Fate of rubber bush (Calotropis procera (Aiton) W. T. Aiton) in adversary environment modulated by microstructural and functional attributes (Postprint)

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Abstract

Calotropis procera (Aiton) W. T. Aiton (Apocynaceae) is a C_3 evergreen species widely distributed in arid and semi-arid regions of Punjab Province, Pakistan. It colonizes diverse habitats including salt-affected and waterlogged areas, deserts/semi-deserts, roadsides, wastelands, graveyards, forests, crop fields, coastlines, and river/canal banks. We sampled 12 populations from different ecological regions to evaluate their growth, physio-biochemical, and anatomical responses to specific environmental conditions. Desert/semi-desert populations exhibited vigorous growth (greater plant height, shoot length, and leaf number), enhanced photosynthetic capacity (higher chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll), and pronounced anatomical modifications including increased stem radius, cuticle thickness, storage parenchyma tissues (cortex and pith), and vascular bundles in stems, along with maximal midrib and lamina thickness, epidermal cell size, cuticle thickness, cortical proportion, abaxial stomatal density, and stomatal area in leaves. These populations displayed high plasticity in structural and functional features that enable survival and tolerance in hot, dry desert environments. Saline-area populations exhibited critical modifications for salt tolerance, including maximal accumulation of organic osmolytes (glycine betaine and proline) and antioxidants (superoxide dismutase [SOD], catalase [CAT], and peroxidase [POD]) at the physiological level, and intensive sclerification, large phloem regions (inner and outer), expanded pith parenchyma cells, and enlarged metaxylem vessels in stems and leaves at the anatomical level. Dry mountain populations showed distinctive features including increased shoot ionic contents (K^+ and Ca^{2+}), thickened collenchyma and sclerenchyma in stems, enlarged trichomes, and numerous small stomata on abaxial leaf surfaces. No single definitive trait

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Full Text

Preamble

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Fate of rubber bush (*Calotropis procera* (Aiton) W. T. Aiton) in adversary environment modulated by microstructural and functional attributes

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Abstract: *Calotropis procera* (Aiton) W. T. Aiton (Apocynaceae) is a C_3 evergreen species widely distributed in arid and semi-arid regions of Punjab Province, Pakistan. It colonizes diverse habitats including salt-affected and waterlogged areas, deserts/semi-deserts, roadsides, wastelands, graveyards, forests, crop fields, coastlines, and river/canal banks. We sampled 12 populations from different ecological regions to evaluate their growth, physio-biochemical, and anatomical responses to specific environmental conditions. Desert/semi-desert populations exhibited vigorous growth (greater plant height, shoot length, and leaf number), enhanced photosynthetic capacity (higher chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll), and pronounced anatomical modifications including increased stem radius, cuticle thickness, storage parenchyma tissues (cortex and pith), and vascular bundles in stems, along with maximal midrib and lamina thickness, epidermal cell size, cuticle thickness, cortical proportion, abaxial stomatal density, and stomatal area in leaves. These populations displayed high plasticity in structural and functional features that enable survival and tolerance in hot, dry desert environments. Saline-area populations exhibited critical modifications for salt tolerance, including maximal accumulation of organic osmolytes (glycine betaine and proline) and antioxidants (superoxide dismutase [SOD], catalase [CAT], and peroxidase [POD]) at the physiological level, and intensive sclerification, large phloem regions (inner and outer), expanded pith parenchyma cells, and enlarged metaxylem vessels in stems and leaves at the anatomical level. Dry mountain populations showed distinctive features including increased shoot ionic contents (K^+ and Ca^{2+}), thickened collenchyma and sclerenchyma in stems, enlarged trichomes, and numerous small stomata on abaxial leaf surfaces. No single definitive trait could serve as a universal indicator of biomass production across these rubber bush populations.

Keywords: *Calotropis procera*; Apocynaceae; phenotypic plasticity; aridity; rubber bush

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1 Introduction

Calotropis procera is a drought-resistant and salt-tolerant xerophytic C_3 plant that thrives in arid and semi-arid regions. It colonizes diverse habitats including high-salinity areas, waterlogged sites, deserts/semi-deserts, river/canal banks, coastlines, forests, railway tracks, wastelands, open barren fields, and roadsides. The plant maintains its evergreen status from young vegetative to mature reproductive stages under open-field conditions (Boutraa, 2010; Menge et al., 2016). In Punjab Province, Pakistan, it shows wide geographic distribution and invades multiple habitats, yet the mechanisms underlying its successful distribution across heterogeneous environments remain unexplored. This study addresses this knowledge gap through comprehensive investigation of population-specific responses to environmental conditions.

