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Biochemical and morpho-physiological insights revealed low moisture stress adaptation mechanisms in cotton (*Gossypium hirsutum* L.)

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Cotton (*Gossypium hirsutum* L.) is a multipurpose crop. Abiotic stresses, especially extreme heat and drought, limit crop growth and thus reduce cotton yield by about 50%. In this study, 30 cotton genotypes were tested against low moisture stress in a pot experiment in triplicates along with control under wire house conditions. At the 3–4 leaf stage, different morpho-physiological and biochemical parameters were measured in order to select the low moisture stress-tolerant genotypes. For the selection of the best performing genotypes, Multi-Trait Genotype-Ideotype Distance Index (MGIDI) was used for the ranking of genotypes on the basis of multiple indices. For biochemical traits, 09 (TPC, TF, TSP, MDA, SOD, POD, CAT, APX, and Proline) out of 24 showed significant genotypic effects and were used for MGIDI. Eight genotypes (N-812 N-1296 N-696 N-377 N-121–896 N-T86, and N-3496) were observed to be best performing than others at 25% selection pressure (SI = 25%). For morpho-physiological traits, 14 out of 15 showed significant genotypic effects and used for MGIDI. Ten genotypes (N-1237 N-812 N-1296 N-696 N-9078 N-377 N-512 N-121 N-375, and N-896) were observed to be best performing at 35% selection pressure (SI = 35%). Six genotypes, i.e. N-812–1296 N-696 N-377 N-121, and N-896 were found common in both MGIDI analysis. In conclusion, three genotypes, i.e. N-696, N-896, and N-T86 proved to be most resilient to low moisture stress. Develop protocols, identified genotypes and markers that can be used for development of climate-smart cotton genotypes.

Keywords Cotton, Low moisture stress, Morpho-physiological markers, Biochemical markers, MGIDI

Cotton (*Gossypium hirsutum* L.) is a main agricultural crop that covers the largest textile manufacturing businesses globally, with a substantial influence on the economy of countries about \$600 billion worldwide¹. Cotton is recognized as a multipurpose crop, and its principal output, cotton lint, is used in the textile sector². In recent years, cotton production has decreased from 13.960 to 11.935 million bales, a decline of 14%³. *Gossypium hirsutum* (90%) (features high yield and early system of cultivation⁴) play critical roles in the production of cotton due to their potential of high yield and cotton textiles manufacturers gained competitive advantage in the market. Cotton is a globally important crop that produces natural fiber. *Gossypium hirsutum* L. was developed to provide a higher yield of fiber and to endure severe environmental conditions⁵. Cotton genetic resources are large, scattered geographically over five continents, and include roughly 45 diploid and 5 tetraploid Malvaceae species^{6,7}. Two allotetraploid cotton species, *G. hirsutum* L. (source of the upland cotton), and *G. barbadense* L. (source of the Egyptian, Sea Island and Pima cotton), were domesticated about 4000 years ago independently^{8,9}. The remaining three tetraploid species of cotton have not been domesticated and are distributed largely: *G. mustelinum* Miers ex Watt is found mostly in Brazil^{10,11}. Nowadays, the tetraploid specie, *G. hirsutum* account for the major production of cotton globally. Evidence suggests that selection by breeders has resulted in significant alterations in cotton cultivars since domestication, including plant habit, lack of developmental responses to light and dark period, early blooming, increased boll size, fiber properties (better fiber strength, length and fineness) and early methods of cultivation¹².

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Unexpected climate shifts and altering weather extremes have resulted in the high global temperatures and unpredictable patterns of rainfall in recent years, posing serious challenges to cotton production sustainability¹³. Abiotic stresses, especially extreme heat and drought, limit crop growth and thus reduce cotton yield by about 50%¹⁴. Drought stress influences the growth of plants by altering morphological, physiological, biochemical and molecular processes¹⁵. Drought stress has developed as a major concern globally that has a considerable impact on production of cotton. Improving the efficiency of water usage and implementing the strategies of water conservation have emerged as critical issues in irrigated agriculture¹⁶. The drought had drastic impacts on the photosynthesis of cotton plants. As the drought intensity increased, the gaseous-exchange metrics net photosynthesis, stomatal diffusive resistance, and transpiration showed a declining trend. As the severity of the drought stress increased, both hydrogen peroxide and MDA levels increased, which indicates the oxidative stress¹⁷.

At different growth phases, drought stress significantly affects cotton, which influences its physiological processes, yield, and fiber quality¹⁸. Drought stress triggers an internal heatwave effect which reduces the photosynthetic activity and stunts growth in plants¹⁹. The vulnerability of cotton to low moisture stress varies across different phases of development, with crucial periods during seedling growth, flowering, and boll development. At the seedling stage which is sensitive, drought stress could lead to physiological changes that include increased lipid peroxidation and decreased chlorophyll concentration, indicating oxidative stress²⁰. Drought stress diminishes relative water content (RWC) in cotton seedlings, which is essential for sustaining cell turgor and overall plant survivability. A decrease in RWC leads to a reduction in leaf expansion, root development and negatively impacts plant growth²¹.

Drought stress frequently results in the gradual increase of the ROS (reactive oxygen species) at the cellular level. Excessive production of ROS causes oxidative stress to the photosynthetic machinery and adversely affects cell activity²². The increased level of ROS considered as a threat to plant cells, but can also act as a secondary messenger in the signal transduction pathway of stress²³. The ability of plants to scavenge ROS and reduce their negative impacts may be related to their drought tolerance²⁴. Drought stress affects plants via stress signaling/sensing and the activation of multiple parallel stress responses (including physiological, morphological, molecular, and biochemical processes)²⁵. The indirect effect on performance is connected to an increase in ROS generation, which harms macromolecules during oxidative stress and reduces yield. It is critical to discover the major components and attributes of plant stress (drought) tolerance network in order to generate more drought tolerant cultivars¹⁶.

Many researchers have explored numerous factors in the cotton plant, including morphological aspects (leaf, root, and stem growth) and physiological parameters (molecular, histological, and anatomical) as essential selection criteria related with resilience to drought²⁶. Nevertheless, none of these features have been consistently proven to be positively related with drought resistance in cotton²⁷. Based on the duration, timing, and intensity of drought stress, multiple strategies can be used to generate cotton types that are more water efficient and drought tolerant. The first part is to comprehend cotton plant reactions and behavior in response to water scarcity and droughts²⁸. In this regard, scientists revealed that by breeding tolerant cotton plants, we may be able to overcome these critical situations²⁹.

Therefore, in this experiment, numerous physiological, morphological, and biochemical parameters were assessed in normal and low moisture stress conditions in order to screen cotton genotypes at the seedling stage, and their comparison was made to determine which trait is more influenced by low moisture stress. Furthermore, this study presents effective breeding strategies for improving low moisture tolerance in cotton.

Materials and methods

Experimental site

The cotton experiment was conducted in the wirehouse of Marker Assisted Breeding Group (MABG), Plant Breeding and Genetics Division, NIAB Faisalabad (started on March 03, 2023).

Cotton germplasm and growth conditions

In this experiment, thirty diverse cotton genotypes developed at Plant Breeding and Genetics Division, NIAB Faisalabad (Table 1) were grown in CRD design for evaluation of their response to low moisture stress at three to four true leaves and the first square stage. No voucher specimens are involved in this research work. The cotton experiment was carried out with three technical replicates, in which each replication consisted of 15 cotton plants (biological replicates) in 05 bags.

All polythene bags filled with 02 kg of soil were watered at 50% of the field capacity before the seed sowing. The soil used in the study was sandy clay loam composed of 45% sand, 33% silt, and 22% clay sourced from the NIAB experimental field. Seeds were planted (on March 07, 2023), in polythene bags. For sowing, the cotton seeds were submerged in the aerated dehydrated calcium sulphate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) overnight and the next morning the cotton seeds were sown at approximately 2 cm depth. All plants were watered at field capacity after one week of sowing.

Treatments

At the first true leaf stage (18–20 days after sowing), plants were divided into two groups, i.e., non-stress (control) and low moisture stress. In control plants, water was maintained at field capacity. While low moisture stress was applied by maintaining the water level at 50% field capacity. The water level was maintained by weighing each bag on alternate days for both stressed and control sets. After stress treatment, various physiological parameters were measured and noted between 11 a.m. and 2 p.m. on the day before harvesting. Then, 50 -day-old plants were harvested (27-04-2023) and data for morphological traits was recorded and leaf samples for biochemical analysis were stored at -40 °C till further analysis. Details of the procedures are as follows.

No.	Genotypes	No.	Genotypes	No.	Genotypes
1	N-868	11	N-3496	21	N-812
2	N-696	12	N-9078	22	N-121
3	N-896	13	N-990	23	N-153
4	N-1096	14	N-7012	24	N-374
5	N-1296	15	N-1237	25	N-375
6	N-1286	16	N-912	26	N-926
7	N-8437	17	N-T86	27	N-213
8	N-T8411	18	N-377	28	N-878
9	N-1496	19	N-135	29	FH-326
10	N-4496	20	N-512	30	FH-490

Table 1. List of cotton genotypes used in the experiment. Genotype 1–28 NIAB, Faisalabad, 29 and 30 from Ayub Agriculture Research Institute (AARI), Faisalabad.

Physiological analysis

Among physiological traits, diffusive resistance (DR), transpiration Rate (TR), and leaf temperature (LT) was recorded with Porometer.

Transpiration rate

The transpiration rate was recorded in a fully expanded fourth leaf of cotton genotypes from the upper surface by using a Li-1600 Steady State Porometer and expressed in unit microgram $\text{cm}^{-2} \text{s}^{-1}$.

Stomatal diffusive resistance

The physiological trait stomatal diffusive resistance of cotton leaves was measured by using a Li-1600 Steady State Porometer which has a sensor (lithium chloride). It was calibrated before recording the data³⁰. This data was recorded from a fully expanded leaf of cotton genotypes of every replicate and expressed in unit s cm^{-1} .

Leaf temperature

The physiological trait leaf temperature was recorded from the upper surface of cotton genotypes of each replicate using a Li-1600 Steady State Porometer. Leaf temperature was expressed in unit °C. The measurements of data were taken at 13 ~ 14 h.

Relative water content

Relative Water Content (RWC) of cotton leaf samples was measured and data values were recorded in percentage³¹. The leaf samples of cotton genotypes were collected for RWC measurement, and leaf fresh, turgid and dry weights were measured by using a sensitive weighing scale. The following formula was used to determine the RWC³².

$$\text{RWC} = (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100$$

SPAD measurements

SPAD (Soil Plant Analysis Development) chlorophyll meter³³ was used for the measurement of SPAD values of the fourth fully expanded uppermost leaves. For each leaf sample, three measurements were taken with a SPAD meter on different sections of the leaf.

Morphological analysis

Different morphological traits like fresh leaf weight (FLW), wilted leaf weight (WLW), turgid leaf weight (TLW), dry leaf weight (DLW), dry root weight (DRW), fresh root weight (FRW), dry shoot weight (DSW) and fresh shoot weight (FSW) were recorded in grams. Fresh root length (FRL) and fresh shoot length (FSL) were recorded in cm.

Biochemical analysis

All biochemical analyses were performed at the MAB Lab-1 of the Plant Breeding and Genetics Division, Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad, Pakistan. Details of different methodologies are given below.

For biochemical analyses and different enzyme estimations, fresh cotton leaves (0.15 g) were crushed in a cold extraction buffer. Specific extraction buffers were used for each enzyme. Samples were centrifuged at $15,000 \times g$ for 10 min at 4 °C. The supernatant was used for the determination of various enzyme activities and other biochemical assays. For each treatment, the analysis was performed in duplicate.

Total phenolic content

Total phenolics assay was performed according to the micro colorimetric method, in which the Folin-Ciocalteu (F-C) reagent was used³⁴. First, 0.05 g weighted cotton leaves were homogenized in the dark for 2 days after adding the cold 95% methanol (500 μl). Then samples were centrifuged for 5 min at 14,000 g. The supernatant was used to calculate TPC. The assay procedure was followed as: F-C reagent (150 μl) was added in 100 μl

of the sample vortex completely, then added 1.2 ml of Na_2CO_3 (700 mM). At room temperature, the cotton samples were incubated for 1 h. The phenolic content of cotton samples was determined using a linear regression equation.

Total tannin content

From the TPC experiment, the supernatant after spectrophotometer values were taken. For measurement of total tannin content (TTC) using the method³⁵. 0.1 g polyvinylpyrrolidone (PVPP) was added to TPC samples and mixed for 2–3 min. The samples were then centrifuged at 14,000 g. Then, the supernatants of all samples were used to measure their TTC by using UV-Vis (765 nm). Each sample was measured in duplicate. Tannic Acid (0.5 mg/ml) was used for the calibration. Results were calculated based on a standard curve and expressed in units as mg TA/ g plant samples.

