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# Multivariate analysis of mutant wheat (*Triticum aestivum* L.) lines under drought stress

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**Abstract**: Drought is a major threat to wheat production worldwide and development of drought-tolerant wheat genotypes may improve the production of this cereal crop in drought affected regions. In the present study, 24 mutant lines were developed by treating a wheat variety 'NN-Gandum-1(NN-1) with ethyl methane sulfonate (EMS). The $M_5$, $M_6$, and $M_7$ generations were evaluated for quantitative traits under well irrigated and rainfed conditions. Furthermore, best performing mutants of $M_6$ and $M_7$ generations were examined for biochemical parameters. A substantial correlation was found among the quantitative traits (plant height, spike length, tillers per plant, number of spikelets per spike and thousand grain weight) which confirmed their high heritability and relative relation. Principal component analysis (PCA) showed that the first two PCs with eigenvalues >1 had more than 50% of the total variability among the mutant lines under normal and water limited conditions. Cluster analysis depicted that considerable variations existed among all mutant lines. Among these mutant lines, some mutants showed significant variation consistently larger than corresponding values of wild type in multigeneration replication. Using drought tolerance indices (Stress tolerance index and geometric mean productivity), high yielding mutant lines under drought stress were selected. Across all these three-mutant generations, mutant lines NN1-M-363, NN1-M-506, NN1-M-700, NN1-M-701, and NN1-M-1621 showed significant improvement as compared to wild type in response to stress conditions. The selected five drought tolerant mutants showed increased accumulation of proline contents, total soluble sugars, total free amino acids, while decreased total chlorophyll content, carotenoids, and total soluble proteins. Results suggested that the morphological traits and yield attributes along with biochemical parameters could be utilized for the evaluation of wheat cultivars under drought stress.

**Key words:** Drought-tolerance indices, EMS mutant, genetic variability, high yield

## 1. Introduction

Production of wheat is hampered predominantly by biotic and abiotic stresses. Drought stress reduces about 60% of agricultural productivity (Ahmed et al., 2018). Consequently, it is inevitable for wheat breeders to develop new varieties having improved tolerance against stresses, high yield potential, and better quality (Pandey et al., 2017). Deployment of a breeding method for the development of elite genotypes is based on availability of genetic diversity among available germplasm (Channaoui et al., 2019). Genetic variability can be enhanced or created using mutagenesis, which can further be utilized for the development of drought-tolerant wheat cultivars. Thorugh mutation breeding, many beneficial traits including seed yield, dwarfness and earliness can be improved which ultimately leads to overcome biotic and abiotic stresses (Lee et al., 2018). The most frequently used chemical mutagen is ethyl methanesulfonate (EMS) which induces random point mutations in the whole genome (Hussain and Rahman, 2019). The hexaploid nature of wheat crop allows accumulation of high frequency of mutations without disturbing its survival. Mutant population could be utilized for the breeding and selection of drought-tolerant varieties and lessening the devastating effects of drought stress on yield in wheat crop (Zulkiffal et al., 2021). Multivariate methods including principal component analysis (PCA) and cluster analysis could be employed for the assessment and evaluation of the genetic variation resulting due to mutagenesis (Ajmal et al., 2013). The use of PCA along with cluster analysis is advantageous because each genotype can be assigned to a separate group and it also reveals the significance of major contributors towards the total difference on each axis of variation (Lever et al., 2017). The correlation coefficient is another statistical tool that gives clear information of the degree of association between different traits which is useful for the

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final selection of genotypes having desired traits (Ghafoor et al., 2013).

The potential tools for the identification of better performing wheat genotypes are an assessment of agromorphological traits and yield components under drought stress conditions (Ahakpaz et al., 2020). In addition, different drought tolerance parameters including stress tolerance index (STI) and geometric mean productivity (GMP) are being utilized for the selection of desired genotypes with adaptive potential against drought stress (Mohammadi and Abdulahi, 2017). However, under both well-watered and water-limited conditions, STI is found more useful for the selection of genotype with better productivity; the higher the value of STI, the more the genotype is considered to be drought tolerant (Eid and Sabry, 2019).

Understanding the adaptive response of important phenotypic traits that contribute to improved productivity during stress is essential in order to comprehend physiological and genetic methods of wheat adaptation (Mwadzingeni et al., 2016). As a response mechanism, some biochemical catalysts are produced that alter the expression and regulation of multiple osmolytes (You and Chan, 2015) and affect plant cellular mechanisms (Passioura, 2012; Hasanuzzaman et al., 2018). Drought-induced production of chemically reactive oxygen species (ROS) that expose cells to oxidative damage, eventually leading to membrane disability (lipid peroxidation), leakage of ions, breakage or cleavage of DNA strand at various levels, degradation of biomolecules, eventually leading to tissue damage and programmed cell death (Huseynova et al., 2016; Pandey et al., 2017). Though, cereals under abiotic stresses accumulate low molecular weight compatible solutes like sugars and proline to improve tolerance to drought stress (Manuchehri and Salehi, 2014). While, total chlorophyll and carotenoid contents decline under water restricted conditions (Khalilzadeh et al., 2016). Estimation of changes in these biochemicals parameters under stress conditions can be helpful to examine stress responses in plants.

In the present study, twenty-four wheat mutants along with their wild type were chosen from the already developed $M_4$ EMS mutant population of NN-Gandum-1. This experiment was aimed to examine the characters contributing towards genetic variation among wheat genotypes under drought conditions.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Seeds of a wheat variety, NN-Gandum-1, were treated with 0.8 % (v/v) optimized concentration of ethyl methane sulfonate (EMS) for 2 h at 35 °C, (Hussain et al., 2018) in the Plant Genomics and Molecular Breeding (PGMB) Lab, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

### 2.2. Mutant population development and advancement

EMS treated seed were sown in the experimental field of NIBGE Faisalabad, Pakistan. Seeds were sown in 100 rows with eight plants in each row, by keeping 30 cm row-to-row distance. Out of 7500 seeds, total of 3634 ($M_1$) plants were germinated. At physiological maturity, the single main spike of each $M_1$ plant was bagged to help self-pollination. At maturity, the $M_2$ seed from each main spike was harvested to sow $M_3$ generation. Mutant generations from $M_3$ to $M_7$ were advanced by harvesting the main single spike of each plant in all generations. At the $M_5$ generation 24 mutant lines (NN-M-1, NN-M-41, NN-M-61, NN-M-83, NN-M-85, NN-M-116, NN-M-205, NN-M-252, NN-M-284, NN-M-293, NN-M-313, NN-M-357, NN-M-363, NN-M-430, NN-M-490, NN-M-506, NN-M-700, NN-M-701, NN-M-764, NN-M-827, NN-M-1621, NN-M-2259, NN-M-2280, NN-M-236) were selected. For the next three consecutive years (2016 to 2018) the seeds of wild type and 24 mutant lines were sown in the experimental field of NIBGE, under normal irrigation ($W_1$) i.e. 4–5 irrigations and drought conditions ($W_2$), i.e. rainfed.

The $W_1$ plants were watered normally to keep a well-watered level whenever required. Plants in $W_2$ conditions, were subjected to the drought by withholding supply of water for about 8–12 days until indication of water stress such as leaf wilting or leaf rolling appeared (Gusmao et al., 2012). As plants reached pre-anthesis stages; according to Bajji et al. (2001) infrequent watering for 8–12 days for stressed set ups of plants while $W_1$ set of plants were watered normally.

The experiment was conducted using a randomized complete block design (RCBD) in three replications. The plot size was 64 $ft^2$ (8 × 8 ft). In each replication, the plot size was 4.65 $m^2$. Standard agronomic practices for wheat cultivation were observed throughout the experiment.

### 2.3. Data collection of morphological, seed quality and productivity traits of wheat mutant lines for three mutant generations ($M_5$, $M_6$, and $M_7$)

Data of plant height (cm), spike length (cm), tillers per plant, number of spikelets/spike, and 1000 grain weight (g) were collected. In addition, seed quality traits such as protein, moisture, and gluten were also measured using Omega Analyzer (http://www.bruinsinstruments.com/OmegAnalyzerG.html). For seed quality trait measurement, one kg seed of each mutant line was used. The yield of all the mutant lines along with the wild type was also recorded after harvesting. All of these parameters were measured for three ($M_5$, $M_6$, and $M_7$) generations under both ($W_1$ and $W_2$) regimes.