Rising ambient CO_2 concentrations and planetary temperature fluctuations are driving global climate change with significant alterations in seasonal precipitation patterns, ultimately increasing aridity in semi-arid regions worldwide (Koutroulis, 2019). High temperature and dry environment are primary factors influencing plant community success and rehabilitation. Understanding survival mechanisms of these communities could improve protection of natural vegetation in these areas (Peñuelas et al., 2018). Water availability represents the major limiting factor for photosynthesis, growth, and productivity in most terrestrial environments, even for plants that thrive under arid conditions, and strongly influences population dynamics (Duan et al., 2018).

Abiotic stresses (drought and salinity) impair plant growth through multiple mechanisms including physiological drought from restricted water supply under osmotic stress, disruption of cellular functions, and impairment of physiological processes like photosynthesis and respiration through ionic imbalance or toxicity (Muhammad et al., 2021). These stresses ultimately cause reactive oxygen species (ROS) accumulation at metabolically active sites, which may damage biological structures and processes (Sachdev et al., 2021). Superoxide radicals, hydrogen peroxide, singlet oxygen, and hydroxyl radicals generated in cells perturb biochemical events by disrupting membranous organelles and metabolites (Ahanger et al., 2017).

To counteract such oxidants, plants produce enzymatic and non-enzymatic antioxidants. The most vital enzymatic antioxidants are peroxidase (POX), superoxide dismutase (SOD), and catalase (CAT), while non-enzymatic antioxidants include carotenoids, polyphenols, glutathione, ascorbate, tocopherols, and anthocyanins that scavenge most ROS molecules (Batool et al., 2013).

Key adaptations of arid plant communities include seasonal leaf shedding, small thick leaves, surface pubescence, organ succulence, thickened epidermis and cuticle, lignification of mechanical tissues, and small, sparse stomata oriented in crypts or on abaxial leaf surfaces (Abd Elhalim et al., 2016), extensive root systems, and greater water-use efficiency. These features specifically minimize transpirational loss, primarily through leaf surfaces, representing a crucial water conservation strategy. Reduced vessel diameter is another key feature that may affect water conduction but minimizes the risk of embolism or cavitation (Kaleem and Hameed, 2021).

C. procera (Apocynaceae), commonly known as giant milkweed or rubber bush, is a perennial woody evergreen shrub 3–5 m tall with silver-green opposite subsessile leaves, purple-tipped flowers, and inflated pale green seed pods (Rivas et al., 2020). Native to tropical and sub-tropical regions of Asia and Africa, it is commonly found in the Middle East. It grows as a wasteland plant and reproduces by seeds only, showing vigorous growth under adverse climate and poor soil conditions (Pompelli et al., 2019). While it can withstand drought and salt-affected environments, the mechanisms behind its success in arid and semi-arid areas remain unclear.

The species rapidly colonizes diverse habitats including roadsides, stream/river banks, lagoon edges, and overgrazed areas through anemochory and zoochory. It dominates sandy soils with low rainfall and is considered an indicator of degraded lands, supporting 80% of wildlife for completion of their life cycles (Khan et al., 2019).

Although this species has great ecological importance in supporting pollinators (honeybees, ants, butterflies) and casual visitors (wasps, moths, dragonflies) in open and deserted environments—which may contribute to its wide distribution—this alone is insufficient to judge its survival potential across diverse habitats. There must be alterations at growth, structural, and functional levels in response to different environmental stresses that determine ecological success across heterogeneous environments. This study addresses three key questions: (1) Are morpho-anatomical and physiological traits of *C. procera* plastic in nature? (2) Does this species modify its structural and functional features according to environmental conditions? (3) What traits determine the ecological success of the species? We evaluated the structural and functional mechanisms of different *C. procera* populations and related their adaptive features to environmental conditions that enable colonization of diverse habitats, particularly in arid and semi-arid areas of Punjab Province, Pakistan. We hypothesized that this species adopts internal (anatomical) and external (morphological) mechanisms to cope with prevailing environmental conditions, and that such adaptive components could be extracted and incorporated as biological markers into sensitive species to revegetate wasteland areas.

2 Materials and Methods

2.1 Climatic conditions of the Punjab Province

The Punjab Province experiences tropical to sub-tropical, arid to semi-arid, dry, and hot environmental conditions. Average annual precipitation ranges from 250 to 1000 mm, with maximum precipitation occurring during the monsoon season (June–August). Northern hilly areas receive more annual precipitation (1000 mm) compared to southern plain areas that receive less than 250 mm. Maximum average temperature reaches 48°C in June, while minimum temperature drops to 4°C in January (Pakistan Meteorological Department). Coordinates and altitudes of sampling sites were recorded using a global positioning system.

2.2 Plant material selection

Each study site was explored over an area of 1 km^2 for plant tagging. Six largest plants (replications; n=6) were subjectively selected at each collection site (habitat), maintaining at least 5 m distance between plants. Sampling consisted of one plant per replication from each population, with sampling points randomly selected within each population. Sampling was conducted during August–October 2020, with youngest shoots selected for anatomical studies.