Total flavonoid content

Total flavonoid content was determined by using the aluminum chloride colorimetric method³⁶. A 1 ml sample (200 μl + 800 μl H_2O) of cotton leaves was added into 50 μl of 2% NH_4Cl solution then added 1 M potassium acetate (50 μl). For 40 min, the mixture was incubated at 40 °C with frequent shaking. Then the optical density of the sample mixture was taken at 415 nm using a UV-VIS spectrophotometer against a blank sample without NH_4Cl . The resulting data was expressed as mg quercetin equivalents/weight of fresh plant material (g)³⁷.

Ascorbic acid

For analysis of ascorbic acid, the 2,6-dichloroindophenol (DCIP) method was used³⁸. For a brief explanation, vitamin C changed DCIP into DCIPH2. This conversion was observed as a decreasing trend by measuring the absorbance at 520 nm using a spectrophotometer. The ascorbate concentration in the samples was determined using a linear regression equation and standard curve.

Total oxidant status

Total Oxidant Status (TOS) in leaf samples was analyzed by using a novel automated³⁹ formulated method. This approach is based on ferric ions being measured with ferrous ions and xylenol orange being oxidized to ferric ions in an acidic medium by the oxidants of the cotton sample⁴⁰. The TOS assay solution contained the reagent R1 [xylene orange (stock solution containing 0.38 g in 500 μl of 25 mM H_2SO_4), 5 ml of glycerol, NaCl (0.4 g) and made volume up to 50 ml with 25 mM H_2SO_4 made volume up to 50 ml, was included in assay mixture], reagent R2 [contained 0.0196 g of ferrous ammonium sulphate, 0.0317 g of o-dianisidine and added in (10 ml) 25mM H_2SO_4], and 140 μl sample extract. After 5 min, the absorption of the colour compound produced was taken at 560 nm by using a spectrophotometer (HITACHI U-2800).

Malondialdehyde content

The lipid peroxidation level in the leaf tissue was determined as malondialdehyde (MDA, a lipid peroxidation product) content analyzed by using the thiobarbituric acid (TBA) reaction method^{41,42}. The absorbance was taken at 520 nm and 600 nm using a spectrophotometer and results were measured by an extinction coefficient of 155 $\text{mM}^{-1}\text{cm}^{-1}$.

Superoxide dismutase

For the analysis of Superoxide dismutase (SOD) activity, cotton leaves were emulsified in a solution containing 50 mM potassium phosphate buffer (pH 7.8)⁴³. To determine SOD activity, the samples were exposed to white light for 10 min after adding reagents [400 μl water, 250 μl of 0.2 M KH_2PO_4 buffer, 100 μl L-methane, 100 μl Triton X, 50 μl nitro blue tetrazolium]. The activity of SOD was measured by analyzing its capability of inhibiting the photochemical reduction of nitro blue tetrazolium (NBT) using a previously developed method⁴⁴. The quantity of enzyme that induced 50% inhibition of NBT photochemical reduction is termed as one unit of SOD activity.

Peroxidase activity

The activity of peroxidase (POD) was analyzed by using the previously described method⁴⁵. To measure POD activity, cotton leaves were crushed in a solution containing 50 mM KH_2PO_4 buffer (pH 7.0). The POD activity was measured by using an assay solution (3 ml). The assay solution was made up of distilled water (535 μl), 200 mM potassium phosphate buffer, 100 μl of 200mM guaiacol, 100 μl of 400mM H_2O_2 , and 15 μl extracted leaf sample. The reaction started with the addition of the extract. The optical density at 470 nm was noted for 0 s, 20 s, 40 s, 60 s. The increase in absorbance was observed and a pink color was produced which indicates an enzyme reaction. One unit of POD activity is defined as a 0.01unit min^{-1} change in absorbance.

Catalase activity

Catalase (CAT) activity was estimated by using the method⁴⁶ with certain amendments. To estimate the catalase activity, the assay solution was made up of 50 mM KH_2PO_4 buffer, 59 mM H_2O_2 , and 100 μl sample (enzyme extract). After the addition of the sample, the enzyme reaction was initiated. A decreasing pattern in absorbance at 240 nm was recorded for 0 s, 30 s, and 60 s. One unit of CAT activity was defined as a 0.01unit min^{-1} change in absorbance. The enzyme activity was expressed on the basis of fresh leaf weight.

Ascorbate peroxidase activity

Ascorbate peroxidase (APX) activity was analyzed by using the previously described method⁴³. To estimate APX activity, cotton leaves were emulsified in a 50 mM potassium KH_2PO_4 buffer (pH 7.0) solution. To prepare the assay buffer 10 mM ascorbic acid, 0.5 M ethylenediaminetetraacetic acid (EDTA), 0.2 M potassium phosphate

buffer (pH 7.0) and 4Mm H_2O_2 were mixed simultaneously. For the determination of APX, 1 ml of assay buffer and 50 μ l sample were added, and the reaction was started when 1 ml of H_2O_2 was added. The ascorbic acid oxidation rate was calculated by measuring the decreasing trend in absorbance at 290 nm for 0 s, 30 s, and 60 s⁴⁷.

Total free amino acids

Total free amino acids were analyzed by using the following method⁴⁸. For the determination of total free amino acids, 50 mM potassium phosphate buffer (pH 7.0) solution. The assay mixture was prepared by 100 μ l sample, 10% pyridine (100 μ l) and 2% ninhydrin (100 μ l). Then, the mixture was warmed in the water bath for 30 min and made a volume up to 5 ml with distilled water. The optical densities of the mixture were recorded at 570 nm using a spectrophotometer.

Total soluble proteins

The estimation of quantitative protein was executed with the previously described method⁴⁹ by using a spectrophotometer (HITACHI, U2800). To estimate TSP, cotton leaf samples were homogenized in KH_2PO_4 buffer media. The assay solution contained sample extract (5 μ l) and 150 mM NaCl (95 μ l) mixed with 1.0 ml Coomassie Brilliant Blue dye (G-250, 100 mg). The solution was kept to develop a protein-dye complex for 5 min. Then, the optical densities were recorded at 595 nm.

Protease activity

For the determination of protease activity, cotton leaves were emulsified in a solution containing 50 Mm KH_2PO_4 buffer (pH 7.8)⁵⁰. The required assay solution was prepared by adding reagents [1% casein, 0.01 N NaOH, Tris base, and distilled water] and its pH 7.8 was maintained with H_3PO_4 . Protease activity was analyzed by casein digestion assay as described previously⁵¹. In this analytical protocol, one unit is that amount of enzyme, which releases acid soluble fragments equivalent to 0.001 A280 per minute at 37 °C and pH 7.8. Enzyme activity was expressed on a fresh weight basis.

Amylase activity

The amylase activity of cotton leaves was analyzed by using the following method⁵². For the estimate of α -amylase, two reagents were used. The first solution was the 3,5-dinitro salicylic acid (DNS), which was prepared by dissolving 1 g of DNS in distilled H_2O , followed by sodium-potassium tartrate tetrahydride and 2 N NaOH. The second solution was a 1% starch solution. The reaction mixture was incubated for 3 min after adding 200 μ l sample extract and 1 ml starch solution, then heated in a water bath for 15 min after adding DNS. Cool down and made volume up to 9 ml with dH_2O . Spectroscopically, optical densities were recorded at 540 nm⁵³.

Esterase activity

Esterase activity was analyzed by using the Fast Blue BB method⁵⁴. The chemical α -naphthyl acetate used as substrate. The reaction mixture contains the assay solution [30mM -naphthyl acetate, 1% acetone, phosphate buffer (0.04 M)] and enzyme extract. The resulting mixture was incubated in the dark for 10 min at 27°C. After 15 min, 1 ml of staining solution (1% Fast blue BB dye and SDS 5% in a 2:5 ratio) was added and incubated in the dark for 20 min at 30°C. The optical densities of the colour compound produced were recorded at 590 nm using a spectrophotometer. Standard curves were prepared by using standards and the enzyme activity (α -naphthyl acetate esterase) was measured in μ M min⁻¹ /g wt.

Total sugar content

The total sugar content in cotton leaf samples were determined using the method and the phenol-sulfuric acid reagent method⁵⁵. For determination of total sugar level, the assay mixture was prepared by 100 μ l sample mixed with 400 μ l distilled water, 250 μ l 5% phenol, and 1.25 ml H_2SO_4 . Then, we placed for 20 min at 27°C and the reading was recorded at 490 nm using a spectrophotometer.

Reducing sugars

For the estimation of reducing sugar in cotton leaf samples using the dinitrosalicylic acid (DNS) method⁵⁶. To estimate the reducing sugar level of cotton leaves, the 100 μ l sample was mixed with 500 μ l DNS and 900 μ l distilled water. Placed in the water bath for 15 min and cooled at 25°C. Then added 4.5 ml distilled water, and the optical densities of the solution were recorded at 540 nm using a UV-VIS spectrophotometer.

Non-reducing sugar

The non-reducing sugars were then estimated as the difference between the reducing sugars and total soluble sugars.

Pigments analysis

The amount of chlorophyll (a and b) was determined by following the pigment analysis method⁵⁷ whereas carotenoids were determined following the method⁵⁸. To extract pigments, 75 mg of cotton leaf samples were emulsified in 80% acetone (80 ml acetone and 20ml H_2O) and incubated at room temperature for 1 day in the dark. After 1 day, it was centrifuged for 5 min at 14,000 rpm. Then, the optical densities of the supernatant were measured at 645, 663, 505, 453, and 470 nm.

Proline

Cotton leaf samples (0.1 g) were emulsified in 3% sulfosalicylic acid solution (2 ml) in test tubes, vortexed for 10 s, and left for 5 min. Free proline assays were performed on filtered extracts using the procedure⁵⁹. For the

assay, proline extract (1 mL) was treated with 2% ninhydrin solution (1 ml per sample) followed by the addition of glacial acetic acid (1 ml). Then, each sample was vortexed for 10 s and heated for 1 h in the water bath at 95–98 °C. After immediately, cooled in an ice bath to terminate the reaction. Toluene 5 ml was added to each sample and kept for 1–2 min at 27 °C. The proline content in the toluene fraction was determined by recording the optical densities at 520 nm using a UV-vis spectrophotometer against a toluene blank. The ultimate findings were expressed as $\mu\text{mol proline/g}$ of frozen or fresh leaf samples⁶⁰.

Statistical analysis

Microsoft Excel and XLSTAT were used for descriptive analysis and all other statistical analysis was performed in RStudio version 1.0.153 including the three-way ANOVA, histogram (by using the “ggplot2” package)⁶¹, and correlation⁶². Principal component analysis (PCA) was performed by using XLSTAT. Best performing genotypes were differentiated using Multi-Trait Genotype-Ideotype Distance Index (MGIDI) as explained in⁶³ with the help of the “metan” package in R⁶⁴ however selection pressure was kept 50%. PCA (principal component analysis) was performed by using the “factoextra” package in R to minimize the number of dimensions in the data⁶⁵.

Results

Physiological parameters

Stomatal diffusive resistance

Under the control conditions, stomatal diffusive resistance was found highest in cotton genotype N-868 ($0.421 \pm 0.03 \text{ s cm}^{-1}$) while it was lowest in N-9078 ($0.298 \pm 0.01 \text{ s cm}^{-1}$). Under low moisture stress, stomatal diffusive resistance was highest in cotton genotypes N-375 and FH-326 ($0.346 \pm 0.008 \text{ s cm}^{-1}$), however, it was found lowest in N-4496 ($0.272 \pm 0.02 \text{ s cm}^{-1}$). Under low moisture stress conditions, stomatal diffusive resistance was non-significantly increased in N-926, N-123, N-878, FH-326, and FH-490 compared to that of control (Fig. S1a).

Transpiration rate

Under low moisture stress, the transpiration rate was found highest in genotype N-4496 ($76.88 \pm 4.07 \mu\text{g cm}^{-2} \text{ s}^{-1}$), while it was lowest in N-1296 ($46.36 \pm 5.6 \mu\text{g cm}^{-2} \text{ s}^{-1}$). Transpiration rate was highest in N-4496 ($71.66 \pm 1.2 \mu\text{g cm}^{-2} \text{ s}^{-1}$) under the control condition while it was lowest in genotype N-377 ($48.85 \pm 4 \mu\text{g cm}^{-2} \text{ s}^{-1}$). Transpiration rate under stress treatment was observed retained in all genotypes except for N-377, N-868, N-896, and N-1296 (Fig. S1b). It was significantly increased in N-377 under low moisture stress conditions.

