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STRUCTURAL AND FUNCTIONAL ATTRIBUTES OF *CITRUS RETICULATA* BLANCO UNDER DIVERSE SOIL AND ENVIRONMENTAL CONDITIONS

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Abstract

Citrus reticulata Blanco (Family Rutaceae) also known as mandarin orange is a small citrus tree. It acquired vital position in fruit industry worldwide, as well as in Pakistan. Citrus plants from six different habitats of Punjab were explored to investigate the structural and functional modifications in response to diverse soil and climatic conditions. *C. reticulata* revealed specific anatomical and physiological changes relating to the rhizosphere and environmental circumstances. Plants from low rain-fall habitats showed thicker stems, larger cortical cells and broader xylem vessels for maximum water conservation. Plants of slightly saline habitats exhibited enhanced accumulation of osmolytes and calcium, narrow vessels, larger pith cells and well-developed waxy layer to prevent water loss under physiological drought. Plants of Bhalwal revealed maximum biomass production and highest fruit yield due to favorable soil conditions like good saturation percentage, higher uptake of K^+ and Ca^{2+}. Some anatomical and physiological modifications such as greater accumulation of osmolytes, efficient photosynthetic attributes, thicker stem and wider vessels result in better growth in Bhalwal. Plants of investigated sites demonstrated moderate capability of structural and functional changes relating to the soil composition because fruit yield of slightly saline, poorly irrigated and low rainfall habitats declined to greater extent. Citrus plants require good soil composition and better environmental conditions, so higher fruit yield can be obtained by cultivating plants in well-irrigated and slightly acidic soil.

Key words: Saturation percentage, Osmolytes, Biomass, Cortical cells, Xylem vessels.

Introduction

Citrus possessed important position in fruit industry worldwide, as well as in Pakistan. Almost 199 thousand ha area of Pakistan is under the cultivation of citrus and 95% of total production is contributed by Punjab province (Anon., 2010). *Citrus reticulata* Blanco is the leading one of all the citrus cultivars in Pakistan, contributing up to 70% of total citrus production (Khan *et al*., 2010). Growth of citrus depends on environmental conditions and various soil physico-chemical characteristics (Iglesias *et al*., 2007). Almost all the ecological regions of Punjab dominated by arid and semi-arid environmental conditions (Hanif *et al*., 2010). Different soil attributes like pH, level of salinity, calcium and potassium affect the distribution of flora (Abbadi & El Sheikh, 2002).

All the growth phases of plants affected by varied soil chemistry that drives diverse plant species towards anatomical and physiological adaptations. Interaction of plants with adjoining environment results in various mechanisms and strategies for successful adaptation under diverse environmental conditions (Soukup *et al*., 2004). Cultivated plants possessed moderate potential to adapt to edaphic and climatic conditions leading to better growth (Naz *et al*., 2009). Newly colonizing plants develop anatomical modifications like thick epidermis, well-developed cortical and sclerenchyma cells (Wahid, 2003). Gas exchange ability enhanced by higher stomatal density and size under waterlogged conditions (Muhlenbock *et al*., 2007). Salt content in soil adversely affected the growth of plants either by cell damage or water uptake inhibition (Munns, 2002). Some plants gathered a higher amount of proline and other osmo-protectants to minimize the side effects of NaCl (Cha-Um & Kirdmanee, 2011). Homeostasis is an adaptive mechanism developed by many plants for selective uptake of ions, dumping off toxic ions and excretion of undesirable ions by leaf surface (Flowers & Colmer, 2008).

Family Rutaceae is the largest among the eudicot with 900 species and 150 genera. The genus *Citrus* is economically most important one in Rutaceae family (Huh *et al*., 2008). Citrus species vary from small shrubs to medium size tree and extensively distributed throughout tropical and temperate regions especially in Australia and Africa (Ladaniya, 2008). No work has done on this species regarding structural and functional adaptations to handle diverse soil and environmental conditions. Anatomical investigation in combination with physiological parameters conducted to report mechanisms of adaptation crucial for newly cultivated species. It is hypothesized that flexibility in anatomical and physiological traits allow this species to flourish successfully in diverse habitats.

Materials and Methods

Samples of evergreen tree *Citrus Reticulata* Blanco collected from different ecological regions of Punjab, Pakistan. Plant samples along with soil were collected from diverse regions of Punjab province, viz., Sillawanli, Kotmomin, Bhera, Bhalwal, Sargodha, Chak 40 NB, Old Botanical Garden UAF and New Botanical Garden UAF Faisalabad during the year 2015-2016. Soil physico-chemical characteristics of *Citrus Reticulata* Blanco rhizosphere are presented in Table 1.

Gas exchange parameters: Net transpiration rate (*E*), CO_2 assimilation rate (*A*), stomatal conductance (*gs*), sub-stomatal CO_2 concentration and water use efficiency (WUE) readings were taken by using LCA-4 ADC portable infrared gas analyzer (Analytical Development Company, Hoddesdon, England) on the largest leaf of each sample. Gas exchange parameters were measured at 9:00am to 11:00am; atmospheric pressure, 99.9 k Pa; molar flow of air per unit leaf area, 403.3 mmol $m^{-2}s^{-1}$; water vapor pressure of chamber ranged from 6.0 to 8.9 mbar, temperature of shoot ranged from 28.4 to 32.4°C, ambient CO_2 concentration 352 µmol mol^{-1}, ambient temperature from 22.4 to 27.9°C, PAR 1711 µmol m^{-2} s^{-1}.

Plant ionic contents: Plant ionic contents were determined by wet digestion following Wolf (1982) method. 0.1gram dried and grounded shoot material was taken in digestion flask and then 0.5ml concentrated H_2SO_4 was added to each flask followed by overnight incubation. After that flask digested on hotplate for at least 30 minutes following several additions of H_2O_2 until transparent digested material obtained. Ca^{2+}, K^+, Mg^{2+} contents in digested material were determined using flame photometer (Jenway, PFP-7).

Organic osmolytes: Fresh plant samples were kept in zipper bags, stored in icebox and brought back to laboratory for further analysis of total free amino acids, proline, soluble proteins and sugars. For the determination of total free amino acids, Moor and Stein (1948) method was followed. One mL of extract was taken in 20mL test tubes first. The next step was to add one mL of ninhydrin into test tube. Test tubes covered with aluminum foils and then heated for 20 minutes in boiling water. After that test tubes were placed in cold water to cool down and diluted with 5mL diluent and then incubated at room temperature for 15 minutes. Reading was recorded by spectrophotometer at 570nm.