2.3 Study layout, soil topography, and coordinates

This study was conducted under natural conditions (semi-arid and arid areas) in Punjab Province, Pakistan, where *C. procera* populations occur. Twelve populations from heterogeneous environments were collected to evaluate their structural and functional responses [Figure 1: see original paper]. Selected regions varied in geographical conditions, habitats, and soil types, including stream/river channels (KHP: canal bank; TBJ: near Tounsa Headwork's; ADS: near Treemu Headwork's), mountainous range (PCK: near mountain spring; PDK: muddy dunes along roadside; NSR: sandy-stone hills), hyper-arid areas (DIS: Thal Desert; AHP: Cholistan Desert; DGK: flat plain with sandy dunes), and salt-affected lands (LSH: saline wasteland; KBL: hyper-saline lake; KKL: near salt lake).

2.4 Soil physical-chemical parameters

Soil from the root zone of each population was analyzed for physical-chemical characteristics at two depths (30 and 60 cm). Soil pH and electrical conductivity (EC) were assessed using a combined pH and EC meter (Cond 720, WTW series; Inolab, Weilheim, Germany) on saturation paste extracts. Soil ionic contents (Na^+, K^+, Ca^{2+}) were analyzed using a flame photometer (Jenway PFP-7, ELE Instrument Co. Ltd., Felsted, UK), while Cl^- content was estimated using a digital chloride meter (Model 926, Sherwood Scientific Ltd., Cambridge, UK).

Coordinates and elevation of each collection site were determined using Google Earth Pro Software.

Population details: - KHP: canal bank (Rahim Yar Khan) P: 102 mm; E: 76 m; 28°09$‘19“N, 70°37’51“E−TBJ : nearTaunsaHeadwork′s(Muzaffargarh)P : 211mm; E : 131m; 30°30’46“N, 70°50’58“E−ADS : nearTreemuHeadwork′s(Jhang)P : 280mm; E : 149m; 31°08’41“N, 72°08’47“E−PCK : nearmountainspring(Khushab)P : 401mm; E : 788m; 32°37’11“N, 72°26’50“E−PDK : muddydunesalongroadside(Jhelum)P : 152mm; E : 204m; 32°35’18“N, 73°02’36“E−NSR : sand−stonehills(Khushab)P : 435mm; E : 288m; 34°00’37“N, 71°59’15“E − DIS : ThalDesert(Layyah)P : 250mm; E : 162m; 31°13’41“N, 71°48’35“E−AHP : CholistanDesert(Bahawalpur)P : 141mm; E : 136m; 30°41’00“N, 71°45’33“E−DGK : flatplainwithsandydunes(DeraGhaziKhan)P : 236mm; E : 125m; 30°02’56“N, 70°38’43“E−LSH : salinewasteland(Bahawalpur)P : 140mm; E : 127m; 29°27’56“N, 71°58’27“E−KBL : hyper−salinelake(Khushab)P : 590mm; E : 743m; 32°37’18“N, 72°12’50“E−KKL : nearsaltlake(Chakwal)P : 480mm; E : 746m; 32°45’22“N, 72°42’48”$E

2.5 Morphological parameters

Three average-sized primary shoots were clipped from each plant base, with averages used for morphological characterization. Parameters recorded included plant height, shoot (branch) length, shoot fresh weight, shoot dry weight, leaf number, and leaf area. Plant height was measured using a clinometer, shoot length with measuring tape. Shoot fresh weight (SFW) was assessed immediately after detachment, then dried in an oven at 65°C to constant weight for shoot dry weight (SDW) measurement using a digital loading balance. Leaf area was calculated using:

$$LA = L \times W \times CF$$

where LA is leaf area (cm^2), L is length (cm), W is width (cm), and CF is the correction factor (0.75).

2.6 Physio-biochemical parameters

2.6.1 Ionic contents Plant samples were oven-dried at 65°C for inorganic ion analysis. Ground dried samples were analyzed for Na^+, K^+, and Ca^{2+} after H_2SO_4 digestion (Wolf, 1982) using a flame photometer (Model 410, Sherwood Scientific Ltd., Cambridge, UK).

2.6.2 Photosynthetic pigments Photosynthetic pigments (chlorophyll a [chl*a*], chlorophyll b [chl*b*], total chlorophyll [total chl], and carotenoids) were assayed using the method of Arnon (1949). Fresh leaf material (0.1 g) was ground in 5 mL acetone (80%) and centrifuged at 14,000 r/min for 15 min at 4°C. Absorbance of the filtrate was recorded at 480, 645, and 663 nm using a spectrophotometer (IRMECO U2020, IRMECO GmbH & Co. KG, Lütjensee, Germany). Final values were calculated using:

$$Chl\ a = [12.7 \times OD_{663} - 2.69 \times OD_{645}] \times \frac{V}{W \times 1000}$$

$$Chl\ b = [22.9 \times OD_{645} - 4.68 \times OD_{663}] \times \frac{V}{W \times 1000}$$

$$Total\ chl = [20.2 \times OD_{645} + 8.02 \times OD_{663}] \times \frac{V}{W \times 1000}$$

$$Carotenoids = [7.6 \times OD_{480} - 1.49 \times OD_{510}] \times \frac{V}{W \times 1000}$$

where OD is optical density, V is total acetone extract volume (mL), and W is fresh sample weight (g).