Drought tolerance indices were estimated using the following equations. The equation for tolerance index (TOL), mean productivity (MP) (Rosielle and Hamblin, 1981), stress susceptibility index (SSI) (Fischer and Maurer, 1978); geometric mean productivity (GMP), and stress tolerance index (STI) (Fernandez, 1992) were used. Here, Yp and Ys express yield while P and S express, mean yield under $W_1$ and $W_2$ conditions, respectively.

$$TOL = YP - YS$$

$$MP = (YP + YS)/2$$

$$GMP = \sqrt{YS \times YP}$$

$$\text{STI} = \frac{(YP \times YS)}{P^2}$$

$$SSI = \frac{\left\{1 - \left(\frac{YS}{YP}\right)\right\}}{\left\{1 - \left(\frac{S}{P}\right)\right\}}$$

**2.4. Biochemical assays on selected mutant lines for two mutant wheat generations ($M_6$ and $M_7$)**

For the estimation of drought-tolerance index, selected mutant lines were further tested for studying their biochemical responses under both the regimes ($W_1$ and $W_2$) for two consecutive wheat growing seasons ($M_6$ and $M_7$). Various biochemical assays such as total soluble proteins (TSPs), total soluble sugars (TSSs), total free amino acids (TFAs), total chlorophyll contents, carotenoid contents, proline contents, were performed for $M_6$ and $M_7$ generations. For the aforementioned assays, fresh leaf samples were collected from the selected mutants and wild type in ice.

The total TSPs (mg $g^{-1}$ FW) was measured by following the protocol described by Lowry et al. (1951). The optical density of the solution was measured at 620 nm using a spectrophotometer. Finally, the concentration of TSPs was estimated by comparing with the standard curve produced at different concentrations of bovine serum albumin (BSA).

For the determination of TSSs (mg $g^{-1}$ FW), a protocol demonstrated by Yemm and Willis (1954) was adopted. The absorbance of the solution was recorded at 625 nm using a spectrophotometer. Finally, the sugar concentration was determined by comparing it with a standard curve generated at different concentrations of glucose (Fales, 1951).

The TFAs (mg $g^{-1}$ FW) were determined by adopting a procedure described by Hamilton and Van Slyke (1943). The absorbance was recorded at 570 nm using a spectrophotometer and the concentration of TFAs was calculated by comparing with the standard curve of leucine.

Total free amino acid = (Graph reading of sample) × (dilution factor)

Dilution Factor = (Volume of sample/weight of sample)

For the calculation of total chlorophyll and carotenoid contents, the protocols described by Arnon (1949) and Davies (1986) were followed, respectively. The absorbance was monitored at 480, 645, 652, and 663 nm with the help of the spectrophotometer. Using the following formulas, chlorophyll (Chla, Chlb, Chlt) and carotenoids contents were calculated:

$$\text{Chl}_a(\text{mg g}^{-1}\text{ FW}) = [12.7(\text{OD } 663) - 2.69(\text{OD } 645)] \times \frac{\text{V}}{1000} \times \text{W}$$

$$\text{Chl}_a(\text{mg g}^{-1}\text{ FW}) = [22.9(\text{OD } 645) - 4.68(\text{OD } 663)] \times \frac{\text{V}}{1000} \times \text{W}$$

$$\text{Total Chl}_{a+b}(\text{mg g}^{-1}\text{ FW}) = [20.2(\text{OD } 645) + 8.02(\text{OD } 663)] \times \frac{\text{V}}{1000} \times \text{W}$$

$$\text{Carotenoides } (\mu\text{g g}^{-1}\text{FW}) = \text{A}^{\text{car}}/\text{E}_{\text{max}}100.$$

Whereas V is the volume of extract and W is the weight of the leaf sample

$$\text{A}^{\text{car}} = (\text{OD } 480) + 0.114\ (\text{OD } 663) - 0.638\ (\text{OD } 645)$$

$$\text{E}_{\text{max}}100 \text{ cm} = 2500.$$

For the determination of proline contents (mmole $g^{-1}$ FW), a published protocol by Bates et al. (1973) was adopted. Proline contents were calculated by drawing a standard curve developed by Analar grade proline.

**2.5 Statistical analysis**

Software Statistix 8.1 was used for the analysis of variance. SPSS16 software was used to calculate the correlation coefficients. Principle component analysis (PCA) and cluster analysis were performed with NCSS 2020 statistical software (2020). The hierarchical similarity in wheat mutant lines was estimated using NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss. Euclidean distance matrix from the PCA values was used to construct a dendrogram.

## 3. Results and discussion

Wheat mutant lines were screened for morphological, physiological, and biochemical traits under rainfed and normal conditions.

**3.1. Morphological traits data of three mutant generations ($M_5$, $M_6$ and $M_7$)**

Substantial variations in plant height, number of spikelets/spike, 1000 grain weight, spike length, and tillers per plant were observed in all three mutant generations (Table 1). Drought stress affects plant height most prominently. Reduced availability of water results in a decrease in photosynthesis and uptake of other essential nutrients

**Table 1.** Descriptive statistics of 5 morphological traits of NN-Gandum-1 mutants among three mutant generations during $W_1$ and $W_2$.

| Traits | Generation | Condition | Mean | SE | Range | CV % | LSD |
|---|---|---|---|---|---|---|---|
| Plant height (cm) | M5 | W1 | 93.69 | 1.07 | 79–112 | 4.24 | 6.52 |
| | | W2 | 71.16 | 0.95 | 59.67–87.33 | 5.72 | 6.68 |
| | M6 | W1 | 61.19 | 1.13 | 46.33–86 | 3.94 | 3.96 |
| | | W2 | 48.00 | 1.03 | 35.67–64 | 5.23 | 4.12 |
| | M7 | W1 | 96.08 | 0.86 | 90.33–112 | 2.82 | 4.44 |
| | | W2 | 89.93 | 0.91 | 77.67–11.67 | 2.43 | 3.59 |
| Spike length (cm) | M5 | W1 | 13.66 | 0.10 | 12.50–14.67 | 7.11 | 1.59 |
| | | W2 | 10.85 | 0.13 | 9.33–12.50 | 9.35 | 1.67 |
| | M6 | W1 | 13.93 | 0.08 | 12.83–14.67 | 7.65 | 1.75 |
| | | W2 | 11.05 | 0.07 | 10–12.33 | 8.66 | 1.57 |
| | M7 | W1 | 11.71 | 0.09 | 10.50–13.5 | 6.06 | 1.16 |
| | | W2 | 11.33 | 0.09 | 9.83–12.17 | 6.17 | 2.19 |
| Tillers per plant | M5 | W1 | 7 | 0.20 | 5–9 | 19.35 | 4.82 |
| | | W2 | 5 | 0.28 | 3–7 | 21.15 | 2.86 |
| | M6 | W1 | 7 | 0.20 | 5–9 | 13.70 | 3.28 |
| | | W2 | 5 | 0.26 | 3–7 | 18.51 | 2.53 |
| | M7 | W1 | 8 | 0.19 | 6–10 | 13.56 | 2.92 |
| | | W2 | 5 | 0.21 | 5–7 | 13.63 | 2.24 |
| No. of spikelets /spike | M5 | W1 | 21 | 0.30 | 19–23 | 6.03 | 2.20 |
| | | W2 | 19 | 0.32 | 13–19 | 6.87 | 2.09 |
| | M6 | W1 | 19 | 0.34 | 15–21 | 9.13 | 2.62 |
| | | W2 | 15 | 0.23 | 11–15 | 10.58 | 2.50 |
| | M7 | W1 | 19 | 0.28 | 7–21 | 7.47 | 2.33 |
| | | W2 | 17 | 0.36 | 5–13 | 7.54 | 1.15 |
| 1000 grain weight (g) | M5 | W1 | 33.84 | 0.44 | 26.11–37.91 | 2.55 | 1.42 |
| | | W2 | 32.15 | 0.44 | 25.35–37.26 | 3.01 | 1.59 |
| | M6 | W1 | 39.23 | 0.96 | 30.44–44.54 | 3.50 | 1.58 |
| | | W2 | 36.76 | 0.79 | 28.67–43.76 | 4.78 | 1.30 |
| | M7 | W1 | 35.78 | 1.15 | 15.95–40.85 | 3.70 | 1.79 |
| | | W2 | 30.25 | 0.79 | 21.31–36.24 | 2.90 | 1.44 |

that subsequently reduces plant height (Sarto et al., 2017). This study revealed that all mutant lines of NN-1 shown variation for plant height with a substantial decrease from 93.69 cm to 71.16 cm ($M_5$), 61.19 cm to 48 cm ($M_6$), and 96.08 cm to 89.93 cm ($M_7$) under limited water conditions. Similar findings on reduction in plant height under drought stress conditions were reported (Iqbal, 2018).