For determination of proline content, fresh leaves were homogenized in aqueous sulfo-salicylic acid solution and then filtered (Bates *et al*., 1973). Filtrate was then mixed with ninhydrin solution, 6M orthophosphoric acid and glacial acetic acid. After that mixture was allowed to extract with toluene to determine proline and calculated with following formula:

-1 fresh weight = (lg proline mL-19 mL of toluene/115.5)/(g of sample)

µ mole proline g^{-1} F. W= µg proline mL^{-1} ×mL of tolulene/115.5g)/ (gram of sample): Bradford (1976) procedure was followed for the determination of total soluble protein by using the Bovine serum albumin as standard. Standard protocol used for the preparation of dye (G250 Commasie Brilliant blue). It was then purified and mixed with the enzyme extract (0.1µL). The reading was taken at 595nm by spectrophotometer. Anthron reagent was mixed with plant extract to determine total soluble sugars (Yemm and Willis, 1954). This solution was transferred to boiling water for ten minutes, then cool down in ice and then subjected to incubation at room temperature. Readings of soluble sugars were recorded at the wavelength of 625 nm on spectrophotometer.

Anatomical attributes: For anatomical studies, 2cm length segment of stem was selected from the base of 3rd internode. Fully developed leaf with midrib was selected for leaf anatomical studies. 2cm piece of petiole from median region was selected for petiole anatomy. Collected samples were first fixed in FAA for two days (48h), then transferred into alcohol for prolonged preservation (Ruzin, 1999). Free hand sectioning method used for the preparation of permanent slides followed by double staining dehydration technique, in which safranin and fast green dyes were used to study various tissues of stem, leaf and petiole. Compound microscope equipped with camera used for photographs and anatomical parameters measurement.

Statistical analysis

Samples collected for morpho-anatomical and physiological studies were taken in triplicate from each habitat. The data was subjected to one way analysis of variance using Microsoft Excel. Moreover, this data also subjected to multivariate analysis (PCA) and correlation matrix by using R statistical software (R Core Team, 2019) to assess relationship between studied parameters.

Results

Soil analysis: The soil of Kotmomin is most alkaline in nature followed by Sargodha's and soil of Bhalwal is slightly acidic (Table 1). Soil collected from Kotmomin showed the maximum electrical conductivity as compared to other habitats, while C-40 soil pressented the minimum values of EC. Soil of Bhalwal surpassed all other habitats regarding saturation percentage, while new botanical garden soil recorded with least values. The maximum and minimum values for Na^+ contents observed in Kotmomin and C-40 respectively. Soil exhibited varied response regarding K^+ contents with highest values recorded in C-40 and lowest in Kotmomin. Ca^{2+} content was reported the maximum in soil of Bhera and the minimum in C-40. The highest annual rainfall was recorded in Sargodha and lowest in Kotmomin.

Morphological characteristics: The maximum number of oranges were observed in plants of bhalwal, followed by sillanwali plants. Kotmomin showed least number of oranges per plant (Table 2).

Gas exchange parameters: *Citurs reticulata* showed similar behavior regarding different gas exchange parameters. The highest net CO_2 assimilation was recorded in plants of bhalwal and its minimum value noted in the plants of Old Botanical Garden (Table 2). Sargodha and New Botanical Garden exhibited similar values of CO_2 assimilation. Maximum and minimum transpiration rate was noticed in Old Botanical Garden and Bhalwal respectively. Plants of Bhalwal surpassed all other habitats regarding stomatal conductance, sub-stomatal CO_2 conc. and water use efficiency. The least values for stomatal conductance and water use efficiency seen in Old Botanical Garden. Sub-stomatal CO_2 conc. was recorded with minimum values in plants of Sargodha.

Table 1. Physical and chemical attributes of soil collected from *Citrus reticulata* Blanco habitats.

Habitat	SW	KM	BH	BW	SGD	C-40	OBG	NBG
pH	7.5	8.3	7.7	6.8	8.1	8.1	7.9	7.8
Electrical Conductivity (dS m^{-1})	2.2	4.2	3.8	2.25	2.46	1.33	2.3	2.2
Saturation Percentage	34.57	32.86	35.2	38.14	36.8	32.3	30.8	29.1
Sodium (mg g^{-1} d.wt.)	117.5	212.8	206	134.8	165.3	66.1	89.6	97.4
Potassium (mg g^{-1} d.wt.)	165.8	64.8	249.3	345.2	173.6	371.6	156.5	142.1
Calcium (mg g^{-1} d.wt.)	208.9	315.3	407.6	199.8	320.7	116.9	162.7	194.6
Annual rainfall (mm)	305	200	267	250	400	387	345	322
Elevation (m a.s.l.)	185	196	197	194	190	189	184	186
Coordinates	31.816157° N 72.538229° E	32.18452° N 73.02576° E	32.477007° N 72.913573° E	32.275141° N 72.904713° E	32.0740° N 72.6861° E	32.103975° N 72.728188° E	31.450366° N 73.134961° E	31.438517° N 73.049133° E

(SW: Sillanwali, KM: Kotmomin, BH: Bhera, BW: Bhalwal, SGD: Sargodha, C-40: Chak-40 NB, OBG: Old Botanical Garden, NBG: New Botanical Garden)

Table 2. Physiological Traits of *Citrus reticulata* Blanco from selected habitats of Punjab.