2.7 Biochemical analyses

2.7.1 Total soluble proteins (TSP) Total soluble proteins were analyzed following Bradford (1976). Approximately 0.25 g leaf sample was crushed in 5 mL potassium phosphate buffer (pH 7.8) using an ice-chilled pestle and mortar. The extract was centrifuged at 12,000 r/min at 4°C. Bradford reagent (5 mL) and 100 L extract were mixed and vortexed for 10 s, with absorbance measured at 595 nm using a UV-spectrophotometer (IRMECO U2020, Lütjensee, Germany).

2.7.2 Proline content Free proline content was estimated following Bates et al. (1973). Fresh leaf sample was homogenized in 3% sulfo-salicylic acid. The extract was incubated with acid ninhydrin and glacial acetic acid at 100°C, then extracted with toluene. Absorbance of the chromophore-containing toluene was measured at 520 nm using a UV-spectrophotometer (IRMECO U2020, Lütjensee, Germany). A standard curve was used to compute final proline content on a fresh weight basis.

2.7.3 Glycine betaine (GB) Fresh leaf sample (0.25 g) was ground in 5 mL distilled water and centrifuged at 12,000 r/min for 15 min. A mixture containing 500 L sample and 1 mL 2 N H_2SO_4 was refrigerated for 90 min, then 200 L potassium triiodide, 2.8 mL distilled H_2O, and 6.0 mL 1,2-dichloroethane were added (Grieve and Grattan, 1983). Absorbance was recorded at 365 nm using a UV-spectrophotometer (IRMECO U2020, Lütjensee, Germany).

2.7.4 Enzymatic antioxidant activities Fresh leaf sample (0.25 g) was ground in 5 mL potassium phosphate buffer (pH 7.8) using an ice-chilled pestle and mortar. The extract was centrifuged at 12,000 r/min for 15 min, with the supernatant stored at −20°C for enzyme activity analysis (SOD, CAT, and POD).

2.7.5 Superoxide dismutase (SOD) SOD activity was determined based on nitro blue tetrazolium (NBT) principle following Giannopolitis and Ries (1977). A homogenized mixture containing 50 L sample extract, 0.1 mL L-methionine, 0.1 mL triton-X, 400 L distilled water, 50 L riboflavin, 50 L NBT, and 1.0 mL potassium phosphate buffer (pH 7.0) was exposed to light for 20 min. Absorbance was recorded at 560 nm using a UV-spectrophotometer (IRMECO U2020, Lütjensee, Germany).

2.7.6 Catalase (CAT) CAT activity was determined following Chance and Maehly (1955) using 3.0 mL reaction mixture containing 1.9 mL potassium phosphate buffer, 1.0 mL H_2O_2, and 0.1 mL sample extract. Absorbance was measured at 240 nm using a UV-spectrophotometer (IRMECO U2020, Lütjensee, Germany).

2.7.7 Peroxidase (POD) POD activity was measured following Chance and Maehly (1955) using 1.00 mL reaction mixture containing 0.10 mL H_2O_2, 0.05 mL leaf extract, 0.10 mL guaiacol solution, and 0.75 mL phosphate buffer. Absorbance was recorded at 470 nm using a UV-spectrophotometer (IRMECO U2020, Lütjensee, Germany).

2.8 Anatomical parameters

For anatomical studies, samples were immediately preserved in leak-proof plastic vials containing formalin acetic-alcohol solution (v/v: 5% formalin, 10% acetic acid, 50% ethanol, 35% distilled water), then transferred after 2–3 days to acetic alcohol solution (v/v: 75% ethanol, 25% acetic acid) for long-term preservation. Stem anatomy was examined using 1.5 cm pieces from the topmost internode of main branches, while leaf anatomy used 1.5 cm pieces from leaf centers including midribs. Free-hand sectioning was applied for permanent slide preparation. Samples were dehydrated using graded ethanol solutions (30%, 50%, 70%, 90%, and 100%) and xylene (100%), then stained with safranin (1%) and fast green (1%) (Ruzin, 1999). Safranin stains secondary and lignified tissues (xylem vessels and sclerenchyma), while fast green stains primary tissues (parenchymatous tissue). Sections were mounted in Canada balsam. Micrographs were captured using a digital camera (Nikon FDX-35, Tokyo, Japan) equipped with a stereomicroscope (Nikon 104, Tokyo, Japan). All parameters were measured using an ocular micrometer calibrated with a stage micrometer.