Leaf temperature

Under the control conditions, the leaf temperature was found highest in cotton genotype N-7012 ($35.80 \pm 0.6 \text{ }^\circ\text{C}$), while it was lowest in N-990 ($34.58 \pm 0.9 \text{ }^\circ\text{C}$). Leaf temperature was highest in genotype N-878 ($36.62 \pm 0.09 \text{ }^\circ\text{C}$) under low moisture stress, however, it was lowest in genotype N-213 ($35.01 \pm 0.3 \text{ }^\circ\text{C}$). Low moisture stress non-significantly affects leaf temperature in comparison to that under control (Fig. S1c).

SPAD measurements

Under the control conditions, the spade value was found highest in genotype N-512 (40.411 ± 0.3), while it was lowest in N-9078 (35.322 ± 0.5). SPAD measurement was highest in N-1286 (40.556 ± 0.7) under low moisture stress while it was lowest in genotype N-153 (31.222 ± 1.6). Non-significant differences in SPAD measurements were found under low moisture stress (Fig. S1d).

Relative water content.

Under low moisture stress conditions, relative water content (RWC) was found highest in cotton genotype N-T86 ($86.49 \pm 1.2\%$), while it was lowest in N-912 ($81.51 \pm 1\%$). RWC was highest in N-121 ($95.87 \pm 0.8\%$) under control conditions and lowest in genotype N-3496 ($81.86 \pm 1.3\%$). Overall, there were no significant genotypic differences in RWC among cotton genotypes under low moisture stress (Fig. S1e).

Morphological parameters

Dry and fresh root weight

Under control conditions, fresh root weight was found highest in genotype N-121 ($2.133 \pm 0.2 \text{ g}$), while it was lowest in N-868 ($0.933 \pm 0.1 \text{ g}$). Fresh root weight was highest in N-512 ($2.66 \pm 0.2 \text{ g}$) under low moisture stress while it was lowest in genotype N-T8411 ($0.87 \pm 0.1 \text{ g}$). Fresh root weight under stress was observed retained in all genotypes except for N-512 and N-T8411, in which it was increased/decreased compared to that under control (Fig. S2a).

Dry root weight was highest in N-512 ($1.578 \pm 0.1 \text{ g}$) under low moisture stress while it was lowest in genotype N-1496 ($0.429 \pm 0.1 \text{ g}$). Under control conditions, dry root weight was found highest in genotype N-812 ($1.419 \pm 0.1 \text{ g}$), while it was lowest in N-868 ($0.719 \pm 0.08 \text{ g}$). Overall, it was observed that low moisture stress had non-significant effects among the cotton genotypes (Fig. S2b).

Fresh and dry shoot weight

Fresh shoot weight was found highest in N-9078 ($18.74 \pm 1.2 \text{ g}$) under low moisture stress while it was lowest in genotype N-T8411 ($5.11 \pm 1.3 \text{ g}$). Under control conditions, fresh shoot weight was found highest in genotype N-812 ($24.21 \pm 1.3 \text{ g}$), while it was lowest in N-868 ($9.30 \pm 1.1 \text{ g}$). However, genotypic differences were found non-significantly in all cotton genotypes under non-stress and stress conditions (Fig. S2c).

Under low moisture stress, dry shoot weight was found highest in genotype N-9078 ($9.91 \pm 0.2 \text{ g}$), while it was lowest in N-T8411 ($2.37 \pm 0.1 \text{ g}$). Dry shoot weight was highest in N-812 ($12.14 \pm 0.07 \text{ g}$) under control

conditions while it was lowest in genotype N-868 (3.95 ± 0.5 g). Dry shoot weight was observed to non-significantly increase/decrease in all cotton genotypes under low moisture stress (Fig. S2d).

Fresh and dry leaf weight

Under control conditions, fresh leaf weight was found highest in cotton genotype N-T86 (0.673 ± 0.02 g) and it was lowest in FH-490 (0.593 ± 0.01 g). Under low moisture stress, fresh leaf weight was highest in cotton genotype N-T86 (0.682 ± 0.02 g), however, it was found lowest in FH-490 (0.578 ± 0.01 g). Fresh leaf weight (Fig. S3a) and dry leaf weight (Fig. S3b) were observed to be least affected by low moisture stress. However, non-significant genotypic differences were observed in all cotton genotypes.

Root and shoot lengths

Under low moisture stress, root lengths were found highest in genotype N-377 (23.88 ± 1.9 cm), while it was lowest in N-990 (16.58 ± 0.7 cm). Root lengths were highest in N-1296 (27.77 ± 0.7 cm) under control conditions while it was lowest in genotype N-213 (14.33 ± 0.6 cm). Under low moisture stress, an increase in root length was observed for all genotypes with the exception of N-1296, and N-1237 in which it was decreased. Overall, the marked retained root length was observed among the genotypes (Fig. S3c).

Under low moisture stress, shoot length was found highest in genotype N-9078 (34.1 ± 1.2 cm), while it was lowest in FH-490 (20.8 ± 1.2 cm). Shoot length was highest in N-812 (40.1 ± 1.3 cm) under control conditions while it was lowest in genotype FH-490 (23.6 ± 1.08 cm). As compared to control conditions, there were no significant genotypic differences for the shoot lengths under low moisture stress. It was found maintained in N-1286, N-T86, and N-213 under stress and non-stress conditions (Fig. S3d).

Biochemical analysis

Total phenolic content

Under control conditions, total phenolic content (TPC) was found highest in genotype N-1296 (26575 ± 2275 $\mu\text{M/g}$ f. wt.), while it was lowest in N-374 (14775 ± 275 $\mu\text{M/g}$ f. wt.). TPC was noted highest in N-4496 (25175 ± 425 $\mu\text{M/g}$ f. wt.) under low moisture stress, however, it was lowest in genotype N-135 (15100 ± 1450 $\mu\text{M/g}$ f. wt.). Under low moisture stress treatment, an increase in total phenolic content was noted in N-9078, N-7012, N-T86, N-374, N-375, FH-326 and FH-490 and it was decreased in N-868, N-696, N-1296, N-377 and N-926 (Fig. S4a). Overall, significant decrease in TPC was found in N-1296 out of 30 cotton genotypes.

Total tannins

Under control conditions, total tannins content (TTC) was found highest in genotype N-1296 (3750 ± 250 $\mu\text{M/g}$ f. wt.), while it was lowest in N-375 (100 ± 25 $\mu\text{M/g}$ f. wt.). TTC was noted highest in N-T86 (4075 ± 125 $\mu\text{M/g}$ f. wt.) under low moisture stress, however, it was lowest in genotype N-696 (625 ± 25 $\mu\text{M/g}$ f. wt.). Under low moisture stress, significantly increased TTC was observed in the 17 cotton genotypes N-868, N-3496, N-990, N-7012, N-1237, N-912, N-T86, N-377, N-135, N-812, N-374, N-375, N-926, N-213, N-878, FH-326 and FH-490 out of 30 (Fig. S4b).

Total flavonoid content

Under low moisture stress, total flavonoid (TF) content was found highest in genotype N-121 (160.9 ± 5.03 $\mu\text{g/ml}$), while it was lowest in N-512 (115.9 ± 1.3 $\mu\text{g/ml}$). Under control conditions, TF content was observed highest in N-896 (162.03 ± 2.9 $\mu\text{g/ml}$), however, it was lowest in genotype N-375 (115.38 ± 0.7 $\mu\text{g/ml}$). TF contents were maintained in 8 cotton genotypes N-1296, N-1286, N-4496, N-1237, N-7012, and N-213, N-878 and FH-490 out of 30 under non-stress and stress conditions (Fig. S4c). Overall, a significant increase in TF content was found in cotton genotypes N-121 and N-T86.

Ascorbic acid

Under low moisture stress, ascorbic acid (AsA) content was found to highest in genotype N-868 (643 ± 5 $\mu\text{g/g}$ f. wt.), however it was lowest in N-4496 (602.2 ± 3.2 $\mu\text{g/g}$ f. wt.). Under control conditions, AsA content was observed highest in N-1237 (642.5 ± 0.5 $\mu\text{g/g}$ f. wt.), however, it was lowest in genotype FH-490 (604.2 ± 4.7 $\mu\text{g/g}$ f. wt.). Under low moisture stress treatment, significant increase in AsA content was found in N-1296 and FH-490 out of 30 cotton genotypes (Fig. S5a).

Total oxidants status

Under control conditions, total oxidants status (TOS) was found highest in genotype N-512 (18085 ± 715 $\mu\text{M/g}$ f. wt.) however, it was lowest in N-T8411 (5300 ± 200 $\mu\text{M/g}$ f. wt.). TOS observed highest in N-153 (15400 ± 100 $\mu\text{M/g}$ f. wt.) under low moisture stress, however, it was lowest in genotype N-912 (6822.5 ± 177.5 $\mu\text{M/g}$ f. wt.). TOS was significantly increased in 7 cotton genotypes N-868, N-T8411, N-1496, N-T86, N-153, N-213 and FH-490 under low moisture stress, while it was retained in 2 genotypes N-1296 and N-926 only (Fig. S5b).

Malondialdehyde contents

Under control conditions, malondialdehyde content (MDA) was found highest in genotype N-912 (476.1 ± 13.1 $\mu\text{M/g}$ f. wt.) however, it was lowest in N-153 (285.6 ± 17.8 $\mu\text{M/g}$ f. wt.). Under low moisture stress, MDA was noted highest in N-812 (472.8 ± 17.6 $\mu\text{M/g}$ f. wt.), however, it was lowest in genotype N-696 (192.3 ± 19.7 $\mu\text{M/g}$ f. wt.). Under low moisture stress in N-990 and N-153, a significant increase in MDA was observed. A significant decrease was noted in N-696 followed by N-896, N-8437, and N-374 under low moisture stress conditions (Fig. S5c).

Superoxide dismutase

Under low moisture stress, superoxide dismutase (SOD) activity was found highest in genotype N-812 (337.02 ± 4.3 Units/g f. wt.) however, it was lowest in N-135 (43.96 ± 7.3 Units/g f. wt.). Under control conditions, SOD activity was observed highest in N-375 (228.76 ± 17.6 Units/g f. wt.), however, it was lowest in genotype N-1496 (19.96 ± 3.1 Units/g f. wt.). Low moisture stress caused a significant increase in SOD activity in 12 cotton genotypes N-1096, N-1296, N-1286, N-1496, N-4496, N-990, N-7012, N-1237, N-912, N-512, N-812 and N-153 out of 30. SOD activity was found maintained only in N-896 under non-stress and stress conditions (Fig. S6a).

Peroxidase activity

Under low moisture stress, peroxidase (POD) activity was found highest in genotype N-896 (12287.7 ± 1098.3 Units/g f. wt.) while it was lowest in N-135 (1764.9 ± 99.9 Units/g f. wt.). Under control conditions, POD activity was observed highest in N-896 (9290.7 ± 699.3 Units/g f. wt.), however, it was lowest in genotype N-812 (2564.1 ± 166.5 Units/g f. wt.). Low moisture stress caused a significant increase in POD activity in N-896, N-1496, N-812, N-121, and N-153. However, a significant decrease in plant POD activity was noted in FH-490 (Fig. S6b).

Catalase activity

Under low moisture stress, Catalase activity was found highest in genotype N-512 (510 ± 10 Units/g f. wt.) while it was lowest in N-T8411 (70 ± 10 Units/g f. wt.). Under control conditions, catalase activity was observed highest in N-696 (300 ± 20 Units/g f. wt.), however, it was lowest in genotypes N-1296, N-T8411, N-7012 and n-377 (70.0 ± 10.0 Units/g f. wt.). Catalase activity was maintained under low moisture stress in all cotton genotypes except for N-1296, N-3496, N-9078, N-990, N-7012, N-812, N-512, N-374, N-213 and FH-490 in which increased activity was observed (Fig. S6c). Overall, a significant increase in catalase activity was observed in N-512 and a significant decrease was observed in genotypes N-696 and N-896.

Ascorbate peroxidase

Under control conditions, ascorbate peroxidase (APX) activity was found highest in cotton genotypes N-1296, and N-375 (420 ± 20 Units/g f. wt.), while it was lowest in N-8437 (140 ± 20 Units/g f. wt.). Under low moisture stress, APX activity was observed highest in N-812 (600 ± 40 Units/g f. wt.), however, it was lowest in genotype N-375 (120 ± 0 Units/g f. wt.). Under low moisture stress in genotype N-812, a significant increase in the APX activity was noted and a significant decrease was found in N-1296 and N-375 (Fig. S7a).