Morphological attributes	SW	KM	BH	BW	SGD	C-40	UAF-OBG	UAF-NBG	F-ratio
No. of oranges per plant	878ab	325e	812b	951a	648c	514d	744bc	723bc	59.77***
Gas exchange parameters									
CO_2 assimilation rate (µmol m^{-2} s^{-1})	12.8b	12.3c	10.7d	13.1a	8.7e	8.6ef	8.4f	8.7e	5.86**
Transpiration rate (mmol m^{-2} s^{-1})	1.8d	2.2b	2.2b	1.3e	2.3c	2.2b	2.5a	2.2b	5.99**
Stomatal conductance (mmol m^{-2} s^{-1})	145.8c	110.1e	109.8e	160.8a	107.8f	116.4d	107.7	154.4b	113.28***
Sub-stomatal CO_2 conc. (µmol mol^{-1})	191.7c	253.6b	190.7c	278.2a	126.3f	171.8cd	139.7e	165.5d	76.97***
Water use efficiency	4.9c	5.8b	5.9a	5.9a	4.7d	3.8f	2.9g	4.0e	4.78**
Shoot and root ionic content									
Shoot Sodium (mg g^{-1} d.wt.)	25.8f	33.9a	32.9b	24.7g	27.3d	26.2e	29.5cd	30.1c	12.97***
Root Sodium (mg g^{-1} d.wt.)	16.7c	18.1a	15.9e	15.8ef	15.4f	16.5c	17.6b	16.2cd	73.83***
Shoot Calcium (mg g^{-1} d.wt.)	20.8cd	18.8 de	23.4c	19.8 d	23.5c	23.7c	26.6a	24.5b	6.15**
Root Calcium (mg g^{-1} d.wt.)	6.4c	6.6c	5.8e	6.1d	7.6b	6.7c	6.6c	8.8a	3.52*
Shoot Potassium (mg g^{-1} d.wt.)	8.4b	4.1	6.4d	10.1a	7.6c	5.1e	6.8d	8.1b	5.35**
Root Potassium (mg g^{-1} d.wt.)	22.4bc	19.1d	22.8bc	17.4e	21.9c	23.4a	23.1b	21.9c	3.42*
Organic osmolytes									
Free amino acids (µg g^{-1} f. wt.)	148.1g	1488.7a	1440.7b	133.8h	785.4f	963.8d	1108.6c	836.3e	29.27***
Proline content (µmol g^{-1} f. wt.)	119.6g	191.6a	147.6d	109.6	139.8e	151.8c	161.8b	132.9f	173.86***
Soluble proteins (µg g^{-1} f. wt.)	874.3c	794.0f	680.8h	1023.8a	845.7d	832.8e	999.9b	802.7f	56.92***
Soluble sugars (mg g^{-1} d. wt.)	27.5b	22.8g	26.1c	28.7a	22.4g	25.2d	23.5f	24.1e	8.44***

Different letters exhibit level of significance between selected habitats

*, **, *** reveals significance at $p<0.05$, $p<0.01$, $p<0.001$ respectively

(SW: Sillanwali, KM: Kotmomin, BH: Bhera, BW: Bhalwal, SGD: Sargodha, C-40: Chak-40 NB, OBG: Old Botanical Garden, NBG: New Botanical Garden)

Ionic content: Shoot Na^+ showed significant variation among all collection sites of Citrus. The maximum and minimum values for shoot Na^+ wer observed in kotmomin and Bhalwal respectively (Table 2). Old and New Botanical Garden possessed almost similar value for shoot Na^+. The highest value for root Na^+ was recorded in plant samples collected from Kotmomin. Root Na^+ varied from 18.1 to 15.4 mg g^{-1} d.wt. in other plant samples. Shoot Ca^{2+} was the maximum in Old Botanical Garden while its minimum values noted in samples of Kotmomin. New Botanical Garden exhibited higher content of root Ca^{2+} with slight differences in other habitsts. Cardinal values for shoot K^+ observed in Bhalwal and Kotmomin. Root K^+ concentration was noticed the maximum in samples of C-40 with a slight decrease in Sillanwali and the minimum values in Kotmomin.

Organic osmolyte: Highest accumulation of amino acids and proline observed in samples of Kotmomin with a slight decrease in Bhakkar for free amino acids and a little reduction noted in Old Botanical Garden for proline (Table 2). Samples of Bhalwal exhibited least values for these two organic osmolytes. A significant variation seen among all habitats of *Citrus* regarding total soluble proteins. Cardinal limits for soluble proteins analyzed in Bhalwal and Bhera. Total soluble sugars were not observed much varied among selected collection sites. Bhalwal surpassed all the habitats regarding total soluble sugars with Kotmomin depicted the minimum values.

Stem anatomy: Stem anatomical attributes illustrated significant variation among samples of selected habitats. The thickest stem was observed in samples of Bhalwal followed by Old Botanical Garden and the thinnest one seen in Kotmomin (Table 3, Fig. 1). Cardinal limits for stem epidermal thickness were noticed in Kotmomin and Bhera. Samples of Sillanwali surpassed all the habitats regarding cortical and sclerenchymatous thickness. Bhera and New Botanical Garden exhibited quite similar thickness of cortex with minimum thickness noted in Sargodha.

Thickness of sclerenchyma decreased a little in sample collected from Bhalwal while the minimum of this character illustrated in samples of Sargodha. Old Botanical Garden possessed the largest vessels of xylem, with a slight reduction in size observed in Sillanwali samples. The smallest xylem vessels seen in Kotmomin. Pith cell area values were the maximum in samples of Old Botanical Garden, decreased in Bhalwal to some extent. All the habitats showed great variation regarding this character, with least values observed in Kotmomin.

Leaf anatomy: The thickest upper and lower epidermis observed in samples of Kotmomin exceeding all other habitats (Table 3, Fig. 2). Upper epidermis slightly decreased its thickness in Sillanwali, with thinnest epidermis noted in Bhera. The minimum thickness of lower epidermis was recorded in Old Botanical Garden. Sillanwali surpassed all the habitats of *Citrus* regarding midrib thickness, lamina thickness and cortical cell area. These parameters declined a little in samples collected from Bhalwal. The thinnest midrib and lamina observed in New Botanical Garden and Old Botanical Garden respectively. Large sized xylem vessels recorded from samples of Bhalwal. Sillanwali depicted a slight decrease in vessel size with smallest vessels recorded in Kotmomin.