2.9 Statistical analysis

One-way ANOVA under complete randomized design (CRD) was applied for statistical analysis (Steel et al., 1997). Means were compared using least significance difference (LSD) following Snedecor and Cochran (1980). Principal component analysis (PCA) was used to assess environmental factor influences on population growth, physiology, and structural responses across habitats. R software v.4.0.1 was used to generate correlation matrices and density heatmaps among studied variables (species and environment) for correlation estimation (R Development Core Team, 2017).

3 Results

3.1 Morphological and physio-biochemical characteristics

All *C. procera* populations exhibited habitat-specific differences in growth attributes . Plant height was greatest in AHP population (4.1 m), followed by DIS (3.5 m), with minimum height (1.0 m) in PDK. Shoot length was maximal in AHP (200.4 cm), followed by DIS (173.8 cm), and minimal in KBL (65.2 cm). KHP population produced the largest leaves (213.2 cm^2), followed by ADS (180.0 cm^2), while LSH had the smallest leaves (46.8 cm^2). DIS population showed the highest leaf number (172.4), followed by ADS (155.4), with PCK showing the fewest leaves (49.2). Maximum shoot fresh weight (664.8 g/plant) occurred in ADS, significantly different from DIS (633.2 g/plant), while minimum shoot fresh weight (410.8 g/plant) was observed in KHP. Shoot dry weight was highest in TBJ (277.2 g/plant), followed by KBL (260.4 g/plant), and lowest in NSR (84.4 g/plant).

3.1.1 Shoot ionic content Substantial variation in ionic contents occurred among populations . Maximum shoot Na^+ (67.6 mg/g dry weight) was observed in LSH, followed by KBL (63.4 mg/g), with minima (15.4 mg/g) in AHP and KHP. Shoot K^+ was highest in NSR (41.1 mg/g), followed by KKL (32.7 mg/g), and lowest in DGK (14.2 mg/g). Shoot Ca^{2+} peaked in NSR (28.2 mg/g), followed by PDK, with minimum (13.8 mg/g) in DGK.

3.1.2 Photosynthetic pigments Chlorophyll *a* content was maximal in AHP (1.85 mg/g fresh weight), followed by DGK (1.72 mg/g), and minimal (0.34 mg/g) in LSH, KBL, and KKL . Chlorophyll *b* was highest in AHP (1.12 mg/g), followed by ADS (1.02 mg/g), with minima (0.16 mg/g) in LSH, KBL, and KKL. Carotenoids were most abundant in DGK (0.12 mg/g), followed by AHP (0.08 mg/g), and least in KKL (0.01 mg/g). Total chlorophyll peaked in AHP (2.97 mg/g), followed by DGK, with lowest values (0.50 mg/g) in LSH, KBL, and KKL. The chlorophyll *a*/*b* ratio was highest in KHP (3.37), followed by TBJ, and lowest in ADS (1.13). The chlorophyll/carotenoid ratio was maximal in TBJ (88.0), followed by NSR, and minimal in LSH (8.3).

3.1.3 Organic osmolytes Proline content was highest in LSH (43.3 mol/g fresh weight), followed by KBL, with minima (13.1 mol/g) in PDK and ADS . Glycine betaine peaked in KBL (5.6 mol/g fresh weight), followed by LSH and KKL, and was lowest in DIS (1.0 mol/g). Total soluble proteins were maximal in KKL (475.6 g/g fresh weight), followed by KBL, and minimal in ADS (91.6 g/g).

3.1.4 Antioxidant enzymes Maximum SOD activity (2.0 U/ g protein) occurred in DGK and LSH, not significantly different from ADS (1.7 U/ g), with minimum (0.7 U/ g) in NSR . CAT activity was highest in KBL (2.0 U/ g), similar to KKL and LSH (1.8 U/ g), and lowest in PCK (0.5 U/ g). POD activity peaked in LSH (2.1 U/ g), followed by KBL, with low activity (0.7 U/ g) in PDK.

3.2 Anatomical characteristics

3.2.1 Stem anatomy Significant variation in stem anatomical attributes occurred among differently adapted populations [TABLE:3; FIGURE:2]. DIS population showed the widest stem radius (1988.3 m), followed by DGK, while the narrowest (1506.7 m) occurred in KBL. Cuticle was thickest in AHP (51.5 m), followed by NSR, and thinnest in ADS and KBL (18.4 m). Epidermal cell area was largest in NSR (663.5 m^2), followed by DIS and KBL, and smallest in PCK (231.3 m^2).

Collenchyma layer was thickest in PCK (100.7 m), followed by DGK, LSH, and KKL, and thinnest in AHP, NSR, and KHP (48.8 m). Cortical region was most enlarged in AHP (198.9 m), followed by DGK, TBJ, and KHP, and greatly reduced in NSR (58.9 m). Cortical cell area was largest in AHP (252.2 m^2), followed by KHP, and smallest in ADS and PCK (35.2 m^2). Sclerification was most intensive in PDK and KBL (790.4 m), followed by TBJ, and minimal in DGK (263.3 m).

