	Baghderai	35° 5' 34''	72° 12'35''
14	Jabar	35°8' 34''	72° 2'10''	14	Chuprial	34° 58'56''	72° 21'39''
15	Chokyatan	35° 12' 20''	71° 52'32''	15	Baidara	35° 2' 55''	72° 18'1''
16	Babyawar	35° 07' 39''	71° 56'21''	16	Bazkhela	34°55' 47''	72° 47'46''
17	Usherai Dara	34° 22' 13''	71° 33'15''	17	Peochar	34°13' 48''	72° 18'34''
18	Barawal	35° 05'11''	71°4 5'38''	18	Bar Thana	34°15' 18''	71° 77'40''
19	Dog Dara	35° 16' 60''	72°1 5'30''	19	Shakar Dara	34° 52'19''	72°24'34''
20	Parakot	35° 28' 34''	72° 45'16''	20	Nalcot	35° 3'53''	72° 18'21''
21	Zeyam	34° 70' 12''	72° 24'78''	21	Bihar	35° 3' 19''	72° 32'4''
22	Siagaonai	34° 20' 18''	71° 30'35''	22	Shawar	34°46' 49''	72° 41'17''

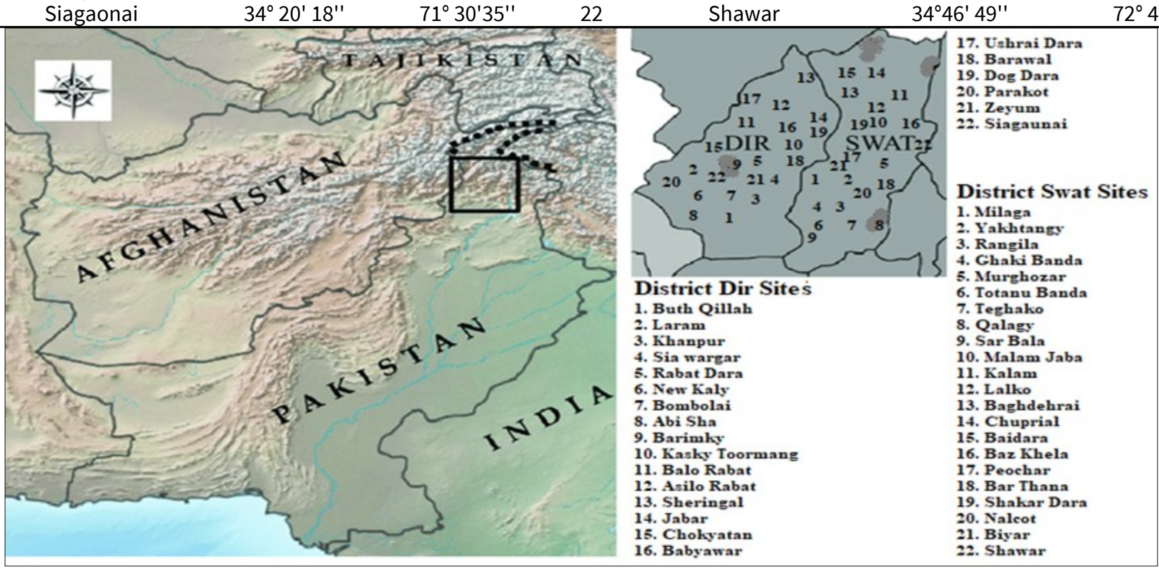

Fig. 1. Location of the sites sample across the districts of Dir and District Swat, Pakistan.

situated at 72° 10' 60.00'' E and 35° 22' 59.99'' N in Malakand Division, Pakistan (Fig. 1). Climatic data on atmospheric temperature and precipitation were collected from the Pakistan Meteorological Department. However, soil temperature was measured with a soil thermometer and light intensity was measured with a light sensor. There is significant variation in the elevations of the sampling sites, ranging from 1062 to 2590.8 m in the Dir district and from 778 to 2804 m in the Swat district. Mean growing season minimum temperature of various sites ranges from 4.0 to 9.8 °C in the Dir district, while in the Swat district, it ranges from 4.2 to 13.7 °C and maximum temperature of various sites ranges from 24.1 to 33.9 °C in the Dir district, while in the Swat district, it ranges from 23.2 to 33.7 °C.

Field sampling

A total of 44 mature walnut plants were labelled in 44 different locations in Dir and Swat in January 2018. Those walnut trees had occurred naturally in the hilly areas of both Districts. Locations' genotypes were named based on location and symbolized by an alphabetical abbreviation. These walnuts were mature, healthy and ready for harvest.

The mean annual presipitation(MAP) is from 1333 to 1870 mm in the Dir district, while in the Swat district it ranges from 1360 to 1660 mm. In District Dir, soil pH ranged from 6.10 ± 0.27 to 6.88 ± 0.25, whereas in Swat it ranged from 6.11 ± 0.15 to 6.90 ± 0.16. Light intensity measurements in Dir ranged from 97 ± 1.1 to 150 ± 1.6 Cd, while in Swat, values ranged from 85.56 ± 1.5 to 155.45 ± 2.2 Cd. The electrical conductivity (EC) of soil in Dir ranged from 0.20 ± 0.17 to 0.61 ± 0.18 Ms cm^{-1} and in Swat, it ranged between 0.22 ± 0.16 and 0.60 ± 0.18 mS/cm. Soil temperature in Dir ranged from 19 ± 0.42 to 28 ± 0.22 °C, while in Swat it ranged from 19 ± 0.35 to 27 ± 0.47 °C (Table 2).

Laboratory measurements

In the Plant Ecology Laboratory of the Botany Department, Abdul Wali Khan University, data on the following parameters were recorded using the standard protocol.

Foliar epidermal study

For anatomical investigation, fresh leaves were collected from two distinct populations and cleaned with distilled water. Narrow, clear sections were prepared using a scalpel and treated with chloral hydrate solution for 3–4 min to decolorize. The sections were then transferred to an alcoholic solution and stained with methyl orange components. At last, through the light microscope, various fragments were investigated and data were recorded (40, 41).

Determination of palisade ratio

The decolorized leaf sections were removed from the chloral hydrate solution, placed on a slide and examined under a microscope. The palisade cells, located beneath the epidermal layer, were observed and data on palisade parenchymatous cells were recorded. The palisade ratio was subsequently calculated.