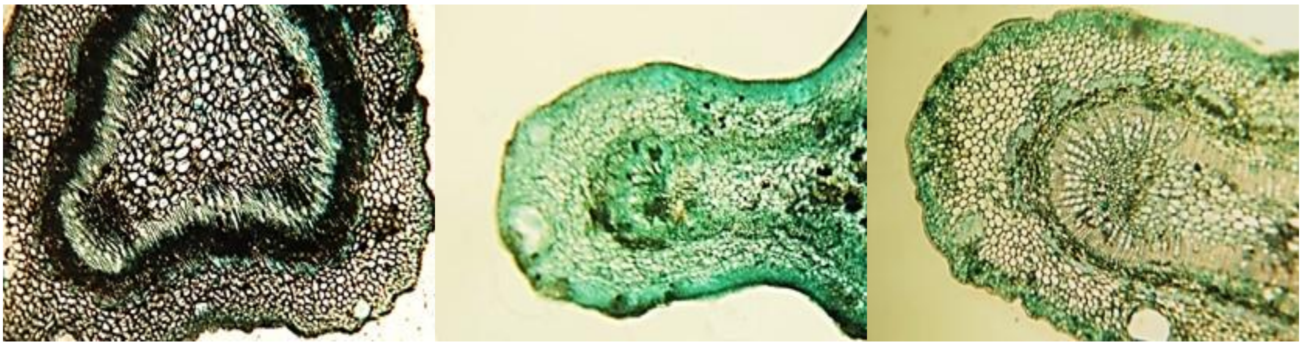
Table 3. Anatomical Traits of *Citrus Reticulata* Blanco from selected habitats of Punjab

Stem anatomical attributes	SW	KM	BH	BW	SGD	C-40	UAF-OBG	UAF-NBG	F-ratio
Stem thickness (µm)	873.1c	549.1e	774.8cd	1324.5a	753cd	615.9d	1046.7b	909.9bc	131.50***
Epidermal thickness (µm)	5.6d	8.3a	4.2de	6.4c	7.3b	4.8de	7.3b	6.4c	8.11**
Cortical thickness(µm)	106.5a	62.7d	98.4ab	83.5bc	57.2de	61.3d	81.1c	91.8b	119.72***
Sclerenchyma thickness (µm)	40.9a	28.2cd	24.4de	37.0b	23.7de	33.9bc	31.0c	25.3d	71.13***
Xylem vessel area (µm²)	172.6a	53.8f	115.6cd	125.3c	106.2de	155.7b	180.1a	92.8e	59.62***
Pith cell area(µm²)	402.5d	372.2de	488.5c	695.8ab	405.3d	550.7cd	748.2a	605.1b	172.56***
Leaf anatomical attributes									
Upper epidermal thickness (µm)	8.6b	9.9a	3.4f	6.3d	8.4b	7.6e	4.8e	6.7d	7.35**
Lower epidermal thickness (µm)	8.3b	9.0a	7.4c	8.3b	6.6de	7.4c	5.7e	7.0d	6.72**
Midrib thickness (µm)	395.4a	314.2	259.5	352.8	336.7	230.9	292.4	204.8	119.56***
Lamina thickness (µm)	165.6a	144.5bc	135.3c	151.9b	132.7c	141.2bc	106.7df	119.5d	75.56***
Cortical cell area (µm²)	115.7a	93.3c	47.4f	101.9b	66.0d	88.5cd	58.6de	54.8e	51.63***
Xylem vessel area (µm²)	182.2b	108.6	122.4f	195.9a	169.2c	116.0ef	146.3cd	137.7d	29.19***
Petiole anatomical attributes									
Petiole thickness (µm)	442.1c	398.5f	438.4cd	379.7	408.3e	421.9cd	485.8a	463.4b	141.98***
Epidermal thickness (µm)	8.2cd	10.6b	7.3d	7.3d	8.9c	12.2a	7.1de	9.2bc	8.28**
Cortical cell area (µm²)	118.4	131.6	139.7	112.3	174.8	149.2	191.3a	108.2	19.47***
Xylem vessel area (µm²)	54.8	69.6f	96.7ef	158.8a	118.3e	131.1d	143.7b	137.4c	29.86***

Different letters exhibit level of significance between selected habitats

*, **, *** reveals significance at $p<0.05$, $p<0.01$, $p<0.001$ respectively

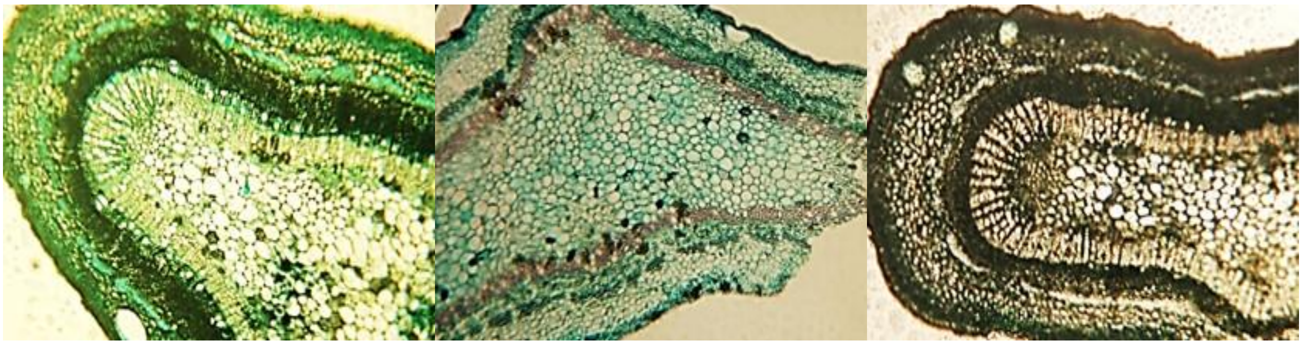
(SW: Sillanwali, KM: Kotmomin, BH: Bhera, BW: Bhalwal, SGD: Sargodha, C-40: Chak-40 NB, OBG: Old Botanical Garden, NBG: New Botanical Garden)



Sillanwali: Irregular stem, intense sclerification, compact cortical cells, greater cortical thickness

Kotmomin: Uneven stem thickness, irregular arrangement of vascular bundles, thinner cortex

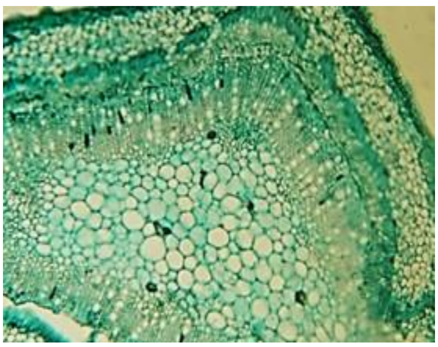
Bhera: Thicker cortex, regular sized pith cells, tightly packed chlorenchyma under epidermis, arranged medullary rays