Spike length is one of the most imperative traits which accommodates a greater number of spikelets per spike (Iqbal et al., 2017). In the present study, a diverse range in spike length 13.66–10.85 cm, 13.93–11.05 cm, and 11.71–11.33 cm was observed in $M_5$, $M_6$, and $M_7$ generations respectively. Similar differences in spike length were also examined in wheat cultivars in earlier studies (Hazra et al., 2019).

Tillers per plant may have a positive or negative impact on final yield under prevailing environmental conditions (Kondić et al., 2017). In the present study, on an average ten tillers per plant under $W_1$ and three tillers per plant in $W_2$ condition were recorded. In previous studies, similar findings on variation in the number of tillers per plants has been reported (Hassani et al., 2017). Similarly, regarding number of spikelets per spike fluctuated from 19–23 in $W_1$ while in $W_2$ it ranged from 13–19. For 1000 grain weight, a highly significant difference was observed between normal ($W_1$) and drought ($W_2$) conditions. The same changes of varied range and deviation in mean were observed in mutant lines during drought stress conditions from normal irrigation for qualitative traits in $M_6$ and $M_7$ populations (Table 1). Hence in this study, mutagenesis enhanced genetic variability in drought-tolerant wheat cultivars as plant height, spike length, tillers per plant improved to overcome drought stress. Correlation matrices revealed a significant relative association among morphological characters, in mutant generations ($M_5$ to $M_7$) (Table 2).

**Table 2.** Correlation coefficient among five traits under normal ($W_1$) and drought ($W_2$) conditions in three mutant generations of NN-Gandum-1.

| Generations | Variables | Conditions | Plant height (cm) | Spike length (cm) | Tiller per plant | No. of spikelets | 1000 grain weight (g) |
|---|---|---|---|---|---|---|---|
| M5 | Plant height (cm) | W1 | 1 | | | | |
| | | W2 | 1 | | | | |
| | Spike length (cm) | W1 | 0.137 | 1 | | | |
| | | W2 | 0.097 | 1 | | | |
| | Tiller per plant | W1 | 0.209** | 0.243** | 1 | | |
| | | W2 | 0.170** | 0.205** | 1 | | |
| | No. of spikelets per spike | W1 | 0.322** | 0.201** | 0.256** | 1 | |
| | | W2 | 0.221** | 0.395** | 0.268** | 1 | |
| | 1000 grain weight (g) | W1 | –0.537* | –0.148 | –0.100 | –0.136 | 1 |
| | | W2 | 0.050 | –0.015 | –0.004 | –0.177 | 1 |
| M6 | Plant height (cm) | W1 | 1 | | | | |
| | | W2 | 1 | | | | |
| | Spike length (cm) | W1 | 0.371** | 1 | | | |
| | | W2 | 0.082 | 1 | | | |
| | Tiller per plant | W1 | –0.003 | 0.257** | 1 | | |
| | | W2 | 0.345** | 0.318** | 1 | | |
| | No. of spikelets per spike | W1 | 0.104 | 0.265** | 0.096 | 1 | |
| | | W2 | 0.404** | –0.053 | 0.273** | 1 | |
| | 1000 grain weight (g) | W1 | 0.100 | –0.200 | –0.090 | 0.117** | 1 |
| | | W2 | –0.125 | 0.181** | –0.238** | –0.303** | 1 |
| M7 | Plant height (cm) | W1 | 1 | | | | |
| | | W2 | 1 | | | | |
| | Spike length (cm) | W1 | –0.349** | 1 | | | |
| | | W2 | 0.014 | 1 | | | |
| | Tiller per plant | W1 | 0.136 | –0.082 | 1 | | |
| | | W2 | –0.010 | –0.352** | 1 | | |
| | No. of spikelets per spike | W1 | 0.240** | –0.013 | 0.362** | 1 | |
| | | W2 | –0.100 | 0.543* | 0.152** | 1 | |
| | 1000 grain weight (g) | W1 | 0.515* | –0.096 | –0.089 | –0.079 | 1 |
| | | W2 | 0.192** | –0.407** | –0.139** | –0.349** | 1 |

** significant at 1% level; * significant at 5% level.

Plant height had shown a positive correlation with spike length, tillers per plant, and number of spikelets per spike, under $W_1$ and $W_2$ regimes in $M_5$ generation. Spike length was positively correlated with tillers per plant and number of spikelets per spike; on the other hand, it showed a negative correlation with 1000 grain weight. Moreover, 1000 grain weight showed a positive correlation with plant height during drought conditions and a negative correlation with all other parameters during both conditions ($W_1$ and $W_2$). In the case of $M_6$ generation, plant height and tillers per plant were negatively correlated under normal ($W_1$) conditions while in water-limited conditions ($W_2$), plant height and 1000 grain weight showed a negative correlation. Moreover, tillers per plant depicted a negative correlation with 1000 grain weight while it was positively correlated with all other parameters during both conditions ($W_1$ and $W_2$). At $M_7$ mutant generation, a positive correlation was observed between 1000 grain weight and plant height but thousand grain weight was negatively correlated with spike length, tillers per plant, number of spikelets per spike at both water regimes ($W_1$ and $W_2$).

The findings of the present study depicted a strong correlation between 1000 grain weight and plant height, which indicates the significance of these traits for enhancing grain yield potential in the mutant wheat population. Similar findings were observed in earlier studies (Singh et al., 2018). Therefore, thousand-grain weight and plant height are the main criteria for selecting high yielding genotypes. On the other hand, among various morphological traits including plant height, the number of spikelets, spike length and tillers per plant; a positive correlation coefficient was observed (Mohsin et al., 2009).

Principal component analysis was performed for estimation of degree of association and variation patterns among various morphological traits. As compared with correlation analysis, multivariate analysis (PCA) offers precise comparisons, estimation of similarities and dissimilarities, and also measures the contribution of various traits to total variability present among germplasm (Panthee et al., 2006). For all three mutant generations ($M_5$–$M_7$) PCA was performed. At $M_5$ generation, PCA assorted all wheat variables into five main factors in both normal ($W_1$) and rainfed ($W_2$) conditions (Figure 1). First, two components contributed 55% of variation with eigenvalues >1. Under normal conditions PC1 showed 38.86% variation; PC2 contributed 21.87% variation whereas in drought conditions PC1 accounted for 34.33% variation and PC2 showed 21.34% variation (Table 3). This method converts large data sets into smaller components [principal components (PC)]; the first PC accounts for the highest variation as compared to subsequent components (Leilah and Al-Khateeb, 2005). In the current study, PC1 accounted for maximum diversity followed by PC2 under both water regimes ($W_1$ and $W_2$). Interestingly, PC1 was found related to plant developmental traits while PC2 was associated with yield-related factors.

This PCA analysis was also operated on $M_6$ and $M_7$ mutant populations. Under both regimes ($W_1$ and $W_2$) first two components of PCA gave >55% of the variation among mutant lines (Tables 3– 5). A positive connection between 1000 grain weight and plant height was also specified by high loading values for them (Figures 1–3). Traits with greater eigenvalues have more impact on clustering (Ahmad et al., 2008).

To segregate these mutant lines into different groups, cluster analysis was performed. At $M_5$ generation, wheat mutants were grouped into six and four clusters when grown under $W_1$ and $W_2$ conditions, respectively. Under both conditions, the average values for 1000 grain weight and plant height were highest in all clusters. While at $M_6$ generation, five clusters of wheat mutants were formed under $W_1$ conditions and six clusters under $W_2$ conditions (Figure 1). A combination of diverse genotypes with varying taxonomic traits clustered together could be the reason for these significant differences (Figures 1–3).