Total free amino acids

Under low moisture stress, total free amino acids were found highest in genotype N-3496 (14.74 ± 0.1 $\mu\text{g/g}$ f. wt.), while it was lowest in N-926 (9.03 ± 0.5 $\mu\text{g/g}$ f. wt.). Under the control conditions, total free amino acids were observed highest in N-868 (12.57 ± 0.5 $\mu\text{g/g}$ f. wt.), however, it was lowest in genotype N-374 (7.74 ± 0.1 $\mu\text{g/g}$ f. wt.). Under low moisture stress, a significant increase was observed in N-3496, N-9078, N-T86, N-153, and FH-490. Total free amino acids were observed to increase/decrease in leftover cotton genotypes, but these differences were non-significant under non-stress and stress conditions (Fig. S7b).

Total soluble proteins

Under the control conditions, total soluble proteins (TSP) were found highest in cotton genotype N-512 (210 ± 3.3 mg/g f. wt.), while it was lowest in N-377 (86.6 ± 5.3 mg/g f. wt.). Under low moisture stress TSP was observed highest in N-812 (220.3 ± 3.6 mg/g f. wt.), however, it was lowest in genotype N-926 (94 ± 2 mg/g f. wt.). Under low moisture stress in genotypes N-377 and N-812, a significant increase in TSP was observed. Significant decrease was found in N-1496, N-7012, N-1237, N-153, N-926, and N-375 (Fig. S7c).

Protease activity

Under control conditions, protease activity was found highest in cotton genotypes N-812 (3230 ± 10 Units/g. f. wt.), while it was lowest in N-1286 (2250 ± 10 Units/g. f. wt.). Under low moisture stress, protease activity was observed highest in N-375 (3160 ± 50 Units/g. f. wt.), however, it was lowest in genotype N-135 (2520 ± 140 Units/g. f. wt.). Under low moisture stress, a significant increase was noted in N-1286 and a significant decrease was found in N-812 (Fig. S8a).

Amylase activity

Under low moisture stress, amylase activity was found highest in genotype N-153 (115.4 ± 4.5 mg/g. f. wt.), while it was lowest in N-868 (9.43 ± 1.8 mg/g. f. wt.). Under the control conditions, amylase activity was observed highest in N-878 (122.2 ± 1.5 mg/g. f. wt.), however, it was lowest in genotype N-512 (11.88 ± 1.6 mg/g. f. wt.). Low moisture stress caused a significant increase in plant amylase activity in N-8437, and N-512 (Fig. S8b).

Esterase activity

Under low moisture stress, genotype N-213 had the maximum esterase activity (116.9 ± 0.3 $\mu\text{M}/\text{min}/\text{g}$ f. wt.), while genotype N-T86 had the lowest (110.14 ± 0.1 $\mu\text{M}/\text{min}/\text{g}$ f. wt.). Under control conditions, esterase activity was highest in genotype FH-490 (117.15 ± 0.7 $\mu\text{M}/\text{min}/\text{g}$ f. wt.), but lowest in genotype N-868 (97.01 ± 6.6 $\mu\text{M}/\text{min}/\text{g}$ f. wt.). Under low moisture stress, an increase in esterase activity was found in 13 genotypes N-868, N-896, N-696, N-1296, N-1286, N-8437, N-3496, N-9078, N-1237, N-926, N-374, N-213 and N-878. Overall, all cotton genotypes had non-significant results for esterase activity (Fig. S8c).

Total soluble sugars

Under the control conditions, total soluble sugar (TSS) was found highest in cotton genotype N-121 (37.03 ± 0.2 mg/g f. wt.), while it was lowest in N-1296 (21.85 ± 0.2 mg/g f. wt.). Under low moisture stress, TSS was observed highest in N-1286 (41.93 ± 1.4 mg/g f. wt.), however, it was lowest in genotype N-8437 (20.11 ± 0.8 mg/g f. wt.). Under low moisture stress, in 11 genotypes N-1206, N-1286, N-4496, N-9078, N-1237, N-912, N-512, N-374, N-926, N-812 and FH-326 a significant increase in TSS was observed (Fig. S9a).

Reducing sugars

Under low moisture stress, reducing sugars was found highest in genotype N-121 (16.44 ± 0.4 mg/g f. wt.), while it was lowest in N-990 (11.31 ± 0.6 mg/g f. wt.). Under the control conditions, reducing sugar was observed highest in N-213 (15.76 ± 0.1 mg/g f. wt.), however, it was lowest in genotype N-696 (8.65 ± 0.6 mg/g f. wt.). Under low moisture stress, a significant increase in reducing sugar was observed in N-696. While, it was maintained in N-896, N-1096, N-1496, N-4496, N-3496, N-9078, N-153 and N-213 (Fig. S9b).

Non-reducing sugars

Under the control conditions, non-reducing sugar was found highest in cotton genotype N-121 (24.38 ± 0.8 mg/g f. wt.), while it was lowest in N-4496 (7.0 ± 0.1 mg/g f. wt.). Under low moisture stress, non-reducing sugar was observed highest in N-1286 (26.17 ± 0.5 mg/g f. wt.), however, it was lowest in genotype N-8437 (3.79 ± 1.2 mg/g f. wt.). Under low moisture stress in 6 genotypes N-1286, N-4496, N-9078, N-990, N-812, and N-512 out of 30 a significant increase in non-reducing sugar was observed. And a significant decrease was found in N-153 (Fig. S9c).

Lycopene

Under control conditions, lycopene was found highest in cotton genotypes N-374 (12.69 ± 0.2 mg/g f. wt.), while it was lowest in N-375 (9.6 ± 0.1 mg/g f. wt.). Under low moisture stress, lycopene was observed highest in N-135 (13.06 ± 0.3 mg/g f. wt.), however, it was lowest in genotype N-868 (10.97 ± 0.1 mg/g f. wt.). Low moisture stress caused an increase in lycopene content among all genotypes except N-868, N-374, N-926, and FH-326 in which it was reduced. However, lycopene content was maintained in N-912 (Fig. S10a).

Chlorophyll a

Under the control conditions, chlorophyll a content was found highest in cotton genotype N-374 (414.08 ± 7.7 ug/g f. wt.), while it was lowest in N-512 (357.1 ± 1.2 ug/g f. wt.). Under low moisture stress, chlorophyll a was observed highest in N-990 (427.5 ± 1.3 ug/g f. wt.), however, it was lowest in genotype N-135 (282.6 ± 20.09 ug/g f. wt.). Chlorophyll a content was generally less affected by the low moisture stress in most of the genotypes and differences were non-significant under non-stress and stress conditions (Fig. S10b).

Chlorophyll b

Under the control conditions, chlorophyll b content was found highest in cotton genotype N-912 (575.5 ± 15.6 ug/g f. wt.), while it was lowest in N-375 (460.1 ± 20.8 ug/g f. wt.). Under low moisture stress, chlorophyll b was observed highest in N-135 (631.05 ± 19.1 ug/g f. wt.), however, it was lowest in genotype N-868 (527.80 ± 7.07 ug/g f. wt.). The decreased chlorophyll b content was found in N-868, N-926, and FH-326. While it was increased in all leftover cotton genotypes non-significantly under low moisture stress (Fig. S10c).

Total carotenoids

Total carotenoid content was highest in cotton genotypes N-374 (15.88 ± 0.1 mg/g f. wt.) and lowest in N-512 (10.61 ± 0.4 mg/g f. wt.) under control conditions. Total carotenoids were found highest in genotype N-7012 (16.5 ± 0.2 mg/g f. wt.) and lowest in genotype FH-326 (12.9 ± 0.3 mg/g f. wt.) under low moisture stress. Carotene content was maintained in N-8437, N-3496, and N-374. Total carotene content increased while showing the least significant differences between non-stress and stress conditions (Fig. S11a).

Total chlorophyll

Under low moisture stress, total chlorophyll was found highest in genotype N-990 (1010.3 ± 0.02 ug/g f. wt.), while it was lowest in N-926 (913.1 ± 33.3 ug/g f. wt.). Under control conditions, total chlorophyll was observed highest in N-374 (987.6 ± 16.3 ug/g f. wt.), however, it was lowest in genotype N-375 (847.2 ± 40.2 ug/g f. wt.). Low moisture stress had the least significant difference in total chlorophyll content among the genotypes under the stress condition compared with that in control plants (Fig. S11b).

Proline

Under the control condition, proline was found highest in genotype N-896 (53.504 ± 1.9 ug/g f. wt.), while it was lowest in N-1096 (32.99 ± 0.2 ug/g f. wt.). Proline was observed highest in N-213 (54.07 ± 1.4 ug/g f. wt.) under low moisture stress, however, it was lowest in genotype N-912 (38.68 ± 0.5 ug/g f. wt.). Under low moisture stress, an increase in proline was found in cotton genotypes N-1096, N-1296, N-8437 N-1496, N-4496, N-1237, N-135, N-926, N-213 and N-878. In comparison with non-stress conditions, the genotypic differences for proline in all cotton genotypes under low moisture stress were observed non-significantly (Fig. S11c).

Correlation analysis

Correlation analysis (Pearson test) performed by using the XLSTAT 2011 version 2.06. It was performed for all the parameters with the alpha of 0.05. On the base of the correlation test, parameters that demonstrated significant correlations with one another (indirect effect) and with the genotypes of low moisture stress tolerance

(direct effect) were selected, in order to classify the cotton genotypes. Correlation maps of all tested traits under control (Fig. 1) and stress conditions (Fig. 2) were computed.

Cluster analysis

Cluster analysis was performed for grouping of the cotton genotypes under low moisture stress and control conditions by using morpho-physiological (Fig. 3) and biochemical traits (Fig. 4).

Principal component analysis

Principal component analysis performed by using all tested traits measured in controlled and low moisture stress conditions.

For the objective of maintaining all potential diversity among the tested cotton genotypes for the parameters under study, principal component analysis was used to reduce the dimensionality of all datasets in the interpretable approach. For further analysis, an important main component is determined by the eigenvalue. The eigen value > 1 represents total variation of approximately 10%⁶⁶. Principal component analysis (PCA) was performed on the data. Eigen value greater than 1 was the best indicator of all the attributes in principal components⁶⁷. Scree plot (Fig. 5) showed that, out of the total 29 principal components, twelve (PC-I to PC-XII) had Eigenvalues greater than 1 and 85.45% of overall cumulative variability was possessed by traits under control conditions. PC-I and PC-II together with a cumulative variability of 29.18%, were the largest contributors to the total cumulative variability in the genetic resource. While under low moisture stress, out of the total 29 principal components, thirteen (PC-I to PC-XIII) had Eigenvalues > 1 and carried 87.04% of the total cumulative variability under control conditions. The greatest contributors to overall cumulative variability in the genetic resource were PC-I and PC-II, with a cumulative variability of 27.46%.