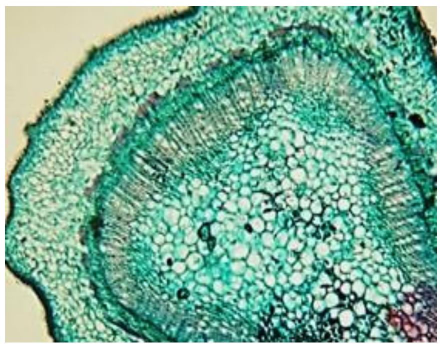
Bhalwal: 2 to 3 layers thick cortex, larger pith cell, regularly arranged vascular bundles, thicker sclerification

Sargodha: Tightly packed pith cells, irregular stem thickness, reduced vascular bundle cell, thin layer of sclerenchyma

Chak-40 NB: Smaller sized cortical cell, regularly arranged medullary rays, thick layer of sclerenchyma



Old Botanical Garden (UAF): Tightly packed and smaller size cortical cell, compactly arranged larger pith cell, vascular bundle in the form of clusters



New Botanical Garden (UAF): Greater cortical thickness, smaller vascular bundle, thin layer of sclerenchyma

Fig. 1. Stem anatomical attributes of *Citrus Reticulata* Blanco from selected habitats of Punjab.

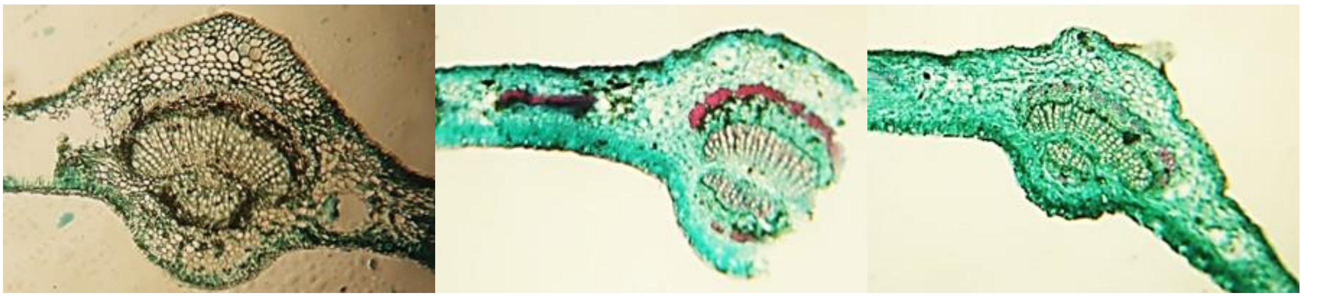
Petiole anatomy: Samples of Old Botanical Garden illustrated the maximum thickness of petiole. A slight reduction in petiolar thickness observed in New Botanical Garden, with least thickness noted in Bhalwal (Table 3, Fig. 3). *Citrus* plants collected from C-40 depicted maximum thickness of epidermis, while minimum thickness noticed in Old Botanical Garden. The largest cortical cells were recorded in samples collected from Old Botanical Garden, a little decrease in cortical cell size noted in Sargodha with smaller sized cells in New Botanical Garden. Cardinal limits for xylem vessel area observed in Bhalwal and Sillanwali.

Multivariate analysis: Principal component analysis (PCAs) demonstrated significant correlation as well as major variation between ionic contents and organic osmolytes of C. *reticulata* (Fig. 4a). Dim-1 and Dim-2 explained 41.9% and 20% (61.9%) respectively of total variations for studied traits in PCAs. The contribution of variables to Dim-1 was largely determined by TSS, TSP, $R.Na^+$ and Pro, whereas contribution of Dim-2 was related to $R.K^+$, $S.K^+$, $R.Na^+$ and TSP. The principal component analysis of gas exchange parameters, ionic contents and organic osmolytes showed 28.4% and 25.4% (53.8%) variability among the traits examined (Fig. 4b). The major contributors to Dim-1 were TSS, TSP, Pro, R.Na and S.Na. whereas Dim-2 consisted of Ci, WUE, and TAA. Apart from this, $S.K^+$ showed slight contribution to Dim-2.

Sillanwali: Thick midrib, larger cortical cells, larger metaxylem, thick layer of sclerenchyma

Kotmomin: Increased wax deposition, thicker midrib, compactly arranged cortex

Bhera: Reduced lamina thickness, irregular arrangement of cortical cell, well arranged vascular region



Bhalwal: Larger epidermal cells, greater midrib thickness, Increased cortical cell area, intense sclerification

Sargodha: Enlarged midrib, tightly packed smaller cortical cell, regularly arranged sclerenchyma

Chak-40 NB: Thinner waxy layer, irregular arrangement of cortical cells, sclerenchyma with reduced lignification

Old Botanical Garden (UAF): Thick bulgy midrib, thin lamina thickness, smaller sized epidermal cells

New Botanical Garden (UAF): Reduced midrib thickness, thin lamina thickness, smaller epidermal and cortical cells

Fig. 2. Leaf anatomical attributes of *Citrus reticulata* Blanco from selected habitats of Punjab.

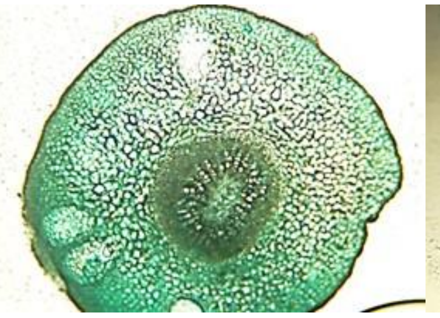
Principle component analysis of ionic content and stem anatomical traits exhibited 29.8% and 22.5% (52.3%) variations among studied traits (Fig. 4c). The major components of Dim-1 were S.PCA, STK, S.XYA, S.Ca^{2+} and R.Na, while major contributor to Dim-2 comprised of S.K^{+}, R.Ca^{2+}, R.Na^{+} and S.CRT. The PCA biplot for ionic contents and petiole anatomical characteristics explained 32.7% and 22.8% (55.5%) variability (Fig. 4d). The Dim-1 consisted of S.Ca^{2+}, R.Na^{+}, P.XYA and P.PTK and Dim-2 consisted of R.K^{+}, S.K^{+}, P.XYA and R.Na^{+}. PCA between ionic contents and leaf anatomical characteristics (Fig. 4e) indicates higher variation; 41.3% and 23.1% (64.4%). The Dim-1 includes L.LTK, L.CCA, L.ELTK and S.Ca^{2+}, however, major components of Dim-2 were R.Na^{+}, S.Na^{+} and L.XYA.