Scanning electron microscopy

Nut samples from various sites in Swat and Dir districts were sectioned longitudinally using a microtome. Each segment was mounted on an aluminium stub with silver paste and placed in a stub holder under vacuum. The nut sections were then examined using a scanning electron microscope (SEM) at 25X magnification. This part of the experiment was done at the Centralised Resource Laboratory, University of Peshawar, Pakistan.

Statistical analysis

The data recorded were tabulated. The experimental work was organized in a randomized design and took three replications. For comparison, data were analyzed using mean values in SigmaPlot. Minitab Statistical Software (version 14.20) and the Statistical Package for the Social Sciences (SPSS Inc., United States, version 23) were used for data analysis. Pearson correlation coefficients were also used. Variance (ANOVA) analysis was used to investigate the effects of various climatic factors, mainly precipitation and temperature, on walnut morphological characters. After calculating the mean values of walnut characters, the correlations among various climatic factors were determined using the given formula.

$$r = \frac{\sum xy}{\sqrt{\sum x^2} \times \sqrt{\sum y^2}} \quad \text{(Eqn. 1)}$$

Where y = variable and r = Correlation coefficient

Results

Leaf anatomy

The shapes and sizes of epidermal cells, pith cells, vascular tissue and palisade cells were examined in leaf anatomy across various altitudes in both populations. A foliar epidermal study was conducted to determine the size and shape of epidermal cells at various altitudes in the two populations. Underneath the epidermal cell, there were palisade cells. Pith cells were composed of a collenchymatous type of cell that was spherical in shape and colourless. In this study, data on surface area, length and width of xylem, epidermal cells, phloem and pith cells were recorded. In general, leaf anatomical traits such as epidermal cell area, xylem cell surface area and phloem cell surface area were larger in Swat, whereas pith cell surface areas were larger in Dir (Table 3; Fig. 2 & 3).

At the districts' Dir, maximum surface area, length and width of pith, phloem and xylem were recorded at the high-altitude location Laram, while lower surface area, length and width were recorded at the low-altitude location Balo Rabat. Significant differences were present in all locations of the District Dir. Similarly, the high surface area, length and width of their epidermis were recorded at the high-altitude location Laram, whereas the low surface area, length and width were recorded at Balo Rabat, which

Table 2. Sampling site of Dir and Swat. Pakistan Meteorological Department.

Parameters	District Dir	District Swat
Sites	22	22
Elevation (m)	1062-2590.8	778-2804
Temperature (MAT) Min (°C)	4.0-9.8	4.2-13.7
Temperature (MAT) Max (°C)	24.1-33.9	23.2-33.7
Precipitation (MAP) (mm)	1333-1870	1360-1660
Soil pH	6.10 ± 0.27- 6.88 ± 0.25	6.11 ± 0.15-6.90 ± 0.16
Light intensity (Cd)	97 ± 1.1-150 ± 1.6	85.56 ± 1.5-155.45 ± 2.2
Soil EC (mS cm^{-1})	0.20 ± 0.17-0.61 ± 0.18	0.22 ± 0.16-0.60 ± 0.18
Soil temperature (°C)	19 ± 0.42-28 ± 0.22	19 ± 0.35-27 ± 0.47

Table 3. Walnut leaf anatomical traits

Parameters (μm)	District Dir	District Swat
Length of the epidermis cell	7.34 ± 0.43 to 8.67 ± 0.15	7.43 ± 0.12 to 9.74 ± 0.32
Width of an epidermis cell	7.24 ± 0.17 to 5.43 ± 0.34	5.41 ± 0.36 to 7.36 ± 0.27
Surface area of the epidermis cell surface areas	31.23 ± 0.34 to 54.16 ± 0.75	31.55 ± 0.44 to 56.12 ± 0.22
Palisade cell ratio	2.3 ± 0.54 to 4.6 ± 0.27*	2.2 ± 0.54 to 4.5 ± 0.32
Xylem cell length	2.35 ± 0.08 to 3.84 ± 0.34	2.42 ± 0.23 to 3.76 ± 0.41
Xylem cell width	2.31 ± 0.67 to 3.59 ± 0.43	2.53 ± 0.08 to 3.42 ± 0.26
Xylem cell surface areas	4.26 ± 0.65 to 10.82 ± 0.44	4.81 ± 0.11 to 10.09 ± 0.32
Phloem cell length	2.54 ± 0.98 to 4.63 ± 0.21	2.65 ± 0.56 to 4.19 ± 0.87
Phloem cell width	2.12 ± 0.64 to 4.22 ± 0.36	2.82 ± 0.17 to 4.34 ± 0.26
Phloem cell surface areas	4.23 ± 0.25 to 15.38 ± 0.78	5.91 ± 0.13 to 20.10 ± 0.11
Length of pith cell	3.54 ± 0.12 to 5.78 ± 0.3	3.07 ± 0.17 to 5.93 ± 0.29
Width of pith cell	3.27 ± 0.11 to 5.54 ± 0.41	3.34 ± 0.04 to 5.75 ± 0.29
Pith cell surface areas	9.08 ± 0.32 to 25.13 ± 0.46	8.04 ± 0.14 to 26.77 ± 0.22