To generate a genotype by trait (G-T) biplot for each genotype and its corresponding trait, the PC-I score (x-axis) was plotted against the PC-II scores (y-axis) under control (Fig. 6) and low moisture stress conditions (Fig. 7). In the genotype by trait (G-T) biplots, the interrelationships between attributes and a visual comparison of multiple traits were effectively demonstrated. The angles formed by the vectors and the distances of the variables from the biplot's origin were used to extract important data. The vector's angle to the principal component axis determines its contribution to that PC. The contribution of vectors to a particular PC increases with its parallelism to the principal component axis. In the correlation plot, the vector length represents the

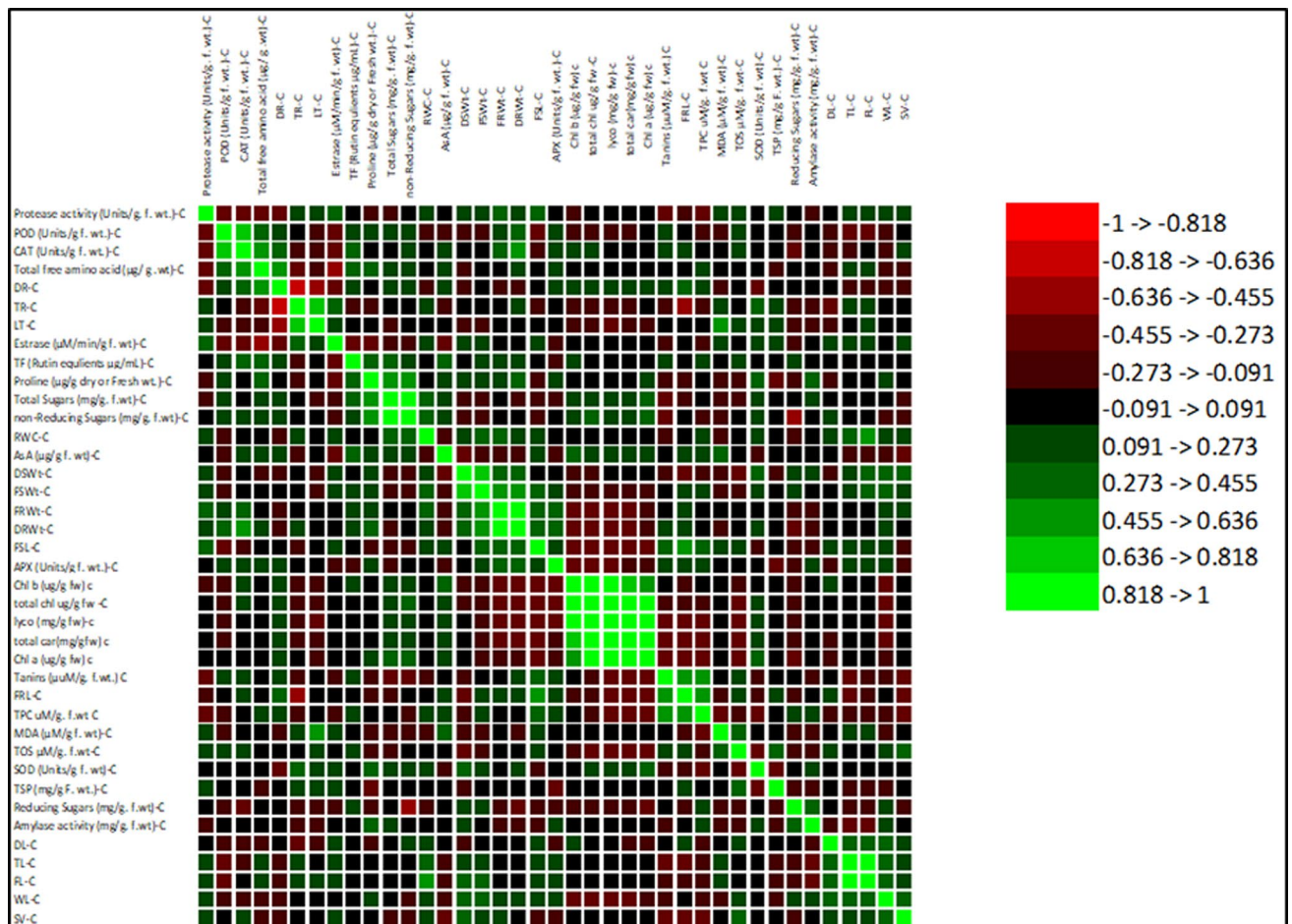


Fig. 1. Correlation of different parameters under control conditions.

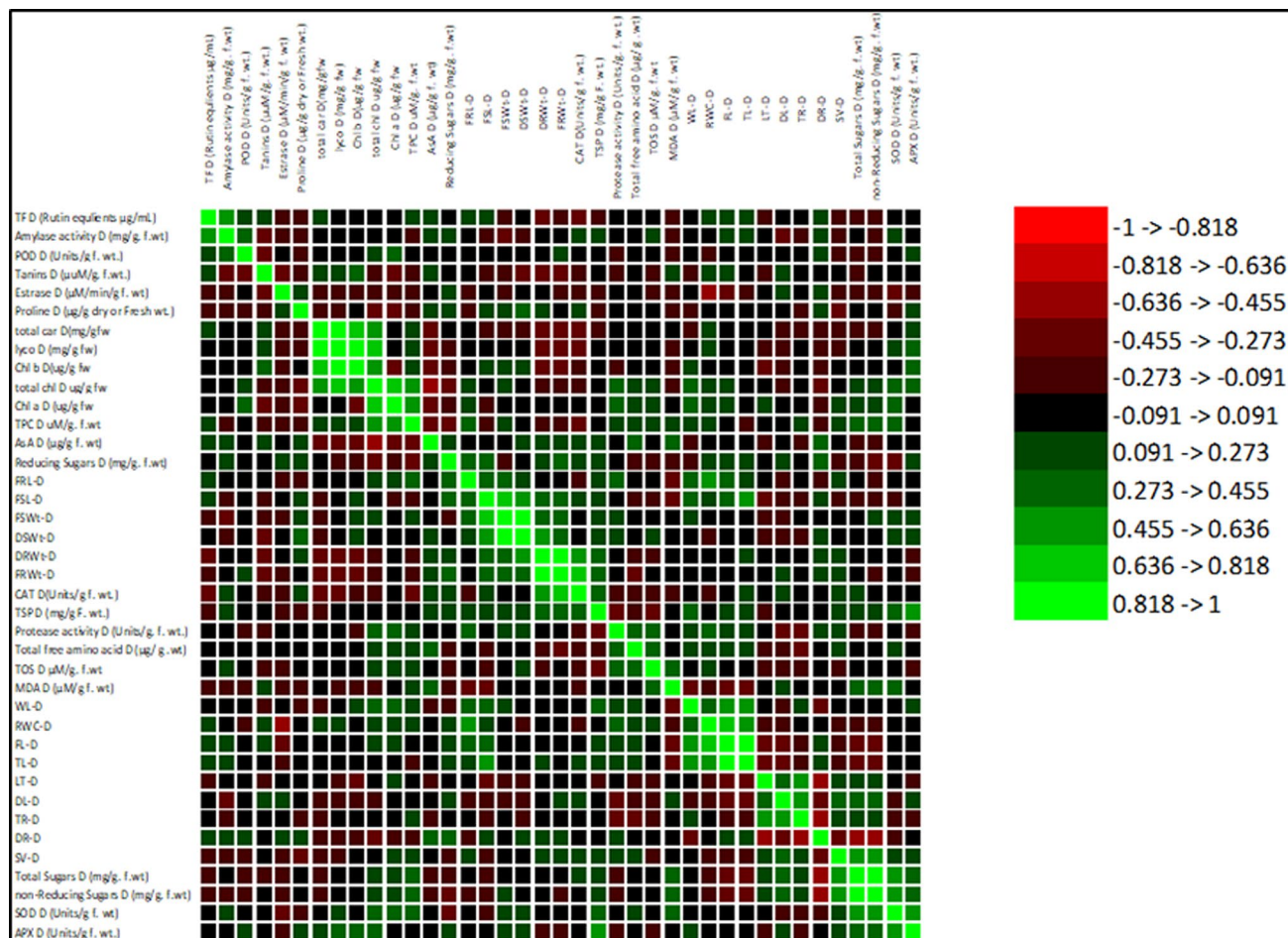


Fig. 2. Correlation of different parameters under low moisture stress conditions.

representativeness quality of the examined PCA dimensions. If the angle between the two vector traits was < 90 , the correlation between that two traits was considered as positive whereas if the angle between the two vector traits was larger than 90 , the correlation was regarded as negative. The angle of 90 between the attributes represented that the attributes were not dependent on one another⁶⁸.

Multi-trait Genotype-Ideotype Distance Index (MGIDI) analysis

For the selection of the best-performing genotypes, MGIDI was used for the ranking of cotton genotypes on the basis of multiple indices as explained earlier⁶⁹. We estimated the MGIDI index for both biochemical and morpho-physiological indices to identify the best-performing genotypes. For biochemical traits, 09 (TPC, TF, TSP, MDA, SOD, POD, CAT, APX, and Proline) out of 24 indices showed significant genotypic effect and used for MGIDI. Eight genotypes (N-812-1296 N-696 N-377 N-121-896 N-T86, and N-3496) were observed to be best performing than others at 25% selection pressure (SI = 25%) (Fig. 8).

For morpho-physiological traits, 14 (except RWC) out of 15 indices showed a significant genotypic effect and used for MGIDI. Ten genotypes (N-1237 N-812 N-1296 N-696 N-9078 N-377 N-512 N-121 N-375, and N-896) were observed to be best performing than others at 35% selection pressure (SI = 35%) (Fig. 9). Six genotypes, i.e. N-812-1296 N-696 N-377 N-121, and N-896, were common and best performed in both MGIDI based on biochemical and morpho-physiological traits.

The broad-sense heritability (h^2) in **biochemical traits** ranged from 0.3681 (SOD) to 0.7938 (POD) and in morpho-physiological traits ranged between 0.355 (TR) to 0.125 (DSW). Traits including TPC, POD, and TSP showed high value of h^2 ($h^2 > 0.6$) that shows that these traits exhibited the good prospect of selection gain. All traits showed desirable gain except TL that showed undesirable gain (Table 2).

Strength and weakness view of variables

A strength and weakness view was provided for all genotypes using biochemical (Figs. 8 and 9), and morpho-physiological traits which comprise total of four factors (FA1, FA2, FA3 & FA4) for each class of traits.

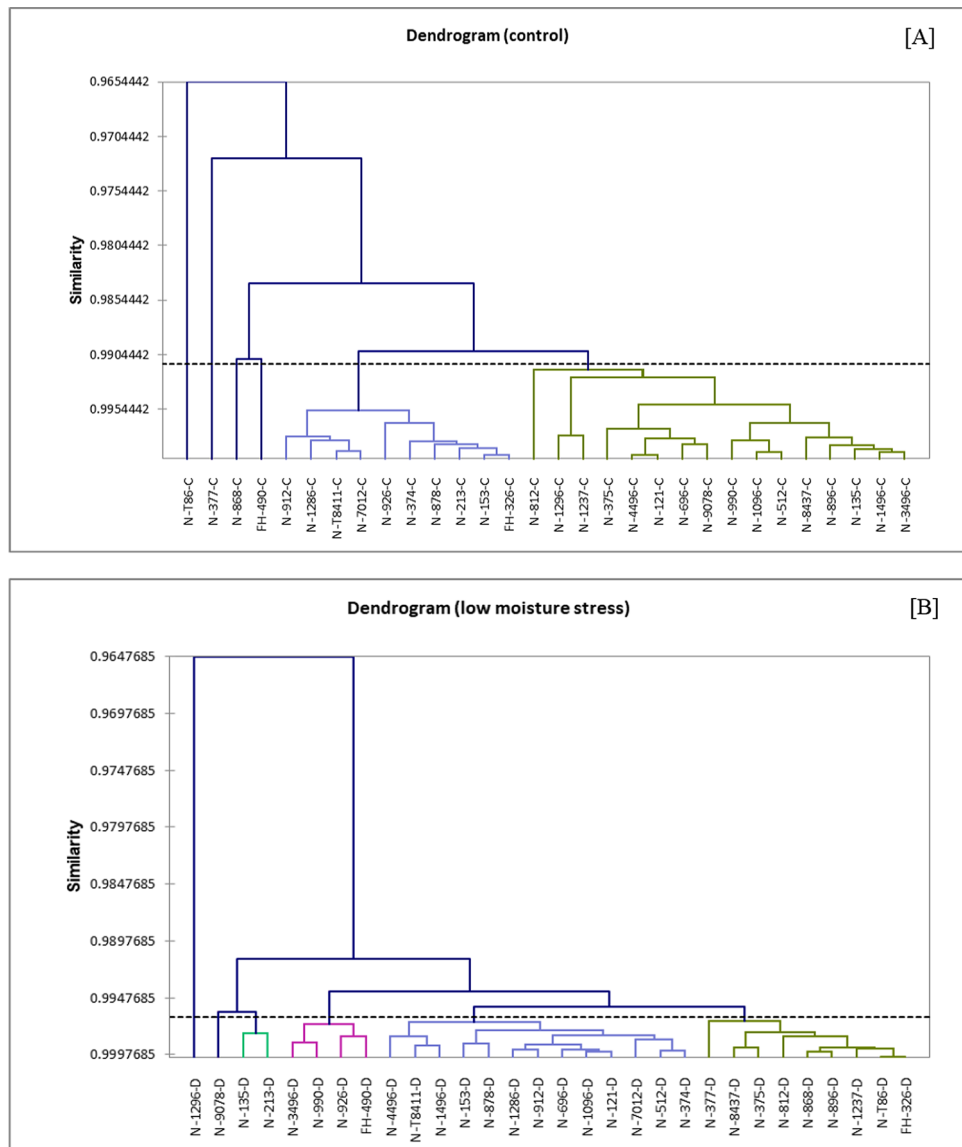


Fig. 3. Clustering of cotton genotypes using morpho-physiological attributes under control (A) and low moisture stress (B) conditions.