Correlation matrix: Correlation studies suggested both positive and negative correlation between different anatomical and physiological attributes of C. *reticulata*. L.CCA, L.LETK, TSP, P.ETK, R.Ca, E, PCCA, WUE, Pro possessed significant positive correlation while P.XYA, P.PTK, S.Ca, S.PCA, Ci and S.XYA were negatively correlated (Fig. 5).

Discussion

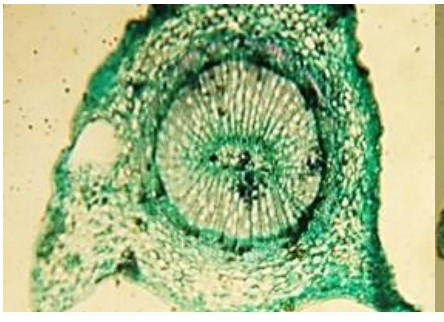
Plants structural and functional response to environmental heterogeneity is very crucial for the survival under abiotic stress (Paula *et al*., 2016). Anatomical features are the most responsive to environmental fluctuations and therefore, develop resistance to biotic and abiotic stresses by acquiring modification with the passage of time (Naskar & Palit, 2015). Leaves and stem are the most vulnerable organs to aerial pressures, so any kind of modification in these organs correlate with environmental changes (Micco & Arrone, 2012). Samples collected from different habitats exhibited variety of alterations in anatomy as well as physiology correlating with soil and climatic condition, most probably due to fixation of adaptive feature during evolutionary process (Badr *et al*., 2020).

Sillanwali: Thin waxy layer, compact cortex and pith cells, moderately sclerified

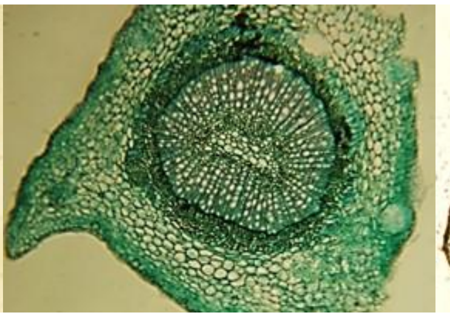


Kotmomin: Well-developed waxy layer, reduced vascular region, intensely sclerified

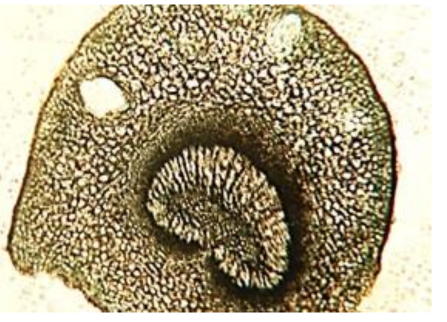
Bhera: Regularly arranged vascular bundle in clusters, thicker epidermis



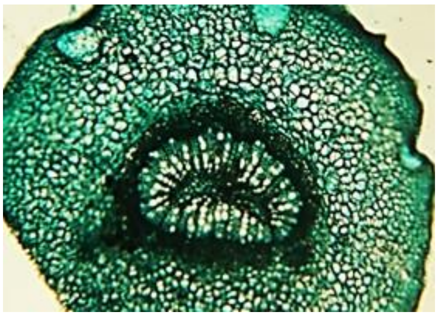
Bhalwal: Decreased petiole thickness, less developed cortical cells, thin layer of sclerified tissues



Sargodha: Well developed cortical cells, broad vascular region, smaller pith cell



Chak-40 NB: Very thin waxy layer, smaller cortical cell, thick layer of sclerenchyma



Old Botanical Garden (UAF): Thick waxy layer, larger cortical cells, intensely sclerified

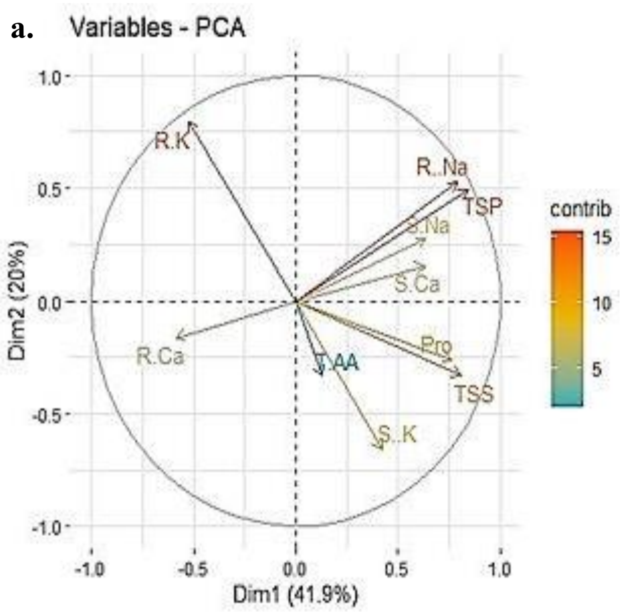
New Botanical Garden (UAF): Increased cortical thickness, narrow vascular region, moderately sclerified

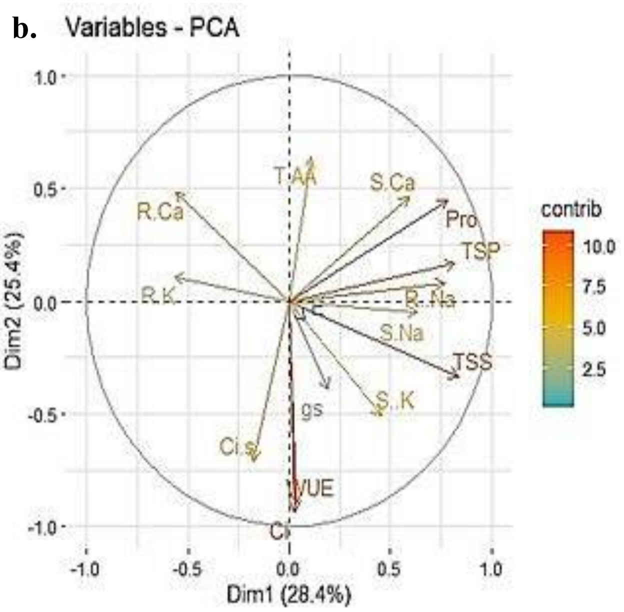
Fig. 3. Petiole anatomical attributes of *Citrus Reticulata* Blanco from selected habitats of Punjab.