During the $M_7$ generation, in total, six clusters were formed during normal conditions. Cluster V contained 10 mutants likewise, 2, 3, 2, and 5 mutants were combined into clusters II, III, IV, and VI, respectively. Maximum tillers/plant, spike length and the number of spikelets/spike, and minimum 1000 grain weight were observed in the mutant lines included in cluster II. Though, under $W_2$ conditions all mutant lines were clustered into five main clusters. Respectively 2, 5, 9, 2, and 7 mutant lines were present in clusters I, II, III, IV, and V. Mutant lines in cluster IV demonstrated maximum plant height. While mutant lines in cluster I depicted minimum tillers per plant and plant height. A few variations for spike length were found in clusters II, III, IV, and V but maximum variation in cluster I was observed. The mutants in cluster II had shown a maximum 1000 grain weight whereas cluster III genotypes revealed a minimum 1000 grain weight and maximum tillers/plant. Therefore, 1000 grain weight is not reliant on tillers per plant. The highest intercluster diversity was shown by the genotypes present in these clusters. Dendrogram created with Ward's method also revealed relationships among different mutant lines

### 3.2. The yield of mutant lines and association of yield-drought tolerant indices

Multiple year's analysis provides a wide range and comparative knowledge about drought tolerance mechanisms and yield potential (Bányai et al., 2020). In this study, after detecting morphological changes in mutant lines, their effect on yield was noticed among $M_5$, $M_6$, and $M_7$ mutant generations. In all the mutant lines, a

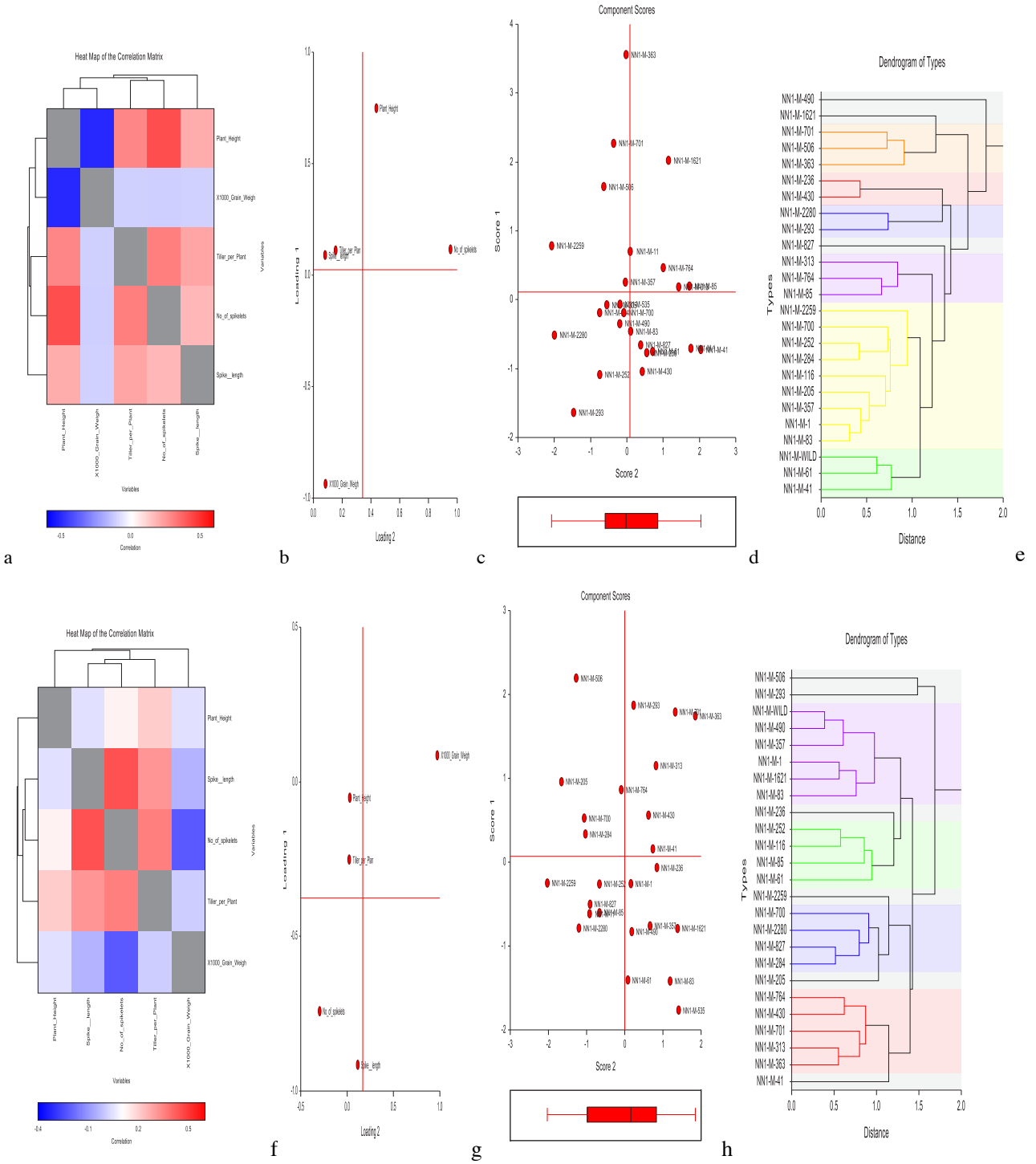


**Figure 1.** Multivariate analysis of 24 wheat mutant genotypes along with NN1 (wild) during $M_5$ generation. (a) Heat map of the correlation matrix under normal conditions and (e) drought conditions; (b) bar chart of absolute component (traits) PCA under normal conditions and (f) drought conditions; (c) Two-dimensional score plot based on PC1 and PC2 under normal conditions and (g) drought conditions; (d) dendrogram generated by Ward's method showing relationship among genotypes under cluster analysis during normal conditions and (h) drought conditions.

**Table 3.** Eigen vector table of $M_5$ generation under normal ($W_1$) and drought ($W_2$) conditions.

| **Eigenvector table $M_5$ normal** | | | | | |
|---|---|---|---|---|---|
| **Variables** | PC1 | PC2 | PC3 | PC4 | PC5 |
| PH | 0.55782 | –0.724237 | 0.039771 | 0.112456 | –0.387413 |
| TGW | 0.349001 | –0.113654 | –0.338686 | –0.728617 | 0.468711 |
| SPKLT | 0.383776 | 0.087867 | 0.75145 | 0.147858 | 0.508385 |
| TPP | 0.438552 | 0.256481 | –0.554663 | 0.601301 | 0.269583 |
| SL | –0.476888 | –0.623747 | –0.106684 | 0.27027 | 0.546892 |
| **Eigenvalue** | 1.942957 | 1.093491 | 0.818254 | 0.726238 | 0.41906 |
| **Individual %** | 38.86 | 21.87 | 16.37 | 14.52 | 8.38 |
| **Cumulative %** | 38.86 | 60.73 | 77.09 | 91.62 | 100 |
| **Eigenvector table $M_5$ drought** | | | | | |
| **Variables** | PC1 | PC2 | PC3 | PC4 | PC5 |
| PH | –0.358865 | 0.239106 | 0.316183 | 0.454762 | –0.712225 |
| TGW | –0.519838 | 0.533432 | 0.34835 | –0.044301 | 0.56737 |
| SPKLT | –0.463065 | 0.107268 | –0.856709 | 0.199606 | 0.016459 |
| SL | –0.606122 | –0.748314 | 0.189841 | –0.190581 | 0.016771 |
| TPP | 0.138461 | –0.294615 | 0.09322 | 0.845616 | 0.412644 |
| **Eigenvalue** | 1.716306 | 1.067043 | 0.883297 | 0.794357 | 0.538997 |
| **Individual %** | 34.33 | 21.34 | 17.67 | 15.89 | 10.78 |
| **Cumulative %** | 34.33 | 55.67 | 73.33 | 89.22 | 100 |