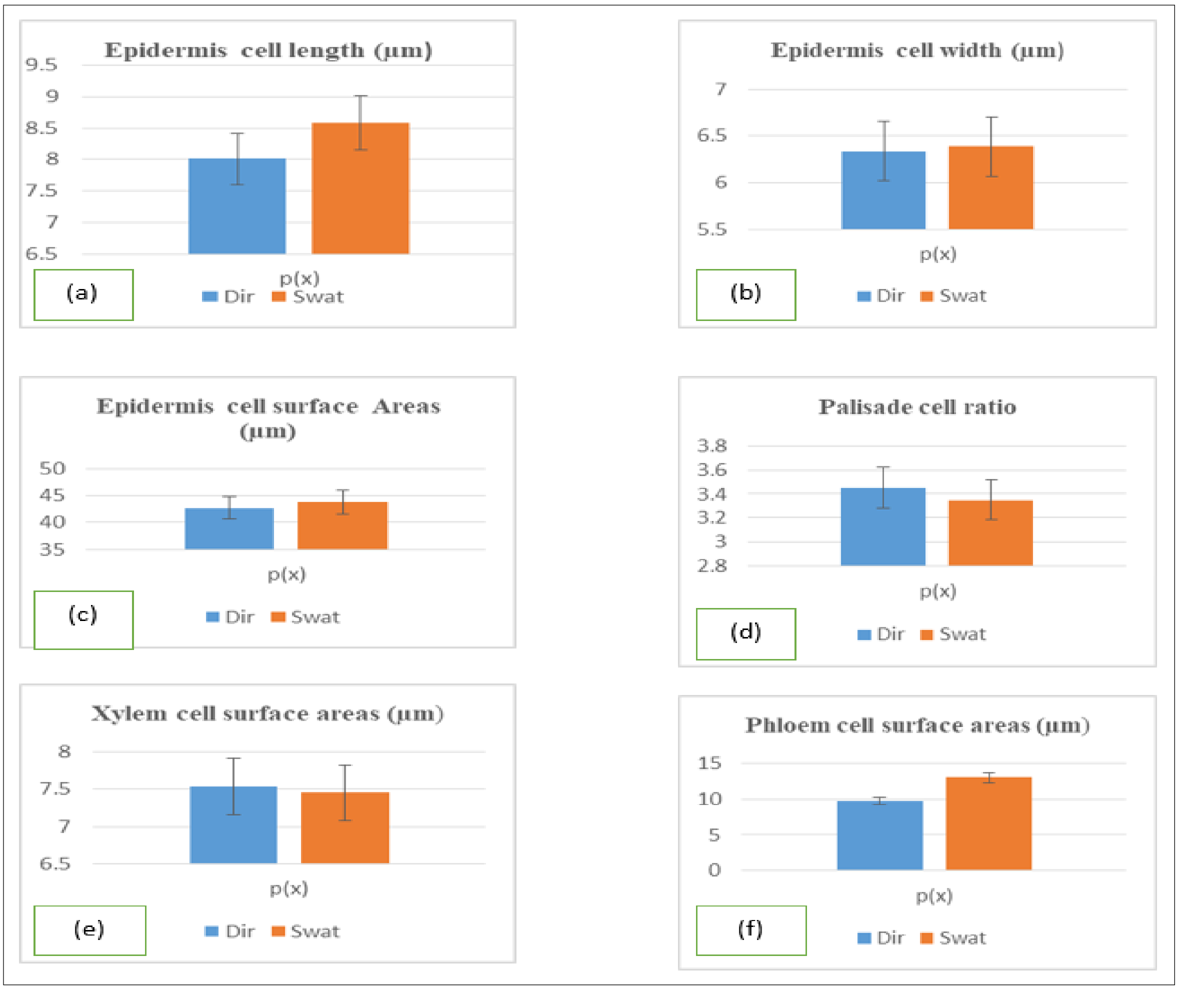

Fig. 2. Histogram of mean values of leaf anatomy traits on the Dir and Swat (a-f). (a) Shows epidermis cell length; (b) shows epidermis cell width; (c) shows epidermis cell surface area; (d) shows palisade cell ratio; (e) shows xylem cell surface area; (f) shows phloem cell surface area.

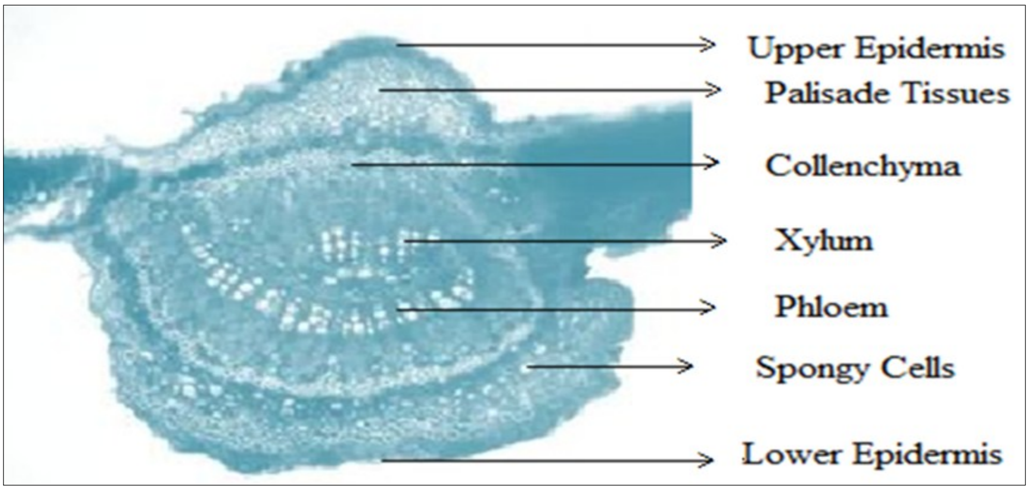

Fig. 3. A typical view of a walnut leaf shows various cells and tissues, including epidermal cells, palisade mesophyll, spongy mesophyll, xylem, and phloem. (Microscope Model: Olympus BX53 and Magnification: 400x).