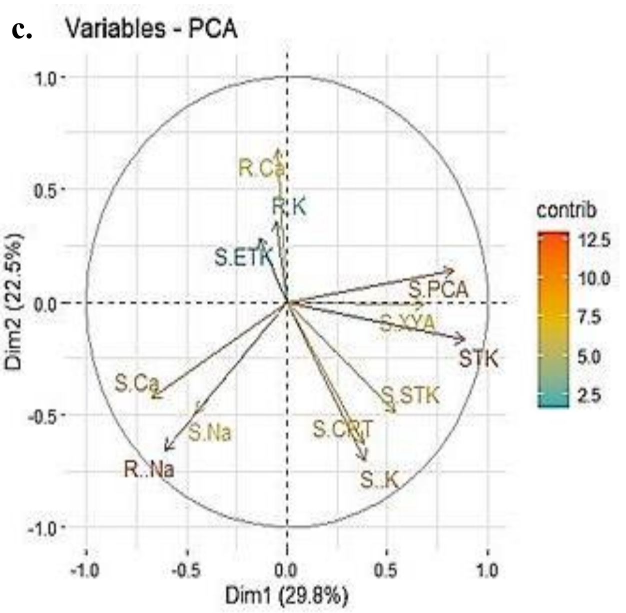
Plants of Bhalwal illustrated maximum fruit yield among all the study sites of Citrus. This habitat didn't get much annual rainfall, but soil is analyzed with good saturation percentage, that is contributing a lot towards better growth (Grigore & Toma, 2007). Some photosynthetic parameters such as CO_2 assimilation rate, WUE, sub-stomatal CO_2 conc. and stomatal conductance also reported maximum in this habitat associated with the higher fruit yield even under environmental stresses (Naz *et al*., 2010). Citrus plants require good soil conditions and slightly acidic pH for their growth. Bhalwal exhibited slightly acidic soil which assumed to be best for oranges production as reported earlier by Liu *et al*., (2015). Transpiration rate was reported the maximum in plants of Old Botanical Garden which lowers the CO_2 assimilation rate and water use efficiency, similar findings were reported by Omamt *et al.*, (2006).

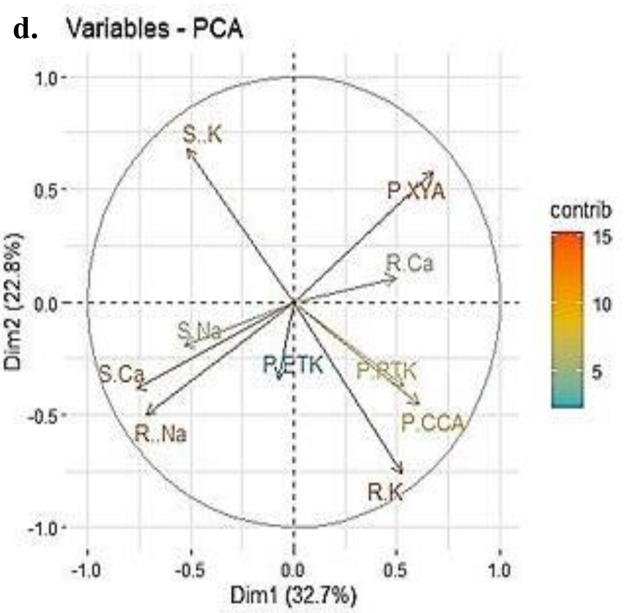
The soil of Kotmomin accumulates higher concentration of salts such as NaCl with greater EC values as compared to other habitats. This habitat receives very low annual rainfall. Fruit production mainly depends upon soil structure, composition, saturation percentage and annual rainfall. The higher concentration of shoot and root Na^+ is negatively impacting the fruit yield, results in minimum fruit production in Kotmomin (Shrivastava & Kumar, 2015). Greater accumulation of Ca^{2+} in plants of Old and New Botanical Gardens contributes to the higher fruit and biomass production because the toxic effects of Na^+ can be mitigated by high Ca^{2+} uptake, same findings has been published by Cabot *et al*., (2009) and Jiang *et al*., (2013). Greater Ca^{2+} concentration has also been associated with better growth as demonstrated earlier by Hameed *et al*., (2013).

Osmo-protectants allow plants to bear a variety of unfavorable environmental influences, such as saltiness and desiccation (Jagesh *et al*., 2010; Ranganayakulu *et al*., 2013). Plants of Kotmomin accumulate high concentration of osmolyte like total free amino acid and proline which indicated its tolerance against increased level of salt (Gupta & Huang, 2014). Total soluble proteins and sugar concentration was reported high in plants of Bhalwal probably to cope with seasonal drought conditions (Gupta & Huang, 2014).