Discussion

Low moisture stress is an adverse environmental condition that has a significant impact on crop yield all over the world. An integrated morphological, physiological, and biochemical approach was used to analyze the low moisture stress responses in cotton, a plant of great economic importance in Pakistan. Water deficit tolerance mechanisms have remained a major concern for plant biologists due to the involvement of multiple parameters⁷⁰. According to previous research, the development and growth of cotton plants severely affected by drought stress⁷¹. Drought resistance is a polygenic trait⁷² that is correlated with various morphological, physiological, and biochemical parameters.

Screening of tolerant cotton genotypes for low moisture stress was the main objective of this research, as well as identification of best performing genotypes under adverse environmental conditions during post-germination seedling development. According to a previous report, tolerant cotton genotypes accumulated more compatible solutes than sensitive genotypes under drought stress. It could be because of higher levels of suitable solutes such as sugars, amino acids, proline, and polyphenols⁷³. Similar trend was observed for solute levels under low moisture stress in tested cotton genotypes in the present study.

Stomatal diffusive resistance can be utilized to interpret the effects of water stress⁷⁴. According to reports, the primary method to control transpiration in plants is stomatal conductance regulation⁷⁵. Stomatal resistance is the ability of mesophyll cells to withstand water evaporation from their outer surfaces, as well as water diffusion across intercellular gaps and the stomatal pore⁷⁶. The increased resistance of mesophyll cells causes a decrease in transpiration⁷⁷. In this experiment, stomatal diffusive resistance was maintained in 14 out of 30 cotton genotypes under low moisture stress conditions. Relative water content (RWC) is a helpful feature for identifying drought

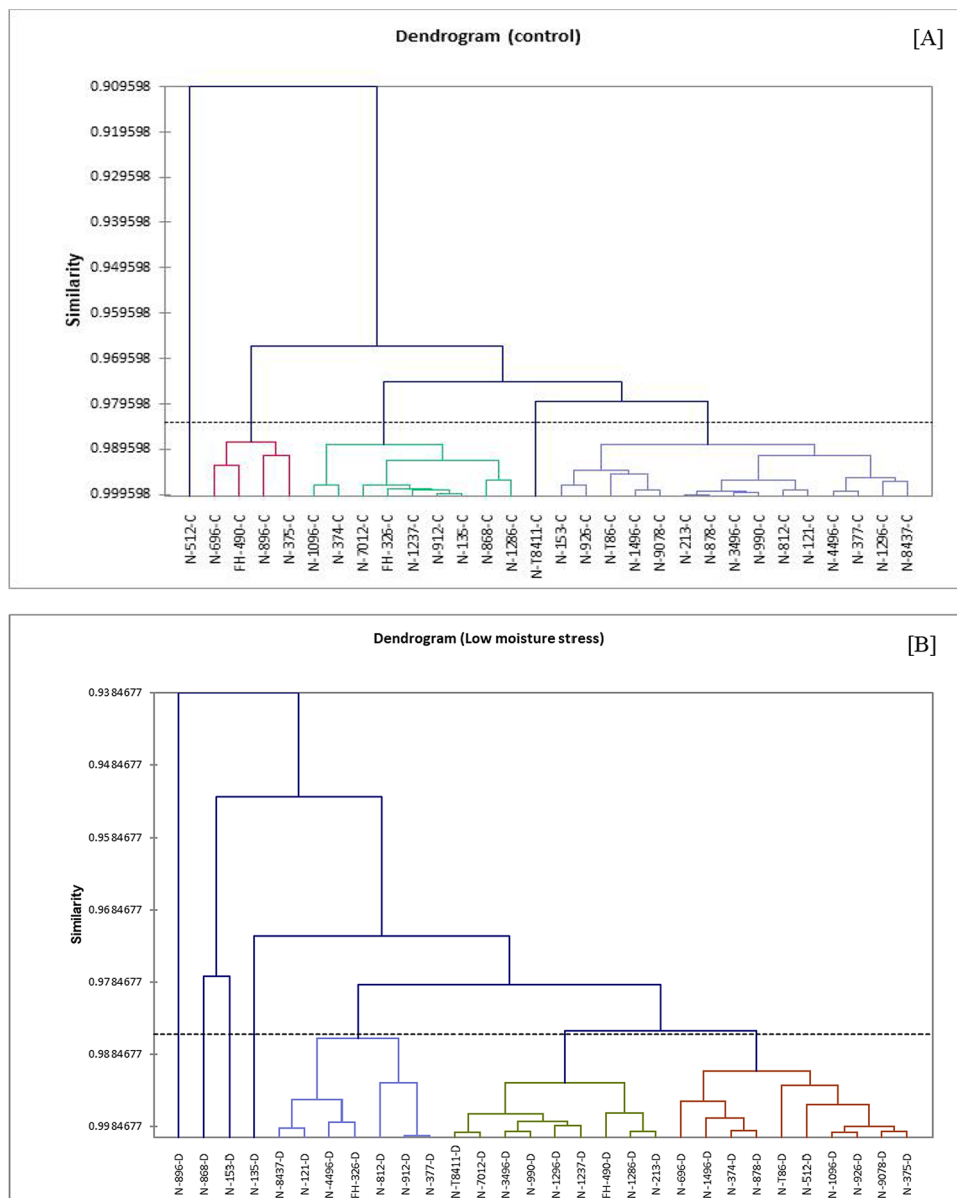


Fig. 4. Clustering of cotton genotypes by using biochemical indices in controlled (A) and low moisture stress (B) conditions.

stress since it is an indicator of water status in plant leaves⁷⁸. Previous studies reported that physiological factors greatly affect the RWC⁷⁹ and drought conditions also have an impact on it⁸⁰. In this study, low moisture stress tolerance was shown among genotypes that retained a high proportion of RWC except for N-121 and N-912 in which it was decreased compared to that under control. Overall, non-significant genotypic differences were observed. A similar pattern of RWC was observed in a previous study³ as cotton genotypes retained a high proportion of RWC.

Genetic variation in transpiration in some crops like sorghum⁸¹ and cotton⁸² in response to drying soil has been reported. In this study, the transpiration rate significantly increased in N-377 under low moisture stress conditions. It was retained in all genotypes except for N-377, N-868, N-896, and N-1296. Genotype N-377 was found to be low moisture stress tolerant based on transpiration rate. As previously reported, tolerant genotypes with a higher fraction of transpirable soil water thresholds can conserve water through decreasing the transpiration rate during the starting days of the drought cycle⁸².

Cotton cultivars had greater leaf temperatures in dry climates⁸³. In this study, leaf temperature was increased under low moisture stress in cotton genotypes N-696, N-926, N-213, N-1096, and FH-490. Overall, low moisture stress had a non-significant impact on leaf temperature compared to that under control.

In a previous study, the non-significant difference in SPAD values in cotton under water stress was reported¹⁸⁴. In this experiment, SPAD values were retained in genotypes N-T86, N-377, and N-121 under low moisture stress. Overall, there was no significant variation in SPAD values between low moisture stress and control in

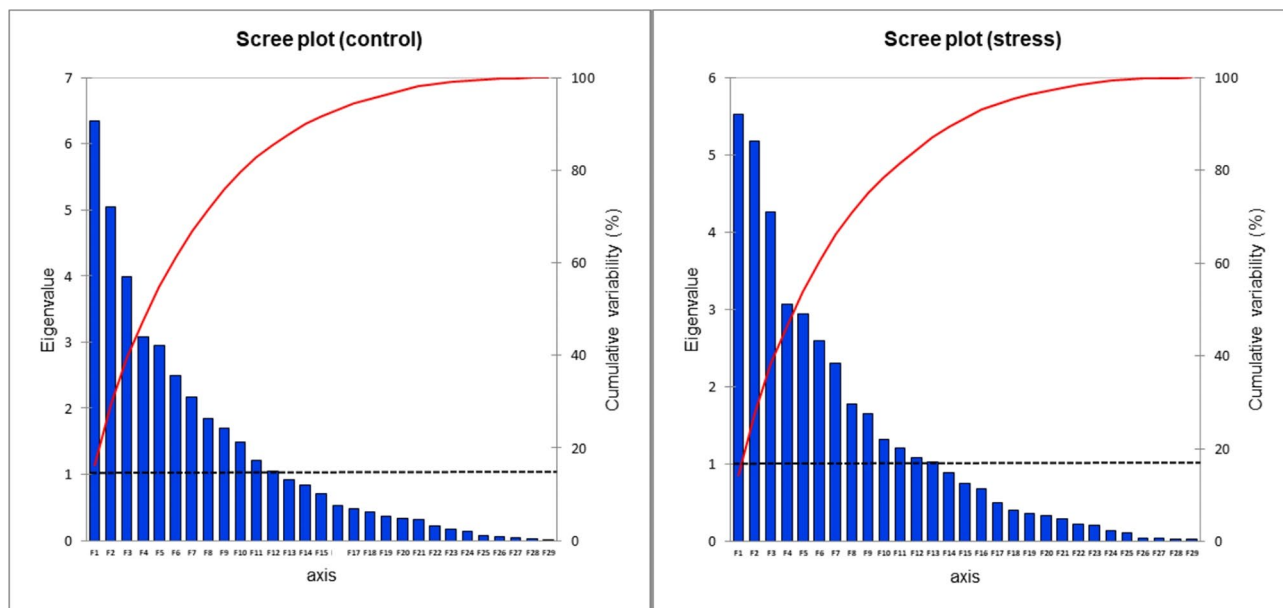


Fig. 5. Scree plots representing eigenvalues and cumulative variability for the trait under study.

cotton genotypes. Plant water status is an important element influencing crop production, and water potential is increasingly being used to estimate plant water status⁸⁵. It has been demonstrated in certain research that increased RL improves a plant's capacity to draw water from the deeper soil⁸⁶. In this experiment, the marked retained root length was observed among the genotypes. Increasing roots, on the other hand, may have a negative impact on shoot growth while more photosynthates move to the roots⁸⁷. In the current study, genotypic differences for shoot length under low moisture stress were non-significant. It was found maintained in N-1286, N-T86, and N-213. It was found that the shoots were more responsive to water deficit stress than the roots. The findings of the present study supported prior research^{88,89}. Fresh root weight under stress treatment was retained in all genotypes except for N-512 and N-T8411. Under low moisture stress treatment, dry and fresh shoot weight was observed increased in cotton genotype N-9078 and it was decreased in N-T8411. However, in dry and fresh root weight, dry and fresh shoot weight, and dry and fresh leaf weight genotypic differences were found non-significant in all cotton genotypes. Previous studies have reported the involvement of additive gene action in the measurements of dry weight, density, and root length⁹⁰.

When there is a water shortage, the leaf functions are greatly harmed, and toxic chemicals, such as ROS, begin to produce at an alarming rate. This happened because of the imbalance between the light capture and utilization of plant photosystems, which results in the formation of singlet oxygen, superoxide anions, hydroxyl radicals, and H_2O_2 ⁹¹. The activity of the cell system is disrupted when ROS affects the cell machinery⁹². Plants, on the other hand, have developed their metabolic machinery to deal with ROS. The plants ability to withstand oxidative stress depends on activation of antioxidant enzymes like POD, SOD, APX, and CAT⁹³. In this research, APX activity generally increased in cotton genotypes under low moisture stress, with a significant increase only in N-812 and a significant decrease in N-1296 and N-375.

Previously reported that SOD activity increased throughout the seedling stage in response to drought stress⁹⁴. In this experiment, low moisture stress caused a significant increase of SOD activity in 12 cotton genotypes N-1096, N-1296, N-1286, N-1496, N-4496, N-990, N-7012, N-1237, N-912, N-512, N-812 and N-153 out of 30. However, it caused significantly decreased SOD activity in N-868, N-696, N-T8411, N-135, N-374, N-926, N-213, N-878 and FH-490. SOD activity was found maintained only in N-896 under non-stress and stress conditions.

Low moisture stress caused a significant increase in peroxidase activity in N-896, N-1496, N-812, N-121, and N-153. While a significant decrease was found in FH-490. Previously, comparable findings have been reported in cotton genotypes under water deficit stress⁹⁵. Catalase activity was maintained under low moisture stress in all cotton genotypes except for N-1296, N-3496, N-9078, N-990, N-7012, N-812, N-512, N-374, N-213, and FH-490 in which increased activity was observed. Overall, a significant increase in catalase activity was found in N-512. However, in genotypes N-696 and N-896, it was decreased significantly. The same findings were also reported in soybeans⁹⁶, wheat⁹⁷, and cotton⁹⁸. In this research, the enzymes CAT, POD, SOD, and APX increased significantly ($p < 0.05$) in cotton genotypes compared to that under control.