**Table 4.** Eigenvector table of $M_6$ generation under normal ($W_1$) and drought ($W_2$) conditions.

| **Eigenvector table $M_6$ normal** | | | | | |
|---|---|---|---|---|---|
| **Variables** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** |
| PH | –0.467306 | 0.487704 | 0.275167 | 0.568878 | –0.38004 |
| TGW | –0.674425 | –0.703475 | –0.084039 | 0.169824 | 0.119882 |
| SPKLT | –0.372383 | 0.219676 | 0.615562 | –0.535388 | 0.384078 |
| TPP | –0.415992 | 0.286943 | –0.598864 | –0.522542 | –0.336046 |
| SL | 0.122707 | –0.369695 | 0.423871 | –0.29639 | –0.762073 |
| **Eigenvalue** | 1.599834 | 1.179253 | 0.966022 | 0.806674 | 0.448216 |
| **Individual %** | 32 | 23.59 | 19.32 | 16.13 | 8.96 |
| **Cumulative %** | 32 | 55.58 | 74.9 | 91.04 | 100 |
| **Eigenvector table $M_6$ drought** | | | | | |
| **Variables** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** |
| PH | 0.524696 | 0.35733 | –0.51550 | 0.068034 | 0.57152 |
| TGW | 0.131411 | 0.473445 | 0.345443 | 0.774804 | –0.197303 |
| SPKLT | 0.527953 | –0.644507 | –0.26242 | 0.330185 | –0.35775 |
| SL | 0.534011 | –0.154631 | 0.735016 | –0.247677 | 0.29888 |
| TPP | –0.378844 | –0.457018 | 0.076198 | 0.474008 | 0.64585 |
| **Eigenvalue** | 1.870134 | 1.270673 | 0.825585 | 0.562204 | 0.471405 |
| **Individual %** | 37.4 | 25.41 | 16.51 | 11.24 | 9.43 |
| **Cumulative %** | 37.4 | 62.82 | 79.33 | 90.57 | 100 |

**Table 5.** Eigenvector table of $M_7$ generation under normal ($W_1$) and drought ($W_2$) conditions.

| **Eigenvector table $M_7$ normal** | | | | | |
|---|---|---|---|---|---|
| **Variables** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** |
| PH | –0.670346 | 0.711525 | –0.112293 | –0.113657 | –0.13726 |
| TGW | 0.414906 | 0.288683 | 0.101286 | –0.851861 | 0.092675 |
| SPKLT | –0.268069 | –0.013629 | 0.739272 | 0.01987 | 0.61728 |
| TPP | –0.309824 | –0.315099 | –0.611956 | –0.265179 | 0.599926 |
| SL | –0.458952 | –0.557603 | 0.236892 | –0.436692 | –0.48127 |
| **Eigenvalue** | 1.735226 | 1.379505 | 0.901442 | 0.639032 | 0.344796 |
| **Individual %** | 34.7 | 27.59 | 18.03 | 12.78 | 6.9 |
| **Cumulative %** | 34.7 | 62.29 | 80.32 | 93.1 | 100 |
| **Eigenvector table $M_7$ drought** | | | | | |
| **Variables** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** |
| PH | –0.166081 | 0.136195 | 0.237767 | –0.322762 | 0.890595 |
| TGW | 0.603205 | –0.689602 | –0.029253 | –0.390711 | 0.084157 |
| SPKLT | –0.055554 | –0.457188 | –0.063059 | 0.805197 | 0.368204 |
| SL | 0.576055 | 0.47519 | –0.598634 | 0.149004 | 0.248577 |
| TPP | –0.523103 | –0.266594 | –0.761756 | –0.269492 | 0.048923 |
| **Eigenvalue** | 1.900133 | 1.249772 | 0.979623 | 0.638409 | 0.232062 |
| **Individual %** | 38 | 25 | 19.59 | 12.77 | 4.64 |
| **Cumulative %** | 38 | 63 | 82.59 | 95.36 | 100 |

significant variation in yield/unit area was noted. In $W_1$ conditions, all mutant lines produced more yield however in $W_2$ conditions yield was reduced. For sorting out stress tolerant genotypes, the current trial was carried out in multiple years in a particular environment to validate genetic bases of stress-tolerant mechanisms because drought stress has the genetics of low heritability. Under water-scarce conditions, yield and drought indices are major indicators for germplasm selection (Khadka et al., 2020).

Regarding drought tolerance indices, minimum values for TOL, SI, and SSI were recorded in NN1-M-1621 and NN1-M-252 in all three generations. While maximum stress tolerance index, mean productivity, and geometric mean productivity were observed for NN1-M-701, NN1-M-252, NN1-M-1621, and NN1-M-700, etc. in all generations ($M_5$, $M_6$ and $M_7$). In wheat crop, genotypes with low values for SSI and TOL and greater values of GMP and STI indexes are considered drought-tolerant genotypes (Anwaar et al. 2020). Based on these drought tolerance indicators, we selected five mutant lines.

In $M_5$ mutant generation, the mean yield was observed 2813.09 kg/ha for selected mutants as compared to wild type 2747.31 kg/ha in $W_1$ conditions. On the contrary wild type produced 2595.48 kg/ha as compared to the average yield (2708.68 kg/ha) of mutant lines in $W_2$ conditions. Likewise, mean yield data of the selected mutants was also greater than that of the corresponding wild type under both water regimes ($W_1$ and $W_2$) in $M_6$ and $M_7$ generation (Table 6; Figure 4).

**3.3. Seed qualitative traits of three mutant generations**

Under both the water regimes a significant variation at $p < 0.001$ was observed for seed qualitative characters in all the mutant lines. The seed quality of wheat should be estimated by measuring gluten content, protein content, zeleny sedimentation, and moisture level (Takač et al., 2021). Minimum decrease of 3.1%, 3.4%, and 3.7% in seed protein contents were noted for mutant lines NN1-M-1621, NN1-M-363, and NN1-M-700 respectively when grown under $W_2$ conditions in $M_5$ generation. Whereas in $M_6$ generation, a reduction of 1.53% in protein contents was observed for wild type. Minimum reduction of 2.35% and 3.13% in seed protein contents were recorded for mutant line NN1-M-701 and NN1-M-363 under $W_2$ conditions in $M_7$ generation (Table 6).

The moisture content is enormously important because it regulates the texture of the wheat grain. In $M_5$ and $M_6$ generation a significant reduction in moisture contents of seed was observed. In $M_7$ generation, the minimum level of seed moisture content was found in NN1-M-701 (2.2