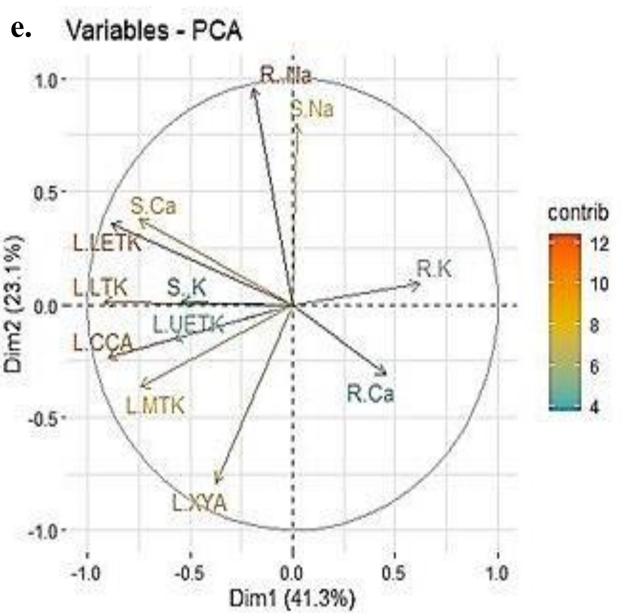


Fig. 4. PCA biplot analysis a) Ionic contents and Organic osmolytes b) Ionic contents, Gas exchange parameters and Organic osmolytes c) Ionic contents and Stem anatomical traits d) Ionic contents and Petiole anatomical traits e) Ionic contents and Leaf anatomical traits (S.Ca: Shoot Ca^{2+}, R.Ca: Root Ca^{2+}, R.K: Root K^{+}, S.K: Shoot K^{+}, S.Na: Shoot Na^{+}, R.Na: Root Na^{+}, TAA: Free amino acids, TSP: Soluble proteins, TSS: Soluble sugars, Pro: proline, gs: stomatal conductance, Ci: sub-stomatal CO_2 concentration, WUE: water use efficiency, STK: stem thickness, S.STK: stem sclerenchyma thickness, S.CRT: stem cortical cell area, S.PCA: stem pith cell area, S.ETK: stem epidermis thickness, S-XYA: stem xylem vessels area, P.CCA: petiole cortical cell area, P.XYA: petiole xylem vessels area, P.PTK: petiole pith thickness, L.MTK: leaf midrib thickness, L.LTK: leaf lamina thickness, L.UETK: leaf upper epidermis thickness, L.LETK: leaf lower epidermis thickness, L.CCA: leaf cortical cell area, L.XYA: leaf xylem vessels area).

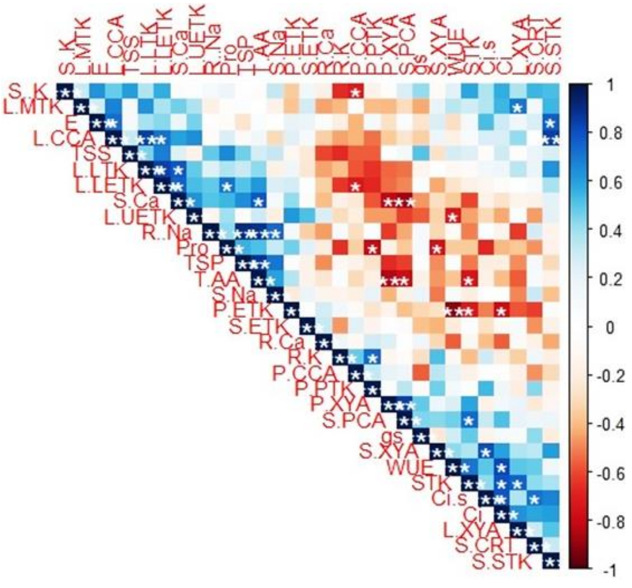


Fig. 5. Correlation between physiological and anatomical characteristics of *Citrus Reticulata* Blanco collected from different habitats (S.Ca: Shoot Ca^{2+}, R.Ca: Root Ca^{2+}, R.K: Root K^+, S.K: Shoot K^+, S.Na: Shoot Na^+, R.Na: Root Na^+, TAA: Free amino acids, TSP: Soluble proteins, TSS: Soluble sugars, Pro: proline, gs: stomatal conductance, Ci: sub-stomatal CO_2 concentration, WUE: water use efficiency, STK: stem thickness, S.STK: stem sclerenchyma thickness, S.CRT: stem cortical cell area, S.PCA: stem pith cell area, S.ETK: stem epidermis thickness, S-XYA: stem xylem vessels area, P.CCA: petiole cortical cell area, P.XYA: petiole xylem vessels area, P.PTK: petiole pith thickness, L.MTK: leaf midrib thickness, L.LTK: leaf lamina thickness, L.UETK: leaf upper epidermis thickness, L.LETK: leaf lower epidermis thickness, L.CCA: leaf cortical cell area, L.XYA: leaf xylem vessels area).

Structural response of comparatively less-tolerant species is more precise for desiccation resistance. Bhalwal exhibited thickest stem that indicated its better growth most probably due to efficient photosynthetic activity and accumulation of some osmo-protectants (Martins & Scatena, 2013). Stem epidermis is outermost covering, which helps the plant to protect from environmental hazards. Well-developed epidermis protects the plants from drought conditions, which is an effective technique in times of water shortages, therefore plants of Kotmomin survive successfully under physiological drought (Akram *et al*., 2002). Sillanwali plants possess larger cortical cells to hold more water which contributed largely to water storage in parenchyma (Farooq *et al*., 2009). Xylem vessels are the dominant water conducting tissues in stem and roots (Corrêa *et al*., 2016), and their size is proportional to the efficiency by which water and nutrients transported (Smith *et al*., 2013). Narrow xylem vessels in Old Botanical Garden are highly valuable to prevent radial water leakage in case of seasonal water shortage (Zhaosen *et al*., 2014). Plants need a significant portion of storage parenchyma to ensure survival in exceptionally severe arid climates (Farooq *et al*., 2009), so plants of Old Botanical Garden possessed larger pith cells.

Larger leaf thickness increased water holding capacity in plants (Naz *et al*., 2016). The thickest midrib and lamina recorded in Sillanwali plants indicates its role to store water under temporary drought conditions may be due to shortage in rainfall Rayner *et al*., 2016). Increased cortical cell area is directly related to enhanced leaf succulence in Sillanwali that is indication to salt tolerance under physiological drought (Han *et al*., 2013). Results demonstrate that greater petiolar thickness in Old Botanical Garden provide support to leaves that help them to expose to sunlight for higher photosynthetic rate (Theerawitaya *et al*., 2015). Thicker epidermis of petiole in C-40 is an adaptation to water shortage by preventing water loss (Adedeji & Jewoola, 2008).

Conclusion

Citrus reticulata Blanco is the leading one among all the citrus cultivars in Pakistan. Present study revealed that citrus develop variety of structural and functional modifications under diverse soil and environmental conditions. Plants were selected from totally diverse areas to evaluate the level of anatomical and physiological adaptation, that ultimately boosts fruit yield, which is essential for world's population increasing. This species has low to moderate potential to adapt to structural and functional soil condition changes. Cultivation of citrus plants in well-irrigated and slightly acidic soil can contribute to enhanced production of fruit to fulfil the requirement of rapidly growing population.

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