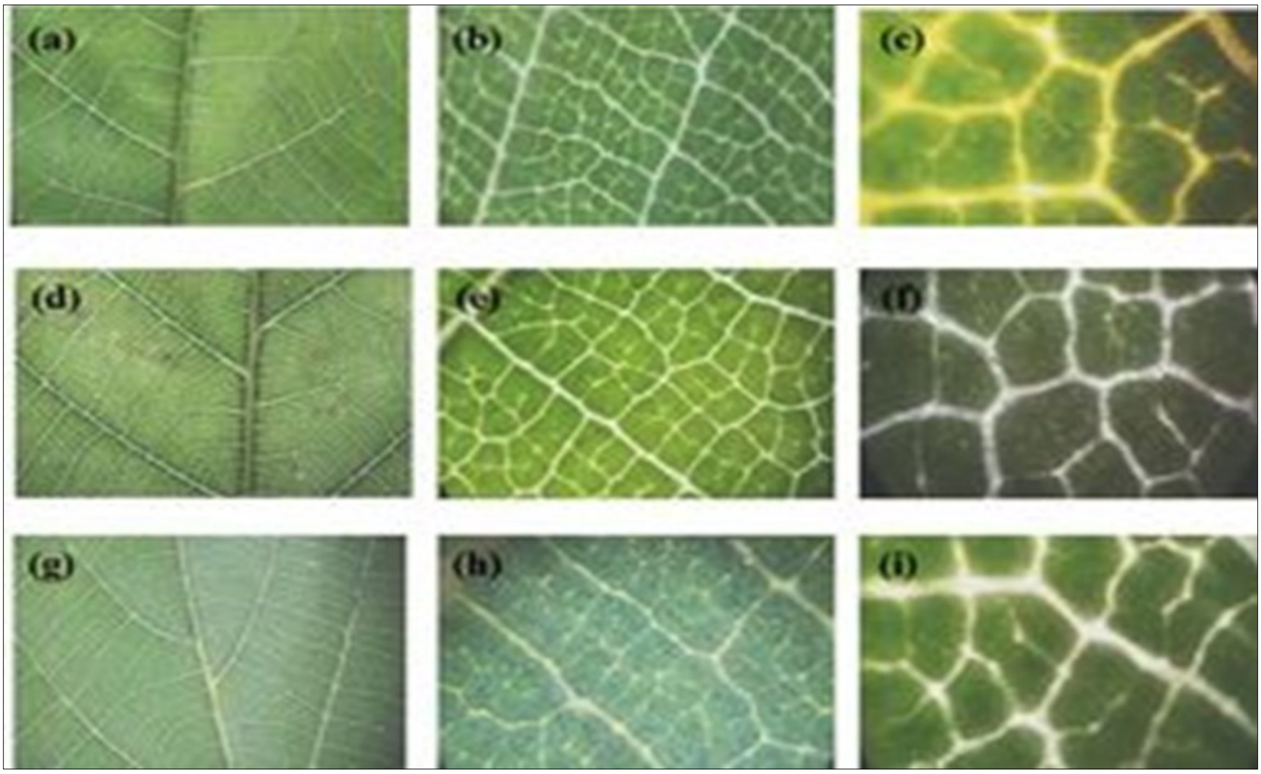

Fig. 4. Showing anatomy of leaves of three different walnut varieties ("a", "b" and "c") for soft walnuts leaf anatomy ("d", "e" and "f") for hard walnut leaf anatomy ("g", "h" and "I") for semi soft leaf anatomy located in districts Dir.

has a lower altitude. At different altitudes, the palisade cell ratio also differs (Fig. 4).

At district Swat, the maximum surface area, length and width of pith, phloem and xylem were recorded at the high altitudinal location Malam Jaba, while the minimum surface area, length and width were recorded in the low altitudinal location Rangila. Significant differences were present in all locations of the district Swat. Similarly, the high surface area, length and width of their epidermis were recorded at the high-altitude location Malam Jaba, whereas the low surface area, length and width were recorded at Rangila, which has a lower altitude. At different altitudes, the palisade cell ratio also differs (Fig. 5).

Anatomy of the nut

In nut anatomy, the sizes and shapes of the pericarp, pellicle and cotyledons were investigated using Vernier callipers, SEM and a simple scale. The pericarp has two parts: an exocarp, which has an outermost green husk surrounding the nut. Endocarp is present inside the shell, which is furrowed, wrinkled or rough and generally hard for the protection of cotyledons. Nuts usually contain two cotyledons, which are parted by a central thinner plate extending from the endocarp's inside layer. Cotyledons contain deep grooves and are folded. Thin skin adjacent to the cotyledons is a pellicle that helps in their safety. Dissected sections of cotyledons show two diverse sheets of the testa, which result from the development inside the integument of the ovule. The nut embryo is enclosed in its pellicle, where the present visible axis.

Pericarp of the nut

The pericarp of a nut has two main parts: the exocarp and the endocarp. Exocarp is the outermost green husk surrounding the whole nut, while endocarp is inner side to the shell which is wrinkled or furrowed, rough and generally hard for the protection of cotyledon. In general, nut anatomical traits both exocarp and endocarp thickness were larger in Swat as compared to the Dir (Table 4; Fig. 6–9).

Nut thin plate and cotyledons of the nut

Nut contains two cotyledons, which are parted by a centre thinner plate lengthening from the endocarp to the inside layer. Nuts' cotyledons have deep grooves and are folded. Dissected sections of cotyledons contain two dissimilar strata of testa, which result from the development inside the ovule integument. In general, the nut anatomical traits, both the nut thin plate and the cotyledons of the nut, were larger in Dir as compared to the Swat (Table 4; Fig.6–9).

Nut pellicle

The thin skin around the cotyledons was a pellicle that helped protect them. In general, the thickness of the pellicle, an anatomical trait of nuts, was larger in Swat than in Dir (Table 4, Fig. 6–9).

In both regions, Dir and Swat, the thickness of the nut endocarp and exocarp was lower at high altitudes than at lower altitudes, whereas nut cotyledon length and width were higher at higher altitudes. Similarly, in both regions, the nut plate length and width were more at high altitude regions as compared to the low

Table 4. Walnut nut anatomical traits

Parameters	Districts Dir	District Swat
Exocarp thickness (mm)	3.29 ± 0.31 to 4.26 ± 0.15	3.42 ± 0.34 to 4.25 ± 0.2
Thickness of endocarp (mm)	1.23 ± 0.42 to 1.55 ± 0.62	1.26 ± 0.54 to 1.58 ± 0.32
Width of Cotyledon (mm)	24.22 ± 0.32 to 34.21 ± 0.32	23.14 ± 0.14 to 30.54 ± 0.23
Length of cotyledon (mm)	29.10 ± 0.21 to 41.20 ± 0.15	28.43 ± 0.42 to 42.42 ± 0.51
Width of plate (mm)	24.12 ± 0.37 to 34.01 ± 0.52	23.17 ± 0.82 to 33.74
Length of plate (mm)	28.15 ± 0.21 to 40.33 ± 0.01	27.35 ± 0.02 to 41.22 ± 0.01
Thickness of plate (mm)	0.16 ± 0.16 to 0.32 ± 0.21	0.14 ± 0.45 to 0.33 ± 0.65
Thickness of pellicle (µm)	32.05 ± 0.75 to 48.54 ± 0.16	35.05 ± 0.32 to 53.05 ± 0.35