Previous studies reported that plant cultivars with greater water deficit tolerance had less MDA content under stressed conditions⁹⁹. In general, MDA is used to identify the lipid peroxidation in crops. MDA is a widely used measure of oxidative lipid damage caused due to abiotic stress. MDA concentration and lipid peroxidation have a positive association, and it may damage cell wall integrity^{100,101}. In the present experiment, Melandialdehyde (MDA) varied significantly in most cotton genotypes. Under low moisture stress in N-990 and N-153, MDA was increased significantly. While, significant decrease was noted in N-696 followed by N-896, N-8437, and N-374. It

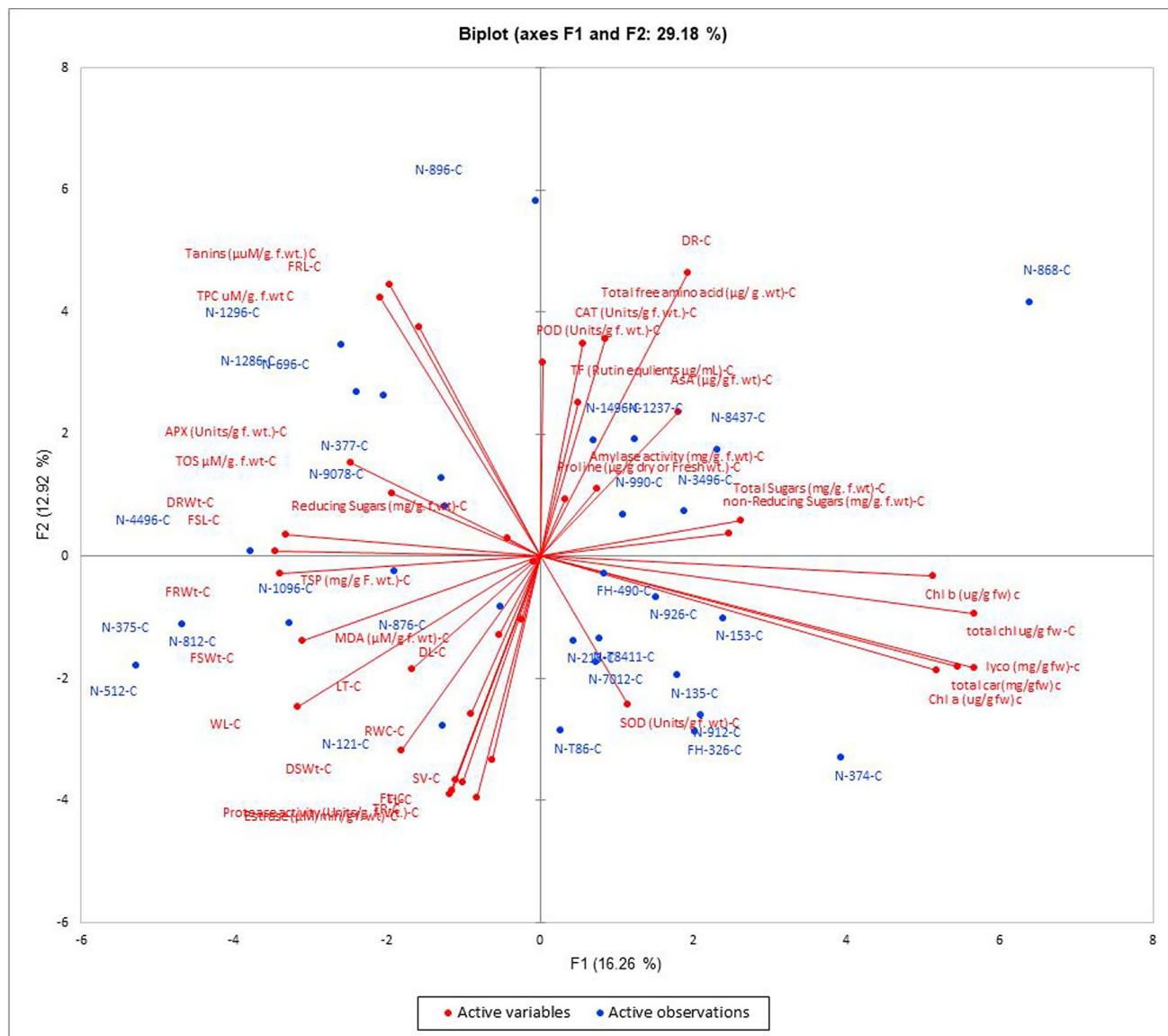


Fig. 6. Principal component analysis of all attributes under control conditions.

demonstrates the inability of other genotypes to deal with the high rate of ROS production. Previous research has found the same susceptibility pattern¹⁰². The antioxidant activity of plants varies. Scientists hypothesized that the variation in antioxidant activity in genotypes could be caused by a variety of variables, including differences in the degree of stomata closure or other reactions that alter the CO₂ fixation degree, or the difference that causes photo-inhibition¹⁰³.

Phenolic compounds are important secondary metabolites in plants that have redox characteristics and are components of antioxidants (non-enzymatic)¹⁰⁴. In response to abiotic stress conditions, plants produce more polyphenols like phenolic acids and flavonoids that help the plant to cope with environmental limitations¹⁰⁵. In this study, the genotypic differences of total phenolic contents under low moisture stress which were observed were non-significant. However, total phenolic contents were maintained in N-896, N-1496, N-4496, N-3496, N-990, N-912 and N-812 under non-stress and stress conditions. Overall, a significant decrease in TPC was found in N-1296 out of 30 cotton genotypes.

It is reported that drought stress increased the total tannin (TT) content, total flavonoids (TF), and total phenolics (TP) in the bean leaves¹⁰⁶ and cotton¹⁰⁷ compared to that under control. In this research experiment, under low moisture stress, a significant increase in the total tannins content was found in 17 cotton genotypes N-868, N-3496, N-990, N-7012, N-1237, N-912, N-T86, N-377, N-135, N-812, N-374, N-375, N-926, N-213, N-878, FH-326 and FH-490 out of 30. However, the significant decrease in TTC was found in 6 genotypes N-696, N-896, N-1296, N-1286, N-8437, and N-4496. Total flavonoid contents were maintained in 8 cotton genotypes N-1296, N-1286, N-4496, N-1237, N-7012 and N-213, N-878 and FH-490 under low moisture stress. Overall, a significant increase in total flavonoid content was observed in cotton genotype N-121. Genotype N-121 was found to be tolerant on the basis of TF contents. Under low moisture stress, ascorbic acid was maintained in

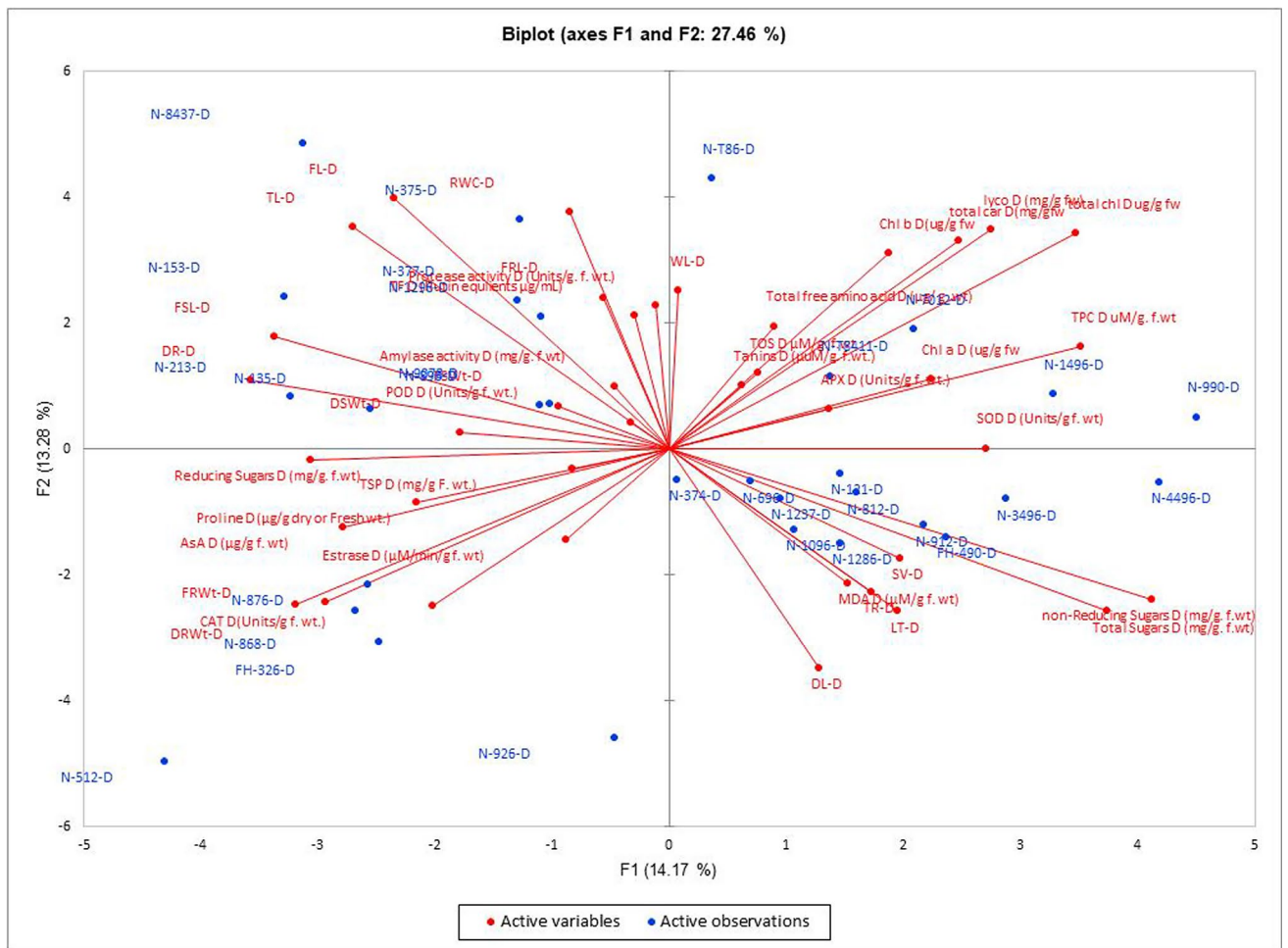


Fig. 7. Principal component analysis of all attributes under low moisture stress conditions.

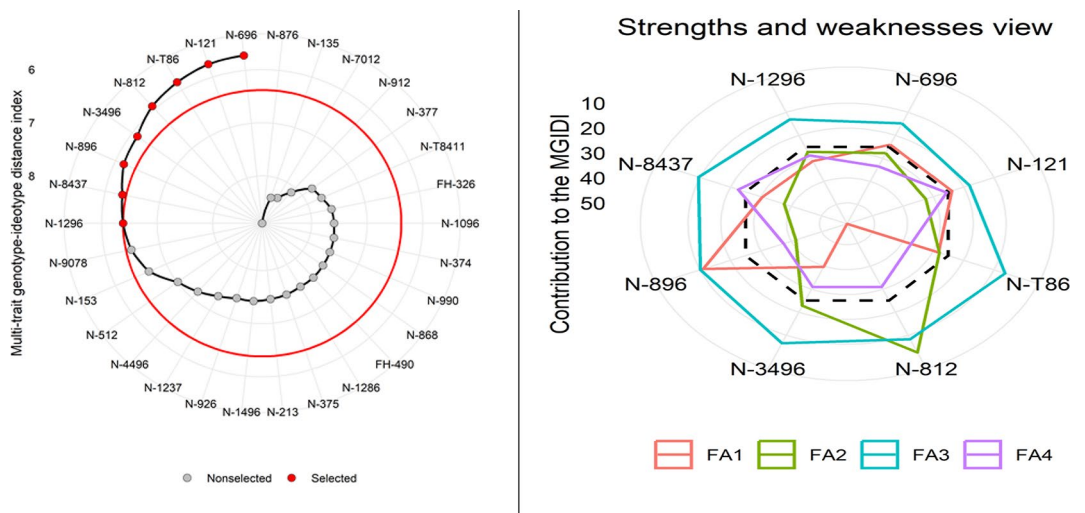


Fig. 8. Ranking of cotton genotypes based on MGIDI index (left) and Strength and weakness view (right) of the stable genotypes identified using biochemical markers. Based on the selection pressure (SI=25%), the red circle shows the cut point.