Metaxylem area was widest in DIS (247.0 m^2), followed by LSH, and narrowest in KBL (23.5 m^2). Outer phloem region was largest in AHP and LSH (588.9 m^2), followed by PCK, and smallest in KHP (58.3 m^2). Inner phloem region was broadest in KKL (503.0 m^2), similar to LSH, and narrowest in NSR and KHP (85.2 m^2). Pith thickness was greatest in DIS (352.1 m), followed by LSH and ADS, and reduced in KBL and TBJ (111.3 m). Pith cell area was largest in LSH and AHP (217.2 m^2), followed by KKL, and smallest in TBJ (41.4 m^2).

3.2.2 Leaf anatomy Populations showed specific leaf anatomical modifications in response to varied environmental conditions [TABLE:4; FIGURE:3]. Midrib thickness was greatest in DIS and KHP (5517.9 m), followed by AHP, and minimal in LSH (1018.3 m). Lamina was thickest in AHP (246.5 m), similar to DGK, and thinnest in PCK (83.2 m). Epidermal cell area was largest in DIS (101.0 m^2), followed by LSH, and smallest in ADS and KHP (16.2 m^2).

Cuticle was thickest in KBL and AHP (35.3 m), followed by KKL and PCK, and thinnest in ADS (13.0 m).

Sclerification was most intensive in KBL (846.5 m), followed by TBJ, and reduced in ADS (147.8 m). Cortical thickness was greatest in DIS (1067.7 m), followed by KHP, and minimal in AHP (262.8 m). Cortical cell area was largest in KKL and DIS (432.0 m^2), followed by KHP, and smallest in LSH (146.6 m^2). Xylem vessels were widest in DIS (633.2 m^2), followed by TBJ, and narrowest in PDK (183.4 m^2). Phloem area was maximal in TBJ and KKL (301.2 m^2), similar to AHP, and minimal in PCK and PDK (101.9 m^2).

Trichome area was largest in PCK (355.2 m^2), followed by AHP, and smallest in DGK (61.9 m^2). Trichome density was highest in AHP (119.2/mm^2), followed by PCK, and lowest in DGK and KBL (40.5/mm^2). Adaxial stomatal size was largest in ADS (957.3 m^2), followed by KHP, and smallest in PCK (432.2 m^2). Abaxial stomatal size was greatest in AHP (881.4 m^2), followed by KHP, and smallest in PDK and PCK (83.8 m^2). Adaxial stomatal density peaked in TBJ (50.0/mm^2), followed by ADS, and was minimal in AHP (23.0/mm^2). Abaxial stomatal density was highest in AHP and PDK (31.0/mm^2), similar to KBL, KKL, and ADS, and lowest in NSR (16.0/mm^2).

3.3 Multivariate analysis

PCA was applied to assess environmental factor influences on growth, physiological, and anatomical attributes of *C. procera* populations [FIGURE:5a–d].

3.3.1 Influence of soil physical-chemical attributes on growth and physiology

Plant height, leaf number, and shoot length were strongly influenced in AHP population, while leaf area was greatly influenced in KHP population rich in soil Ca^{2+}. Shoot dry weight was influenced by soil EC, Na^+, and Cl^- in LSH and KBL populations, whereas shoot fresh weight was greatly influenced by soil K^+ in DIS population. For plant physiology, SOD, shoot K^+, shoot Na^+, and proline showed strong impacts of soil Na^+ in KKL population, while GB, CAT, and POD reflected effects of soil EC and Cl^- in KBL population. Photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and total chlorophyll) showed strong association with AHP population rich in soil K^+, while carotenoids associated with PCK population enriched in soil Ca^{2+}.

3.3.2 Influence of soil physical-chemical attributes on anatomy

For stem attributes, sclerenchyma thickness showed positive relation with KBL population, while collenchyma thickness related positively to LSH population under varying soil EC, Na^+, and Cl^-. Cuticle thickness and stem radius associated with DGK population, whereas cortical cell area, cortical region thickness, and epidermal cell area associated with soil pH in DIS population. Among leaf anatomical attributes, cuticle thickness was strongly influenced in KKL population, while trichome area and density were influenced by soil Ca^{2+} in PCK population. In NSR population, metaxylem area and lamina thickness associated with soil K^+, adaxial and abaxial stomatal areas in TBJ population, while adaxial stomatal density and cortical region thickness showed close association with AHP population.

3.4 Correlation matrices and density heatmaps

Soil physical-chemical traits and morpho-physiological traits exhibited significant ($P<0.01$) correlation [Figure 6a: see original paper]. Soil Cl^-, Na^+, and EC showed strong positive correlation with osmolytes (proline and GB) and antioxidant enzymes (CAT and POD). Shoot Na^+ was positively correlated with organic osmolytes and cellular antioxidants. Soil and shoot K^+ and Ca^{2+} were negatively correlated with antioxidants. A clustered heatmap between soil physical-chemical traits and morpho-physiological traits revealed that soil EC, Na^+, and Cl^- exhibited strong relation with shoot Na^+, shoot dry weight, POD, and SOD, while negative association occurred between photosynthetic pigments and soil traits. Distinct clustering was observed between soil traits and morpho-physiological characters [Figure 6b: see original paper].