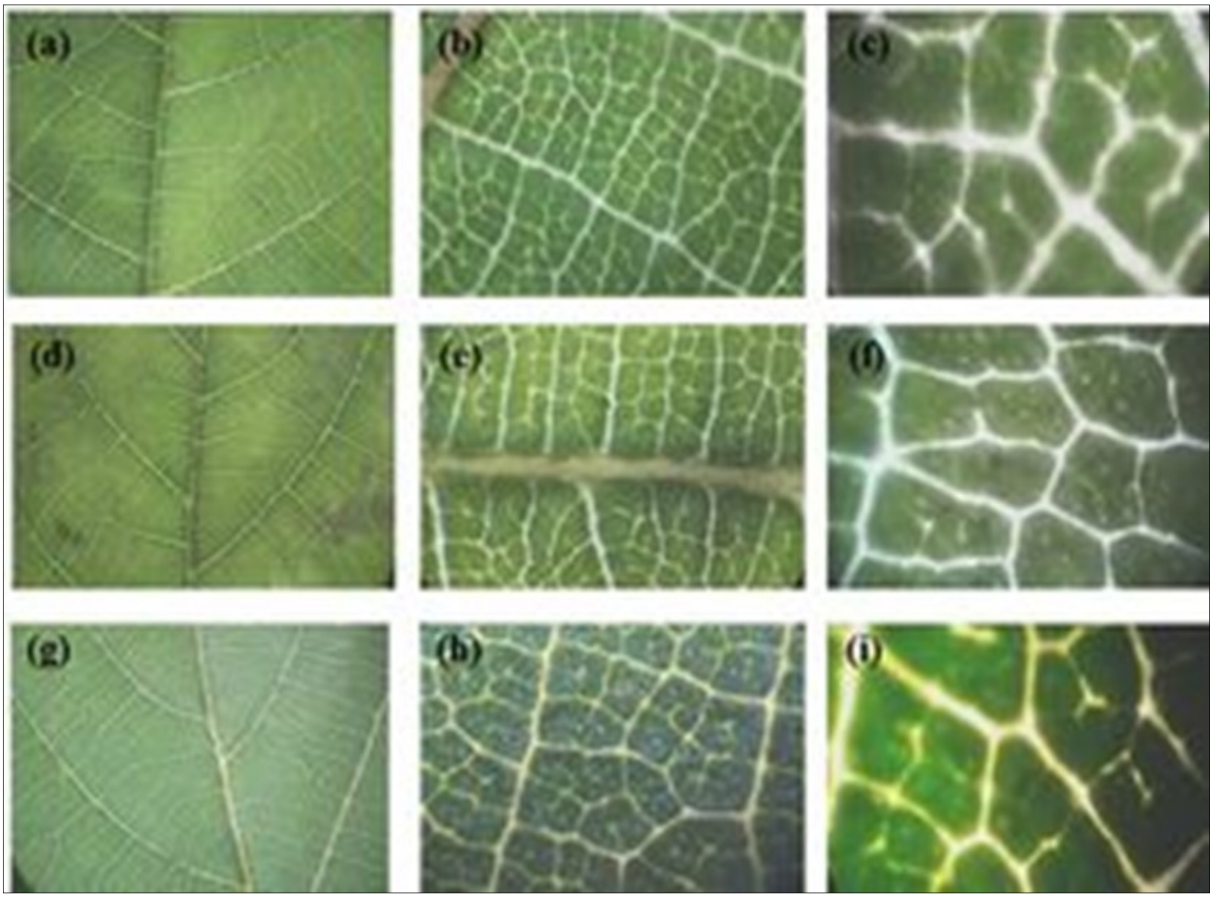

Fig. 5. Showing anatomy of leaves of three different walnut varieties (“a”, “b” and “c”) for soft walnuts leaf anatomy (“d”, “e” and “f”) for hard walnut leaf anatomy (“g”, “h” and “I”) for semi soft leaf anatomy located in district Swat.

Fig. 6. Histogram of mean values of walnut anatomical traits on two populations i.e. districts Dir and district Swat (a-f). (a) Shows exocarp thickness; (b) shows endocarp thickness; (c) shows cotyledon length; (d) shows plate length; (e) shows plate thickness; (f) shows pellicle thickness.