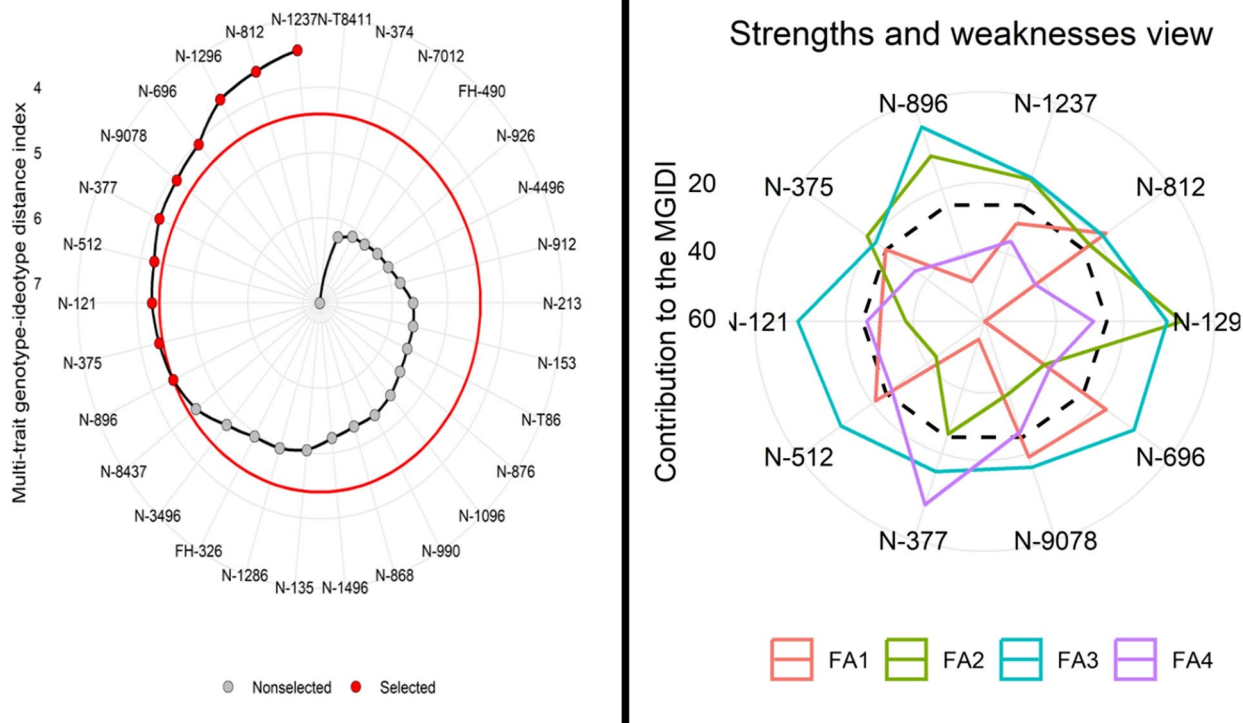


Fig. 9. Ranking of cotton genotypes based on MGIDI index (Left) and Strength and weakness view (Right) of the stable genotypes identified using **morpho-physiological traits**. Based on the selection pressure (SI = 35%), the red circle shows the cut point.

many cotton genotypes. Significant increase in ascorbic acid was found in N-1296 and FH-490. These findings were similar to the results of previously reported studies in different plant species^{106,108,109}.

Total oxidants status (TOS) was significantly increased in 7 cotton genotypes N-868, N-T8411, N-1496, N-T86, N-153, N-213 and FH-490 under low moisture stress, while it decreased significantly in N-1096, N-896, N-912, N-512 and FH-326. Significant differences were observed in all cotton genotypes. TOS was retained in 2 genotypes N-1296 and N-926 only. Increased TOS was reported in cotton species under drought stress³. However, significantly increased total free amino acids were observed in N-3496, N-9078, N-T86, N-153, and FH-490. Genotypes N-T86 and FH-490 were found to be tolerant on the basis of total free amino acids and TOS.

In this experiment, compared to non-stress conditions, total soluble proteins varied significantly in many cotton cultivars except N-868, N-3496, and FH-490 in which these were retained. Under low moisture stress in genotypes N-377 and N-812, a significant increase in TSP was found. Significant decrease was found in N-1496, N-7012, N-1237, N-153, N-926, and N-375. These findings were supported by previously reported studies^{3,110}. The same findings trend was also reported in chickpeas that high temperature and low water create a decrease in the production of protein, which could be due to a variety of variables. As a result, plant development and crop output are reduced in susceptible genotypes¹¹¹.

One of the major plant response mechanisms for achieving tolerance for stress through carbon storage, osmo-protection, and acting on ROS is carbohydrate accumulation under stress conditions^{112,113}. In this study, total soluble sugar varied significantly in most cotton genotypes except N-135 in which it was maintained. Under low moisture stress, in 11 genotypes N-1206, N-1286, N-4496, N-9078, N-1237, N-912, N-512, N-374, N-926, N-812 and FH-326 a significant increase in TSS was noted. Significant decrease was found in N-153. Results revealed that in 6 genotypes N-1286, N-4496, N-9078, N-990, N-812, and N-512, a significant increase in non-reducing sugar was observed. Significant decrease was found in N-153. However, significantly increased reducing sugar was found in N-696. While, it was maintained in N-896, N-1096, N-1496, N-4496, N-3496, N-9078, N-153 and N-213.

Another important biochemical reaction of plants in response to abiotic stimuli (heat, salt, flooding, drought, etc.) is the modulation of various hydrolytic enzymes like esterase and proteases¹¹³. In this study, protease activity varied significantly in many cotton genotypes except N-896, N-4496, N-7012, N-912, N-213, and N-878 in which it was maintained. Under low moisture stress, a significant increase was found in N-1286. Significant decrease of protease and esterase activity was observed in N-812. Genotype N-812 was found sensitive to stress on the basis of protease and esterase activity. However, an increase in esterase activity was found in 13 genotypes N-868, N-896, N-696, N-1296, N-1286, N-8437, N-3496, N-9078, N-1237, N-926, N-374, N-213 and N-878. Previously, it was also found that tolerant genotypes of cotton (*Gossypium hirsutum*) had a significant increase in esterase activity under low moisture conditions⁹⁵. Low moisture stress caused a significant increase in plant amylase activity observed in N-8437, and N-512. However, a significant decrease in-amylase activity was found

VAR	Factor	SD	SDperc	h ²	SG	SGperc	Sense	Goal
Biochemical traits								
TF	FA1	2.7009	2.0996	0.3987	1.0769	0.8372	Increase	100
MDA	FA1	-18.1990	-4.9752	0.4535	-8.2538	-2.2564	Decrease	100
SOD	FA2	15.1222	11.1283	0.3681	5.5665	4.0963	Increase	100
APX	FA2	35.7634	15.8948	0.4246	15.1837	6.7483	Increase	100
PROLINE	FA2	0.5951	1.3383	0.4282	0.2548	0.5730	Increase	100
TPC	FA3	552.5186	2.7914	0.6243	344.9528	1.7427	Increase	100
POD	FA3	336.4063	6.5430	0.7938	267.0489	5.1940	Increase	100
CAT	FA3	8.3347	6.4610	0.4465	3.7217	2.8851	Increase	100
TSP	FA4	7.4687	5.4351	0.6620	4.9445	3.5982	Increase	100
Morpho-physiological traits								
FRW	FA1	0.162055	10.70247	0.19732	0.031977	2.111813	Increase	100
DRW	FA1	0.1029	10.32675	0.216743	0.022303	2.23825	Increase	100
FSW	FA1	0.989516	7.672924	0.167946	0.166185	1.288638	Increase	100
DSW	FA1	0.28025	3.737729	0.125397	0.035142	0.468699	Increase	100
DR	FA2	0.000941	0.288304	0.221199	0.000208	0.063773	Increase	100
TR	FA2	-1.32542	-2.00083	0.355681	-0.47143	-0.71166	Decrease	100
LT	FA2	-0.00866	-0.02448	0.13157	-0.00114	-0.00322	Decrease	100
FL	FA3	0.001063	0.168317	0.196099	0.000208	0.033007	Increase	100
WL	FA3	0.003902	1.483376	0.143762	0.000561	0.213253	Increase	100
TL	FA3	0.001374	0.186527	0.142538	0.000196	0.026587	Decrease	0
SV	FA3	0.10613	0.285032	0.197998	0.021014	0.056436	Increase	100
DL	FA4	0.003516	3.617682	0.145068	0.00051	0.524811	Increase	100
FRL	FA4	1.258234	6.226426	0.315001	0.396344	1.961328	Increase	100
FSL	FA4	1.847448	5.813477	0.280318	0.517873	1.629623	Increase	100

Table 2. Linkage of factors to traits, selection Differential (SD), selection Differential percentage (SD%), heritability (h^2), selection gains (SG), selection gain percentage (SG%), objectives and goal. Significant values are in bold.

in N-868, N-896, N-1096, N-1296, N-1286, N-4496, N-3496, N-9078, N-1237, N-912, N-T86, N-377, N-213, N-878, FH-326 and FH-490. However, an increase in esterase activity was found in 13 genotypes N-868, N-896, N-696, N-1296, N-1286, N-8437, N-3496, N-9078, N-1237, N-926, N-374, N-213 and N-878. In general, all remaining cotton genotypes had non-significant results for amylase, esterase, and protease activity.

According to reports, photosynthesis is the most essential factor influencing crop output during drought conditions¹¹⁴. While photosynthesis depends upon the chlorophyll contents of the plant. In this study, chlorophyll a content under low moisture stress in most cotton genotypes was increased. However, it was decreased in N-868, N-135, N-153, N-374, and FH-326. Low moisture stress caused an increase in chlorophyll b content and the total chlorophyll in all cotton genotypes. While, chlorophyll b content was decreased in genotype N-868, N-926, and FH-326, and total chlorophyll in N-868, N-374, and FH-326. Low moisture stress had the least significant genotypic difference in chlorophyll b, chlorophyll a, and total chlorophyll content in cotton genotypes compared to control plants. Lycopene content was found maintained in N-912.

Increasing proline content may be helpful for both drought stress mitigation and osmotic adjustment. Previous studies found that under the water-deficit conditions, proline content in plants increased^{115,116}. In this study, Proline was observed highest in N-213 under low moisture stress, however, it was lowest in genotype N-912. Under low moisture stress, an increase in proline was found in cotton genotypes N-1096, N-1296, N-8437 N-1496, N-4496, N-1237, N-135, N-926, N-213 and N-878.

Total carotenoids increased in cotton genotype N-7012 under stress treatment but decreased the most in FH-326. Carotenoid content was maintained in N-8437, N-3496, and N-374. The results showed that total carotenoids increased but the differences were non-significant between non-stress and stress conditions. Similar findings were also reported earlier in the cotton cultivars¹¹⁷.

MGIDI, an innovative approach has been illustrated for the selection of best-performing genotypes using multiple attributes⁶⁴. Several researchers have used this index in different crops including, wheat^{63,118,119}, guar¹²⁰, barley¹²¹, and soybean¹²². A Monte Carlo simulation research was used to assess the efficacy of the MGIDI index, evaluating its rate of accuracy in determining characteristics with intended outcomes to both conventional and current indices under different circumstances^{69,123}. In this study, results showed that eight genotypes (N-812–1296 N-696 N-377 N-121–896 N-T86, and N-3496) were observed to be best performing by MGIDI for morpho-physiological and biochemical traits. Previously, MGIDI has also been used for similar implications in tomato under control and water deficit stress conditions¹²⁴. Further, using MGIDI protocol, the best-performing wheat lines under salt stress have also been identified⁶³.

Conclusions

The MGIDI index for both biochemical and morpho-physiological indices was used to identify the best performing genotypes and get insights into involved tolerance mechanisms. Among biochemical traits, TPC, TF, TSP, MDA, SOD, POD, CAT, APX, and Proline proved most efficient indices for selecting cotton genotypes having low moisture stress tolerance. Six genotypes, i.e. N-812–1296 N-696 N-377 N-121, and N-896, were found common and performed best in terms of adjustments in biochemical and morpho-physiological attributes. Three genotypes, i.e. N-696, N-896, and N-T86 proved to be most resilient to low moisture stress and can prove to be a valuable resource for breeding programs aimed to develop climate smart cotton varieties.

Data availability

Data is provided within the manuscript or supplementary information.

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Author contributions

A.S. executed the experiment as well as the collection of data, analytical work, data organization, presentation of resulting data in the form of graphs, write-up, and revision of the manuscript after the biochemical, physiological and morphological analysis. A.H. planned and designed the experiments, and performed the statistical analysis of data, supervised overall research work and finalized the manuscript. M.H. did the arrangement and provided the seeds of cotton genotypes, contributed to designing and planning of wirehouse experiment, reviewed the finalized manuscript. All authors have equally contributed to the manuscript and approved the final submission of the article.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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