Soil physical-chemical characters significantly influenced stem structural attributes [Figure 7a: see original paper]. Positive correlation occurred between Na^+ and inner phloem area, stem radius, pith thickness, and pith cell area. Strong negative influence of Ca^{2+} was observed on epidermal cell area, pith cell area, and sclerenchymatous thickness. Soil Cl^-, Na^+, and EC indicated strong association with outer phloem area, inner phloem area, and cortical cell area. Negative association occurred between epidermal cell area, cortical cell area, outer phloem area, inner phloem area, and stem radius with pith thickness [Figure 7b: see original paper].

Correlation matrix between soil physical-chemical attributes and leaf structural traits indicated significant ($P<0.01$) correlation [Figure 8a: see original paper]. Strong positive correlation occurred between trichome area and density with Ca^{2+}. Phloem area, Ca^{2+}, Na^+, and adaxial stomatal density showed significant negative correlation. Leaf anatomical traits exhibited strong clustering with soil K^+ and Ca^{2+}, while sclerenchymatous thickness grouped with midrib thickness, epidermal cell area with lamina thickness, and abaxial stomatal area with adaxial stomatal area. Soil Cl^-, Na^+, and EC strongly influenced phloem area, cortical cell area, abaxial stomatal area, adaxial stomatal area, and abaxial stomatal density. Density patterns varied among all studied traits [Figure 8b: see original paper].

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4 Discussion

Substantial variation in growth attributes among populations reflected great environmental heterogeneity. These characteristics were genetically fixed over prolonged evolutionary time and represent their respective habitats (Paccard et al., 2013). Growth attributes serve as important criteria for estimating population response and tolerance potential to prevailing stressful conditions (El-Hendawy et al., 2017). In this study, individuals adapted to hyper-arid areas (AHP and DIS) achieved greatest height and shoot size compared to other habitats, apparently due to reduced anthropogenic activities (cultivation, cutting, burning), decreased grazing impact, and episodic water resources from annual precipitation and flooded irrigation. Previous reports confirm that *Calotropis* is a drought-resistant and salt-tolerant plant that grows across various soil types. However, populations in salt-affected lands showed reduced growth attributes including plant height, shoot growth, leaf number and size, and biomass production, likely resulting from salinity's depressive effects. Many researchers report that while *C. procera* can tolerate moderate salinity, it cannot sustain high soil salinity (Boutraa, 2010; Hassan et al., 2015).

Leaves are primary photosynthetic organs exceptionally vital for growth and biomass accumulation (De Faria et al., 2018). Large leaf formation in KHP represents a principal strategy to prevent seasonal water shortage from low precipitation and canal irrigation. According to Tezara et al. (2011) and Rivas et al. (2020), plants maintain high photosynthetic rates, stomatal control, and efficient metabolic performance under drought stress. DIS population produced exceptionally high leaf numbers but smaller leaf size. Numerous narrow leaves may aid carbon acquisition and water-use efficiency by controlling transpiration rate (Mutwakil et al., 2017). Fresh and dry biomass production provides a good criterion for judging plant tolerance to abiotic stresses (Zokaee-Khosroshahi et al., 2014). ADS showed maximum shoot fresh weight, while TBJ showed maximum shoot dry weight, reflecting robust growth and complete dominance in their respective habitats.

Abiotic stresses such as salinity, waterlogging, high temperature, and irradiance impose substantial changes in tissue ionic contents, photosynthetic pigments, organic osmolyte levels, and antioxidants (Hasanuzzaman et al., 2020). High Na^+ accumulation in tissues occurs in various glycophytes and halophytes (Sekmen et al., 2013), as observed in LSH population. These plants utilize such ions to maintain cell osmotic potential and osmoregulatory processes under osmotic stress (Rouached et al., 2010). Remarkable shoot K^+ and Ca^{2+} concentrations in NSR population reflected soil enrichment with these beneficial ions, which regulate cellular osmotica and metabolic processes (Alzahrani et al., 2019). Higher photosynthetic pigment concentrations (chl*a*, chl*b*, and total chl) in AHP population, and large carotenoid quantities in DGK population, appear directly associated with photosynthetic efficiency under stressful environments (Maimaitiyiming et al., 2017).