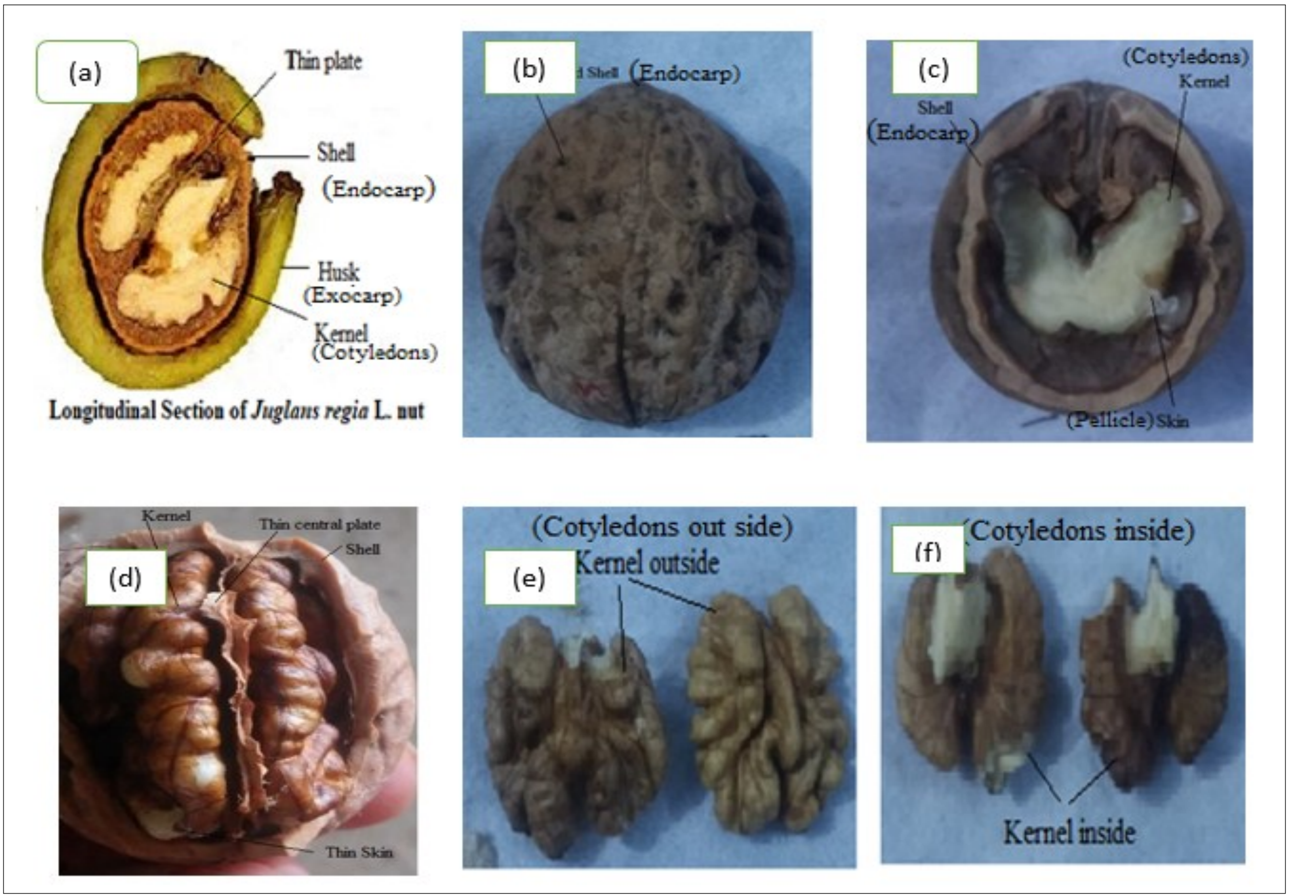

Fig. 7. Various anatomical sections of walnut nut: (a) Longitudinal view of the nut; (b) outer wrinkled shell; (c) internal view showing endocarp, pellicle, and cotyledons; (d) thin partition plate between the two cotyledons; (e) separated cotyledons outside the shell; (f) external view of the two cotyledons of a nut.

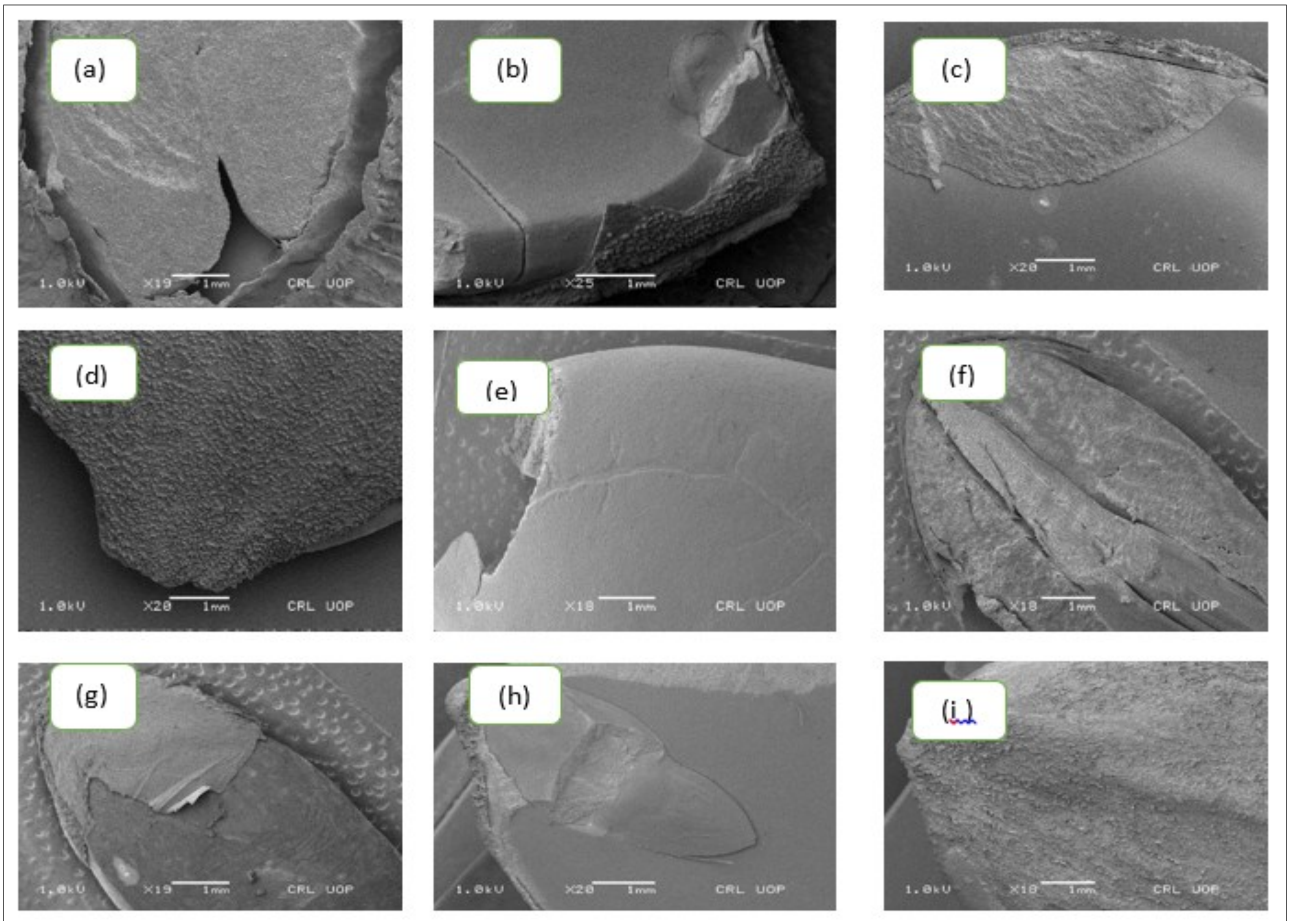

Fig. 8. Showing district Swat scanning electron micrograph of *Juglans regia* L. (a-i), (a) showing sunken collapse; (b and c) showing equatorial view and collapse surface; (d and i) showing close view of exine; (e and h) showing collapse surface; (f) showing equatorial view; (g) showing polar view of nuts.