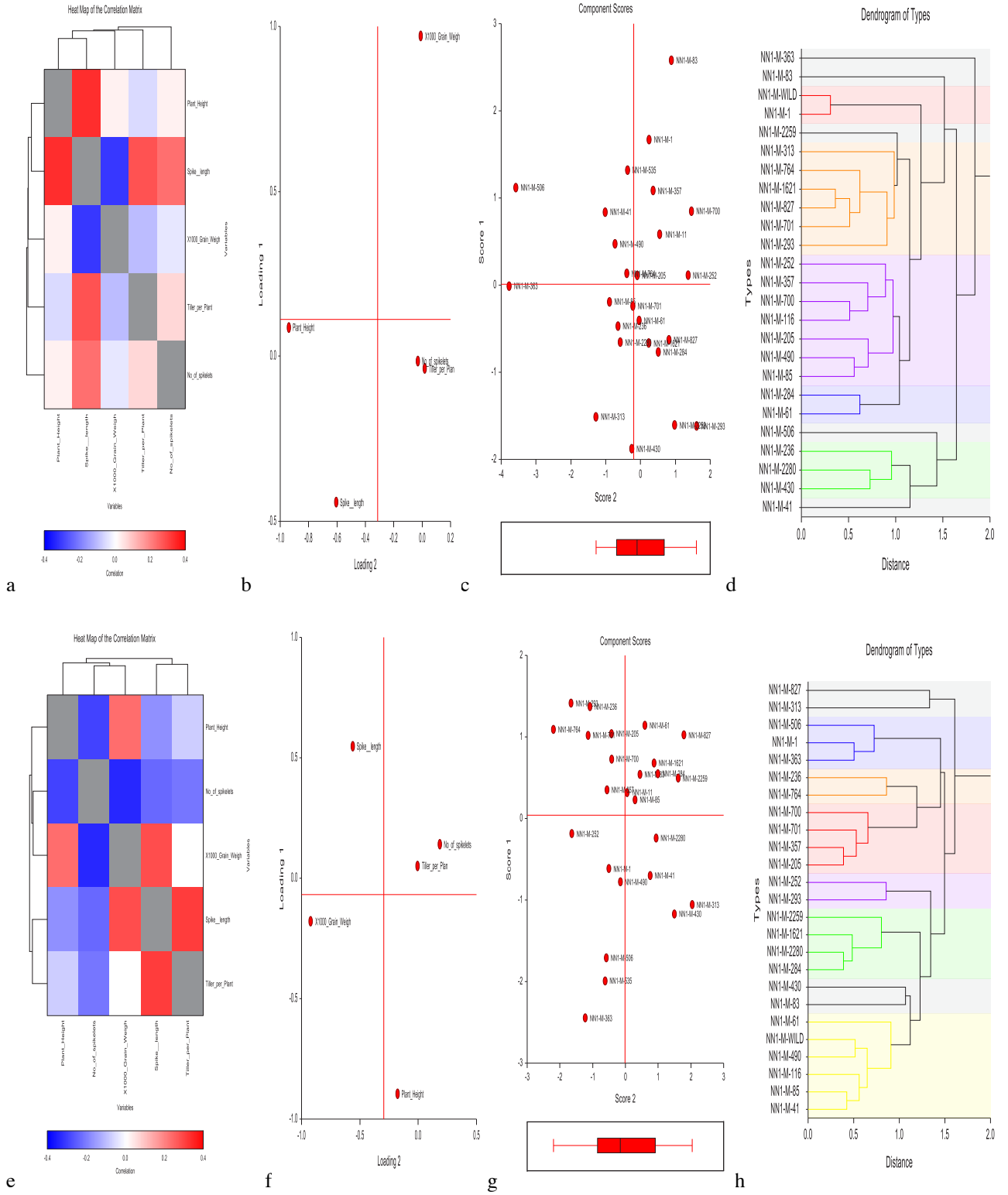


**Figure 2.** Multivariate analysis of 24 wheat mutant genotypes along with NN1(wild) during $M_6$ generation. (a) Heat map of the correlation matrix under normal conditions and (e) drought conditions; (b) bar chart of absolute component (traits) PCA under normal conditions and (f) drought conditions; (c) two-dimensional score plot based on PC1 and PC2 under normal conditions and (g) drought conditions; (d) dendrogram generated by Ward's method showing relationship among genotypes under cluster analysis during normal conditions and (h) drought conditions.

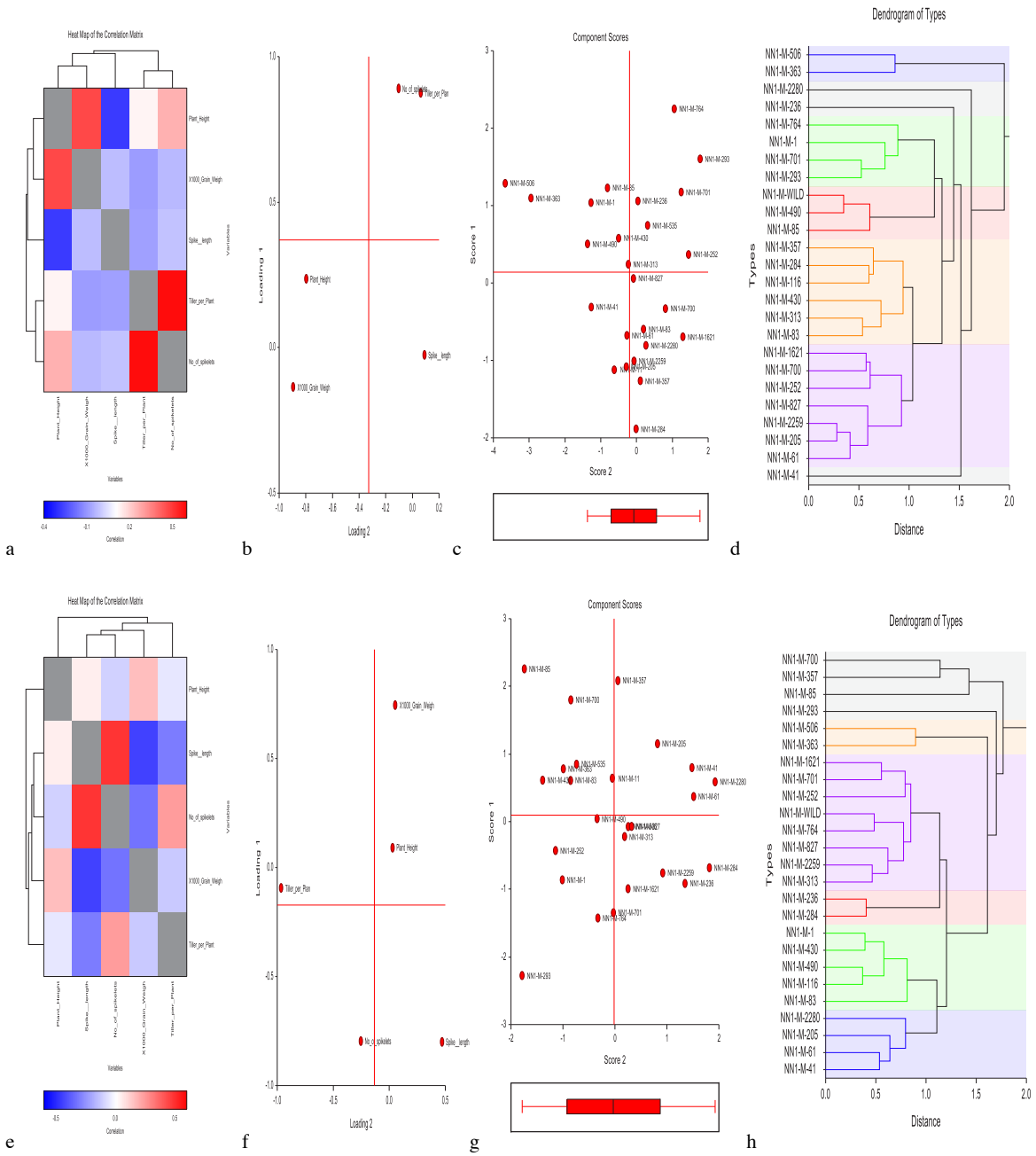


**Figure 3.** Multivariate analysis of 24 wheat mutant genotypes along with NN1(wild) during $M_7$ generation. (a) Heat map of the correlation matrix under normal conditions and (e) drought conditions; b) bar chart of absolute component (traits) PCA under normal conditions and (f) drought conditions; (c) two-dimensional score plot based on PC1 and PC2 under normal conditions and (g) drought conditions; (d) dendrogram generated by Ward's method showing relationship among genotypes under cluster analysis during normal conditions and (h) drought conditions.

%) under $W_2$ conditions. Wheat mutant lines were also examined for seed gluten contents. Minimum reduction in seed gluten contents was observed 2.2% (NN1-M-1621), 2.4% (NN1-M-700), 2.6 % (NN1-M-701), and 5.3% (wild type) in $M_5$ generation. Whereas a mean reduction of 6.7 % was observed at $M_6$ generation level. For seed gluten contents, during $M_7$ mutant population, reduction ranged from 1.9% to 17.5% (Table 6). Minimum decrease in zeleny sedimentation was observed for mutant lines NN1-M-700, NN1-M-363, NN1-M-1621 and NN1-wild during water

**Table 6.** Percentage reduction in seed quality parameters under normal ($W_1$) and drought ($W_2$) conditions among three mutant generations.