Salt-tolerant species accumulate substantial organic osmolytes to offset ROS and free radical effects on photosynthetic apparatus and respiratory organelles, ultimately preventing oxidative stress. Osmoprotectants also maintain protein structure, membrane integrity, and osmotic events in plants (Muchate et al., 2016). In our results, populations from salt-affected lands (LSH, KBL, and

KKL) were rated as more tolerant due to maximal accumulation of proline, glycine betaine, and soluble proteins. Plants evolve antioxidant mechanisms (enzymatic and non-enzymatic) to mitigate and detoxify ROS effects. Enzymatic antioxidants (SOD, CAT, and POD) function in chain reactions to eliminate superoxide and H_2O_2 (Sheikh-Mohamadi et al., 2017). All populations showed varying antioxidant metabolism levels relative to prevailing stress conditions, with saline environment populations (LSH, KBL, and KKL) surpassing others in antioxidant activity. Maintaining high antioxidant levels may supplement salinity or drought stress tolerance by improving protective strategies against oxidative stress (Zhang et al., 2013).

Hameed et al. (2012) found that desert plants adapted to limited water availability experience less severe water stress impacts compared to high-moisture habitats, particularly in tolerant species. Differently adapted *C. procera* populations showed specific responses indicating capability to survive in desert environments. All populations exhibited great diversity in stem anatomical tissues (dermal, mechanical, vascular, and storage). Desert and semi-desert populations showed maximum stem anatomical parameters: cuticle thickness, cortical region thickness, cortical cell area, outer phloem area, and pith cell area in AHP; and stem radius, pith region thickness, and metaxylem area in DIS. Desert plants modify structural and functional features to cope with multiple abiotic stresses and successfully survive harsh climatic conditions. Previous studies report that cuticle or epidermis thickness serves as the first line of defense in arid plants to minimize water loss under water-scarce conditions (Liu et al., 2020). Another adaptation is enlarged stem area from deposition of storage parenchyma and large vascular bundles, which aids water conservation and survival during prolonged dry periods with low soil moisture (Su et al., 2019). Therefore, AHP population showed enormous succulence through storage parenchyma (cortex) for survival. Large phloem region formation represents another principal strategy that may enhance photosynthetic translocation and partitioning to other plant parts (Hussain et al., 2021).

Vascular bundles with large metaxylem vessels play key roles in efficient water and mineral translocation. Tissue sclerification is a prominent feature in plants surviving saline and drought stress (Hameed et al., 2020), as observed in stems of KBL and NSR populations. Tissue sclerification provides mechanical strength to metabolically active tissues, protecting against collapse and desiccation when water becomes a vital commodity due to low soil water potential or water table (Abd Elhalim et al., 2016). Thick collenchyma layer formation under the epidermis and around cortical tissues is a critical feature of PCK population, representing a habitat-friendly strategy to overcome prevailing stress. Collenchyma acts as supporting tissue in dry environment plants by preventing surface water loss (Iqbal et al., 2021).

Leaves are highly responsive to climatic conditions due to direct environmental exposure (Drake et al., 2019). Leaf thickness showed differential responses: desert populations exhibited very thick leaves regarding lamina (AHP) and midrib thickness (DIS and KHP). Leaf thickness likely results from increased storage parenchyma for additional water storage, representing a critical adaptation for desert species to cope with severe drought (Grubb et al., 2015). In addition to thick epidermis and cuticle, trichome size and number, stomatal size, shape, orientation, and regulation are vital for dry environment survival. Most desert species orient stomata on abaxial leaf surfaces to reduce transpiration when leaves are directly exposed to sunlight (Silva et al., 2014). Similar results were observed in AHP population with more stomata on abaxial surfaces. Moreover, small stomata show efficient translocation compared to larger ones due to lower water requirements for turgor maintenance (Rudall et al., 2013).

Intensive sclerification around cortical and vascular regions in KBL population made leaves stiffer and more fibrous. Cuticle thickening additionally contributed to leaf stiffness while protecting against solar irradiance and pathogen infection (Akcin et al., 2015). Large metaxylem formation in KKL leaves may represent adaptability to maintain water supply under low soil water potential. Trichomes act as insulating agents against high wind pressure, rain splashes, and temperature, maintaining maximum humidity on leaf surfaces in dry environments (Dolatabadian et al., 2011). Few but large trichomes in PCK population indicate tolerance strategy to ensure survival under limited water supply. ADS population growing along river/canal banks possessed large adaxial stomata. As stomata are vital for transpiration and gas exchange, their opening allows maximum water utilization and carbon sequestration, maintaining plant vigor by producing more energy under saturated environmental conditions (Buckley, 2019).

5 Conclusions

Survival responses in *C. procera* populations result from complex, differential reactions to prevailing stress conditions. Maximum modulation of growth, photosynthetic, and anatomical features in DIS and AHP populations strongly favored life cycle completion and indicated tolerance potential under harsh environments. Saline area populations showed enhanced metabolite and antioxidant levels to cope with osmotic stress. Beneficial ion (K^+ and Ca^{2+}) accumulation in NSR population and maximum biomass production (shoot fresh and dry weight) in ADS population demonstrated their success in respective habitats. Overall, growth, physio-biochemical, and anatomical indices observed in these populations serve as excellent indicators of survival and stress tolerance. However, further research is needed to investigate habitat effects on functional attributes of *C. procera* populations at molecular and genetic levels.

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