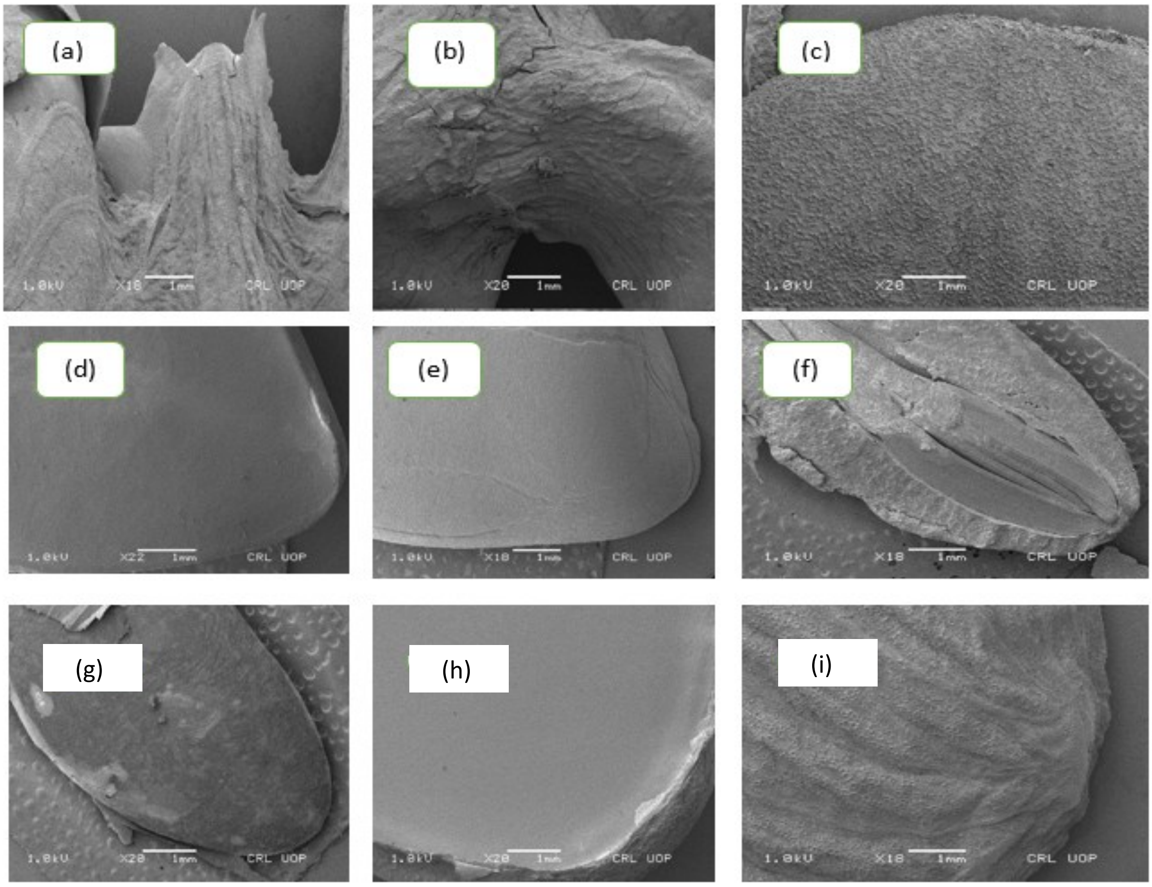

Fig. 9. Showing district Swat scanning electron micrograph of *Juglans regia* L. (a-i), (a and b) showing sunken collapse; (c and i) showing equatorial view and collapse surface; (d and e) showing close view of exine; (e and h) showing collapse surface; (f) showing equatorial view; (g) showing

altitude regions, while the pellicle thickness and plate thickness were less at high altitude sites. Significant differences exist in all locations (Table 4; Fig. 6–9).

Discussion

Effect of climatic drivers on anatomical traits of two populations of walnuts

Anatomy of the leaf

Environmental factors can significantly impact plant growth, altering leaf morphology, anatomy and physiological functions (42). Environmental conditions play a crucial role in shaping species distribution, favoring plants that adapt to local conditions while others face extinction. For instance, high-altitude plants often develop thicker epidermal cells and increased palisade tissue layers (43, 44). The Dir and Swat districts exhibit distinct environmental and geographical differences. Notably, Dir has a higher mean annual temperature, precipitation, soil EC and soil temperature, whereas Swat has higher soil pH, light intensity and altitude (Table 2).

Comparison of leaf anatomy between Dir and Swat revealed significant variations. Swat had larger epidermis cell width, length and surface area, as well as larger phloem and pith cell dimensions. In contrast, Dir had a larger palisade cell ratio, xylem cell dimensions and phloem cell length, reflecting adaptations to differing environmental conditions (Table 3). Our findings suggest anatomical features play a key role in adapting to diverse environments. Previous studies support this, showing that low temperatures impact young leaf cells, driving adaptations to specific conditions (45). Leaf growth, cell size and development are enhanced by warmer temperatures and an ample water supply (45). Low temperatures in Dir may contribute to larger palisade cell ratios, xylem cell dimensions and phloem cell lengths, potentially an adaptive response to the environment.

Walnuts' deep root systems enable efficient water transport and absorption, supporting adaptations that maintain optimal leaf anatomy, including epidermis and palisade tissues, to balance transpiration demands. Temperature showed a strong negative correlation with leaf anatomy parameters, whereas light intensity and altitude showed a strong positive correlation (Table 5). Precipitation showed a positive correlation with leaf anatomical characters. Soil EC had a mixed effect, negatively correlating with palisade cell ratio and epidermal cell size, but positively correlating with phloem cell area, pith cell size and xylem cell size. Soil temperature, however, showed a predominantly negative correlation with palisade cell ratio, epidermal cell area, phloem cell size, pith cell area and xylem cell size (Table 5). Thus, our results show similarities with (44).

Walnut leaves in Dir and Swat districts adapt to their environment, developing larger epidermis, pith cells and vascular bundles in areas with specific conditions: high light intensity, altitude, precipitation and soil EC, but lower soil temperature and annual max temperature, often in slightly acidic soils. Our results showed resemblances with previous studies, which examined that the palisade cell ratio and epidermal cells develop larger in higher altitude places (43, 44). It was also reported that leaf morphology correlates with precipitation and temperature (46). However, our

Table 5 Correlation between climatic factors and walnut leaf anatomical characters