| Traits | Protein content (% reduction) | | | Moisture content (% reduction) | | | Gluten content (% reduction) | | | Zeleny sedimentation (% reduction) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Genotypes** | $M_5$ | $M_6$ | $M_7$ | $M_5$ | $M_6$ | $M_7$ | $M_5$ | $M_6$ | $M_7$ | $M_5$ | $M_6$ | $M_7$ |
| **NN-M-1** | 11.0 | 7.0 | 8.0 | 14.6 | 9.5 | 10.8 | 3.2 | 7.3 | 14.3 | 6.1 | 13.2 | 13.1 |
| **NN1-M-41** | 10.7 | 9.5 | 8.5 | 11.8 | 5.3 | 11.0 | 5.8 | 9.5 | 7.1 | 5.5 | 18.9 | 12.7 |
| **NN1-M-61** | 7.4 | 8.9 | 10.1 | 14.2 | 11.8 | 23.2 | 3.5 | 7.3 | 7.3 | 14.7 | 16.7 | 9.0 |
| **NN1-M-83** | 13.5 | 20.4 | 26.0 | 14.4 | 22.6 | 6.0 | 7.3 | 11.6 | 9.5 | 11.3 | 13.8 | 19.0 |
| **NN1-M-85** | 7.5 | 9.9 | 5.7 | 9.7 | 11.9 | 11.9 | 8.3 | 7.0 | 11.9 | 9.7 | 16.6 | 14.5 |
| **NN1-M-116** | 9.9 | 16.8 | 16.1 | 11.0 | 14.9 | 16.5 | 4.2 | 9.5 | 15.0 | 16.4 | 7.9 | 8.4 |
| **NN1-M-205** | 17.5 | 9.1 | 11.5 | 9.3 | 11.0 | 29.3 | 4.9 | 5.1 | 7.3 | 15.1 | 18.9 | 15.6 |
| **NN1-M-252** | 11.0 | 10.2 | 8.7 | 7.6 | 7.6 | 12.2 | 8.5 | 9.3 | 17.5 | 12.2 | 8.1 | 12.2 |
| **NN1-M-284** | 9.4 | 10.6 | 13.4 | 15.6 | 10.2 | 19.1 | 7.2 | 7.1 | 7.9 | 13.1 | 5.4 | 5.3 |
| **NN1-M-293** | 13.9 | 7.0 | 21.8 | 10.6 | 15.6 | 8.0 | 4.4 | 9.5 | 17.1 | 15.1 | 14.2 | 5.6 |
| **NN1-M-313** | 7.7 | 8.9 | 14.6 | 9.3 | 16.6 | 12.3 | 5.0 | 10.1 | 13.6 | 8.0 | 4.7 | 6.3 |
| **NN1-M-357** | 18.8 | 15.9 | 9.7 | 6.9 | 17.3 | 14.5 | 8.8 | 2.8 | 11.9 | 15.5 | 6.5 | 16.4 |
| **NN1-M-363** | 3.4 | 3.4 | 3.1 | 3.6 | 2.7 | 4.5 | 2.9 | 2.4 | 2.7 | 2.4 | 1.7 | 2.5 |
| **NN1-M-430** | 15.4 | 12.3 | 20.8 | 13.7 | 11.2 | 22.7 | 5.5 | 8.5 | 17.3 | 12.9 | 10.4 | 8.6 |
| **NN1-M-490** | 9.1 | 12.0 | 5.9 | 13.3 | 17.7 | 11.1 | 7.3 | 8.3 | 4.2 | 12.7 | 20.8 | 17.0 |
| **NN1-M-506** | 4.3 | 3.7 | 3.5 | 3.4 | 3.5 | 4.9 | 1.5 | 2.4 | 2.5 | 1.9 | 2.8 | 2.9 |
| **NN1-M-700** | 3.7 | 2.6 | 3.8 | 3.2 | 2.4 | 3.4 | 2.4 | 4.0 | 2.4 | 2.0 | 4.0 | 2.0 |
| **NN1-M-701** | 4.1 | 2.5 | 2.3 | 4.3 | 4.4 | 2.2 | 2.6 | 3.5 | 1.9 | 2.6 | 3.3 | 3.0 |
| **NN1-M-764** | 14.9 | 16.1 | 9.4 | 6.6 | 10.2 | 14.3 | 7.3 | 5.1 | 9.8 | 13.1 | 14.3 | 13.1 |
| **NN1-M-827** | 9.6 | 12.7 | 14.7 | 10.0 | 24.0 | 16.3 | 3.8 | 9.5 | 4.5 | 15.1 | 12.4 | 12.0 |
| **NN1-M-1621** | 3.1 | 4.8 | 3.4 | 4.3 | 4.5 | 2.4 | 2.2 | 3.6 | 2.8 | 2.4 | 2.6 | 3.5 |
| **NN1-M-2259** | 13.3 | 13.1 | 12.7 | 16.5 | 20.4 | 11.8 | 5.6 | 9.3 | 9.5 | 13.9 | 13.3 | 14.7 |
| **NN1-M-2280** | 14.3 | 15.7 | 9.8 | 14.6 | 13.8 | 17.2 | 7.4 | 4.7 | 14.6 | 8.5 | 9.8 | 12.1 |
| **NN1-M-236** | 8.3 | 8.7 | 6.8 | 13.7 | 10.2 | 14.7 | 6.8 | 3.9 | 7.3 | 15.3 | 17.3 | 19.8 |
| **NN1-wild** | 4.6 | 1.5 | 4.8 | 4.4 | 3.6 | 4.9 | 5.3 | 5.1 | 2.5 | 3.1 | 3.8 | 4.0 |
| **Mean** | 9.9 | 9.7 | 10.2 | 9.9 | 11.3 | 12.2 | 5.2 | 6.7 | 8.9 | 9.9 | 10.5 | 10.1 |
| **Maximum** | 18.8 | 20.4 | 26.0 | 16.5 | 24.0 | 29.3 | 8.8 | 11.6 | 17.5 | 16.4 | 20.8 | 19.8 |
| **Minimum** | 3.1 | 1.4 | 2.3 | 3.2 | 2.4 | 2.2 | 1.5 | 2.4 | 1.9 | 1.9 | 1.7 | 2.0 |
| **SD** | 4.5 | 5.0 | 6.2 | 4.3 | 6.3 | 6.9 | 2.2 | 2.8 | 5.2 | 5.2 | 6.0 | 5.6 |

restricted conditions (Table 6). Bouachra et al. (2017) described association in bread making ability and zeleny sedimentation. The knowledge hence attained will be useful for improving wheat quality for their intended use in bakery products. Furthermore, on five selected lines biochemical assays were performed in $M_6$ and $M_7$ generation.

### 3.4. Biochemical assays of selected mutants for two mutant generations ($M_6$ and $M_7$)

The current study indicated that drought stress significantly decreased the total soluble proteins in both generations of wheat (Figure 5). The current study reveals a decline (16%–37%) in soluble proteins during dehydration, which are same as the findings of Rodriguez et al. (2002).

The wheat plant accumulates high levels of soluble sugars for mitigating water deficit. Soluble sugars work as osmo-protectants for leaf osmotic adjustment and protect the cells from dehydration (Guo et al., 2018). Similarly, in the current study an increase of 23%–69% in soluble sugar content due to desiccation was elucidated by all five mutant lines in both generations (Figure 5). Wheat genotype NN1-M-701 showed the highest increase, i.e.

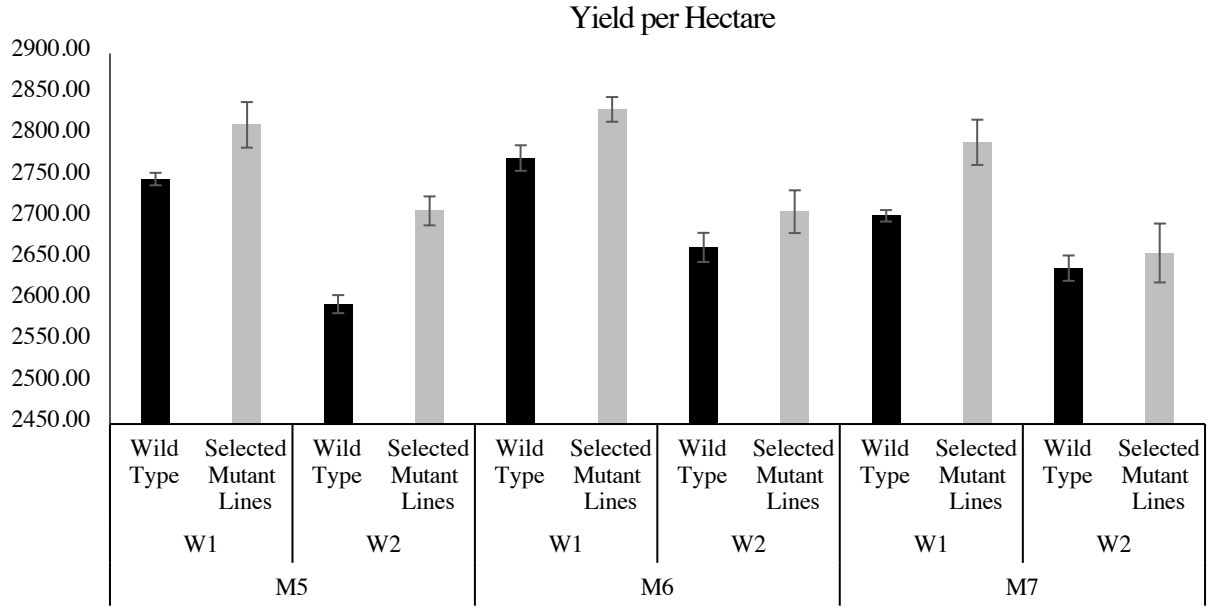


**Figure 4.** Comparison of yield per hectare of selected mutant lines vs. wild type among three mutant generations during $W_1$ and $W_2$.

64% and 69% in $M_6$ and $M_7$ generations, respectively. At $M_6$ generation, wheat genotypes NN1-wild, NN1-M-506, and NN1-M-252 depicted an increase of 62%, 57%, and 40%, respectively, while for $M_7$ generation, both the mutants and wild type showed a positive response to total soluble sugars.

Free amino acids and sugars contents can be used as a vital selection criterion for drought tolerance. The increase (37%–76%) in TFAs in present study indicates stress tolerance in mutants by detoxification of reactive oxygen species (ROS) and perform the functional role as a compatible osmolyte (Noreen et al., 2017).

Similarly, a remarkable increase in total free amino acids was recorded for all the mutants in both generations. At $M_6$ generation, 72% increase was observed for NN1-M-506. While next year, 75% and 76% increase in TFAs were estimated for NN1-M-701 and NN1-M-506 respectively. TFAs accumulated more in $M_7$ as compared to $M_6$ generation. However, relatively less accumulation of TFAs was observed in NN1-M-1621 under rainfed conditions.

Drought limits photosynthesis by retarding the synthesis of photosynthetic pigments. In the present study, for total chlorophyll contents, NN1-M-wild depicted 17% reduction while NN1-M-506 (23%) for $M_6$ generation and showed better performance during dehydration (Figure 5). Photosynthetic pigments production reduces under drought stress because of splitting of thylakoid membranes due to desiccation of cells (Kalaji et al., 2016).

A dynamic part is played by carotenoid contents to scavenge oxygen, hence, the relative quantity of carotenoid in plants approves the comparative tolerance of the plant to stress. In the current experiment, a reduction in chlorophyll and carotenoid contents was observed in mutant genotypes in both generations. In the current experiment, maximum reduction (15% and 16%) in carotenoid content was observed in genotypes NN1-M-506 and NN1-M-701, respectively, for $M_6$ generation. NN1-wild showed a reduction of 20% in the $M_6$ generation while 36% reduction was recorded for the $M_7$ generation. The production of carotenoid contents in plants is affected by the genetics of the plant as well as the prevailing environments (Bollinedi et al., 2019).

Accretion of proline in wheat leaves sustains osmotic pressure of cell linked with a decrease in oxidative stress and high photosynthetic ability. Similarly, in the present study, water stress- induced proline contents accumulation in all mutants. Maximum increase (76%) was recorded for NN1-M-701, followed by 73% in each of NN1-M-506, and wild type under rainfed conditions (Figure 5). These mutants are found highly reactive due to the rise in proline contents during water-limited conditions. The present results were consistent with the findings of Quilambo (2004) that water deficiency enhanced synthesis of proline and resultantly increase proline content in leaves. Due to the effect of mutagenesis, all selected EMS mutants showed an increase in total soluble sugars, total free amino acids, and proline content while decreased total chlorophyll content, carotenoids, and total soluble protein even in water-limited conditions as mutagenesis helps in the improvement of traits as well as genetic variation in mutant genotypes of wheat to cope drought stress.

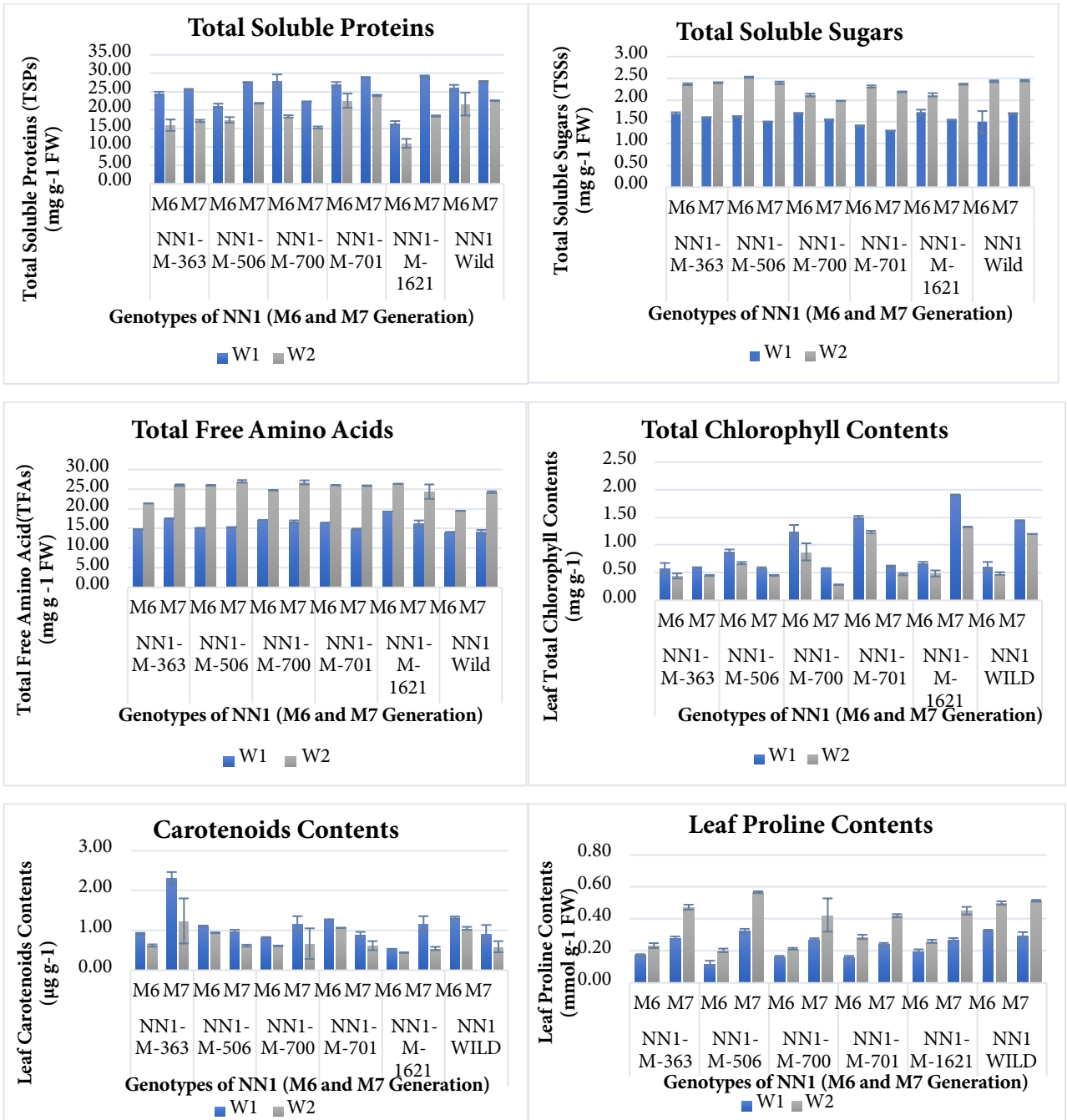


**Figure 5.** Biochemical assay (total soluble protein, total soluble sugar, total free amino acid, total chlorophyll contents, carotenoids contents, leaf proline contents) in NN-Gandum-1 genotype under well-watered (control, $W_1$ regime) and rainfed (stressed, $W_2$ regime) conditions. Values presented are means ± S.D of three replicates per genotypes in both $M_6$ and $M_7$ generation. Different color indicates stress condition, blue bar for W1-well watered (control) and grey bar for W2-rainfed (stressed) conditions.

## 4. Conclusion

In conclusion, EMS-based induced mutagenesis is helpful to create novel genetic diversity in complex traits like tolerance to drought. Significant variations in phenotype existed in the mutant population of NN-Gandum-1 for morphologically imperative traits linked with yield and biochemical parameters under drought stress. The correlation analysis studies highlighted the need to use the correlated traits as a means of indirect selection for the improvement of wheat crops. Additionally, the nonappearance of correlation between traits allows various valuable recombinants to be advantageous in breeding

plans. The created novel germplasm can be explored at length by the national and international wheat community for understanding the genetics to tolerate/resist drought stress in wheat.

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