Environmental factors	Districts	Coefficient of Correlation				
		Epidermal cell area (µm)	Palisade cell ratio (µm)	Xylem cell area (µm)	Phloem cell area (µm)	Pith cell area (µm)
Temperature	Dir	-.909**	-.912**	-.974**	-.887**	-.965**
	Swat	-.920**	-.963**	-.857**	-.928**	-.940**
Precipitation	Dir	.167	.177	.396	.348	.250
	Swat	.221	.111	.231	.334	.270
Altitude	Dir	.930**	.945**	.969**	.900**	.980**
	Swat	.855**	.890**	.894**	.951**	.891**
Soil pH	Dir	-.145	.056	.075	.101	-.080
	Swat	-.119	.051	.174	.036	-.008
Light intensity	Dir	.947**	.810	.847**	.804**	.945**
	Swat	.904**	.817	.863**	.849**	.967**
Soil Temperature	Dir	-.039	-.075	-.407*	-.457*	-.475*
	Swat	-.309	-.309	-.494*	-.426*	-.426*
Soil EC	Dir	-.052	-.082	.216	.128	.111
	Swat	-.071	-.026	.316	.126	.126

Correlation is significant at the 0.05 level (2-tailed), while ** Correlation is significant at the 0.01 level (2-tailed).

results are in contradiction to those of investigations that noted that epidermal cell size was smaller at high altitudes (47, 48). It was also found that latitude and temperature were the most important predictors of variation in leaf features, whereas precipitation had a smaller effect (49).

Nut Anatomy

Walnuts exhibit notable diversity in anatomical traits, particularly in nut features, reflecting adaptations to local environments. Studies highlight significant variations in walnut traits between the Dir and Swat regions, underscoring the wide-ranging diversity within these areas (50).

Shell thickness plays a crucial role in protecting the kernel from external environmental stresses, serving as a defense mechanism. In Dir, the maximum thickness of endocarp and exocarp was observed in a region of Balo Rabat, whereas the cotyledon maximum length and width were recorded in the Laram. Similarly, in district Swat, the maximum thickness of the endocarp and exocarp was observed in the Rangila region, while the maximum lengths and widths of the cotyledons were recorded in Malam Jaba (Table 4; Fig. 4–7). Our results were consistent with a previous study that reported similar endocarp thickness values (51). However, studies conducted in north-eastern regions (52), East Anatolia (53) and other parts of Turkey (54) recorded comparatively lower endocarp thickness values. Thus, it may have a different effect on the anatomy of different species.

In the Dir and Swat regions, walnut traits vary with altitude: low-altitude sites have thicker endocarp, exocarp and plates, but smaller cotyledons and pellicles, which are associated with low light, high precipitation and high soil temperature. Conversely, high-altitude sites have thinner shells but larger cotyledons and pellicles, which are linked to high light, high precipitation and low soil temperature. These results all showed resemblances to previous studies, which reported that seed traits vary across habitats and other climatic factors (55, 56). It was further observed from separate studies that wild walnut plants exhibit considerable variation in traits such as nut size (ranging from small to large), shell thickness (from thin to thick) and shape (from oval to round) (57, 58). Several investigators observed that the traits of the walnut are affected by water stress (59), temperature (60), rainfall, light (61) in various ages. In addition, it was reported that air and soil temperatures affect the seed exocarp (62).

Correlations among anatomical traits may reveal fundamental biological developments that are of considerable interest in walnut genetic diversity (63). In the Dir and Swat districts, altitude and temperature positively correlate with nut anatomical characters. Light intensity has mixed effects, negatively correlating with shell thicknesses but positively with cotyledon dimensions. Soil temperature mostly shows negative correlations, except for pellicle and plate thickness. Precipitation's effects are mixed, while soil EC positively correlates with shell and cotyledon dimensions but negatively with pellicle and plate thickness. Coefficient correlation between all environmental factors, i.e., precipitation, altitude, temperature, light intensity, soil pH, soil EC and soil temperature, to

Table 6. Correlation between climatic factors and walnut nuts' anatomical characters

Environmental factors	Districts	Coefficient of Correlation					
		Exocarp thickness (mm)	Endocarp thickness (mm)	Cotyledon length (mm)	Cotyledon width (mm)	Plate thickness (mm)	Pellicle thickness (µm)
Temperature	Dir	.957**	.968**	.891**	.828**	.983**	.976**
	Swat	.947**	.968**	.817**	.830**	.966**	.964**
Precipitation	Dir	.370	.274	.169	-.007	-.332	-.289
	Swat	.208	.126	.283	-.191	-.224	-.229
Altitude	Dir	.972**	.982**	.680**	.880**	.972**	.977**
	Swat	.847**	.894**	.640**	.841**	.913**	.902**
Soil pH	Dir	.080	.019	.113	.114	.094	.077
	Swat	.114	.017	.168	.059	.097	.044
Light intensity	Dir	-.963**	-.927**	.566**	.599**	-.918**	-.927**
	Swat	-.956**	-.988**	.556**	.550**	-.974**	-.964**
Soil Temperature	Dir	-.023	-.023	-.230*	-.345	.354	.101
	Swat	-.039	-.092	-.217*	-.242	.352	.121
Soil EC	Dir	.251	.051	.171	.130	-.013	-.073
	Swat	.250	.027	.229	.227	-.007	-.230

Correlation is significant at the 0.05 level (2-tailed), while ** Correlation is significant at the 0.01 level (2-tailed).

all characters of leaf anatomy, i.e. endocarp thickness, thickness of exocarp, width of cotyledon, cotyledon length, pellicle thickness and thickness of plate, was noted in Table 6. Separate studies also observed a positive correlation between altitude and seed characters across species and within species (64, 65). Based on the existing results, a wide range of genetic diversity has been described, revealing wide-ranging anatomical differences in walnut areas of the Khyber-Pakhtunkhwa, Pakistan region.

Conclusion

The study investigated the impact of climatic factors on *Juglans regia* L. (walnut) nuts and leaves anatomy in Dir and Swat districts. Results showed significant anatomical variations due to differences in altitude, temperature and light intensity. Swat had larger leaf epidermis, phloem and pith cells, while Dir had larger xylem cells and a palisade cell ratio. Nut exocarp and endocarp thickness were lower at high altitudes, while cotyledon length and width were greater. The study highlights the adaptive importance of anatomical features and the need for further investigation and conservation of local genetic resources in Pakistan's mountainous areas.

Acknowledgements

The authors are grateful to the local inhabitants of different communities of Barcharai Mountain, District Dir Lower, Pakistan, for helping in the identification of local names of the plant species.

Authors' contributions

MK designed the study conception and framework. IK supervised the study. HUR performed the experimental research. M & RZ interpreted the results. MR drafted the manuscript. RU encouraged the first author to conceive the presented idea. FU and MI performed data analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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