EMS-based mutants are useful for enhancing drought tolerance in spring wheat

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Abstract
Sustainable wheat production in drought prone areas can be achieved by developing resilient wheat varieties. In the present study, chemical mutagenesis was used to induce mutations in a cultivated wheat variety ‘NN-Gandum-1’. In total, 44 mutants were selected based on their high yield potential for exposing to well-watered (W 1) and rainfed (W 2) conditions for one season. Then, 24 mutants were selected and were exposed to W 1 and W 2 regimes. On the basis of least relative reduction in physiological parameters under W 2 regime, five mutants were selected for conducting exome capturing assays. In total, 184 SNPs were identified in nine genes (ABC transporter type 1, aspartic peptidase, cytochrome P450, transmembrane domain, heavy metal-associated domain, HMA, NAC domain, NAD (P)-binding domain, S-type anion channel, Ubiquitin-conjugating enzyme E2 and UDP-glucuronosyl/UDP-glucosyltransferase). Maximum number of mutations were observed in chr.2D, which contained mutations in three genes, i.e. ABC transporter type 1, NAD (P)-binding domain and UDP-glucuronosyl/UDP-glucosyltransferase which may have a role in conferring drought tolerance. The selected mutants were further tested for studying their biochemical responses under both the regimes for 2 years. The extent of membrane damage was estimated through malondialdehyde and hydrogen per oxidase, and tolerance to drought stress was assessed via antioxidant enzymes in leaves. The selected mutants under drought stress increased the accumulation of proline content, total soluble sugars, total free amino acids, while decreased total chlorophyll content, carotenoids and total soluble protein. These mutants can further be explored to understand the genetic circuits of drought tolerance in wheat.

Keywords Triticum aestivum L. · EMS · Biochemical and physiological assay · Exome capture · SNPs · Drought

Introduction
Wheat (Triticum aestivum L.) is cultivated in more than 60 countries on 219.52 million hectares with an annual production of 758.27 million metric tons (https://apps.fas.usda.gov/psdonline/circulars/production.pdf). Cereals have central role in global food security, predominantly in developed countries. Huge efforts have been made to improve/sustain wheat production by developing advanced varieties with improved genetics. However, there are still many factors which significantly decrease the wheat production (Abhinandan et al. 2018).

Water deficit is predominant among the abiotic stresses that risk the cultivation of cereal crops. Around 60% of wheat production is primarily affected by the limited supply of water (Mendanha et al. 2020). Under the present scenario of climatic change, it could be inferred that water stress will aggravate in future (Noya et al. 2018). Currently, improving
yield potential and breeding drought-tolerant wheat varieties are in progress to meet the increasing food demand of growing population of the world (Sallam et al. 2019). With the onset of first green revolution (thirst agriculture revolution) between 1950 and 1960s, substantial advancements towards increasing wheat production were made by introducing dwarfing genes into the old wheat cultivars using conventional breeding techniques (Conway 1998). This improved germplasm containing the dwarfing genes was widely used for developing new wheat varieties, as a result, the wheat production increased significantly. There are still many areas left undiscovered which together reduce the wheat production globally especially drought stress (Senapati et al. 2019). To survive water stress via conventional breeding is challenging as it is difficult to regulate the multigenic response of abiotic stress tolerance (Khan et al. 2019). To combat dehydration, plants bring several changes in their physiological, morphological, biochemical and molecular mechanisms (Senapati and Semenov 2020).

Understanding the adaptive response of important phenotypic traits that contribute to improved productivity during stress is essential in order to comprehend physiological as well as genetic methods of wheat adaptation. Various antioxidants (both enzymatic and non-enzymatic) play an important role in plants to combat dehydration by controlling oxidative damage (Ren et al. 2020). It was observed that concentration of various antioxidant enzymes like peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) increased significantly to mediate the effects of reactive oxygen species (ROS) for mitigating the oxidative stress in wheat (Caverzan et al. 2014). Hence, enzymatic antioxidants have an important role in coping drought stress and thus can be used in screening drought-tolerant wheat varieties (Tabarzad et al. 2017).

For improving the genetics of wheat cultivars for coping drought, it is important to create allelic diversity followed by screening the wheat material under rainfed conditions. Mutagenesis had been an effective strategy to develop improved wheat genotypes and cope drought stress (Sharma and Zheng 2019). Mutations can be spontaneous or induced artificially by exposing the biological material with physical or chemical mutagens (Mba et al. 2010). Either for forward or reverse genetics, chemical mutagenesis is a standard choice for analysing gene function. EMS (ethyl methane sulphonate) is extensively applied chemical mutagen which creates abrupt point mutations in plant genome (Brini and Masmoudi 2014). Resultantly, mutated wheat lines and dwarf mutants have been produced, which are characterized to be drought resistant (Hussain and Rahman 2019).

With the genetic advancements, NGS (next-generation sequencing) tools can be used to assess genetic variation and polymorphism (Jia et al. 2018). Like other polyploids, wheat genome can withstand neutral mutations as these accumulated changes in genes have no significant effect on survival of wheat plant (Krasileva et al. 2017). For many plant species, complement of variant can be mapped via WGS (whole genome sequencing) (Schneeberger 2014) and these causal mutations can be recognized by identifying mapping regions (Mo et al. 2018). Wheat has large genome size of around 17.6 GB and sequencing the whole wheat genome is quite expensive (International Wheat Genome Sequencing Consortium 2014). In fact, exome capturing approach can be used to lessen the cost of WGS (Hodges et al. 2007). Exome capturing has been used to detect and sequence genetic variants among coding sequence (CDS) within wheat genome (Saintenac et al. 2011), and SNPs discovered by the exome capture assay can be helpful to detect mutations in putative candidate genes, mapping markers could also be developed (Rimbert et al. 2018).

In the present investigation, five mutants (NN1-M-363, NN1-M-506, NN1-M-700, NN1-M-701 and NN1-M-1621) were selected from large mutant population of a wheat cultivar NN-Gandum-1 (NN-1). These mutants were subjected to exome capturing to detect SNPs relevant to drought resistance during M4 generation. The genetic diversity among wheat genotypes and drought-tolerant mutant lines were further studied by applying different biochemical parameters for 2 years (M4 and M5 generation). The information generated through these studies can be used for exploring the genetic circuits of drought tolerance in wheat which will help in improving drought-tolerant wheat cultivars.

Materials and methods

Experimental growth conditions

A high yielding wheat variety ‘NN-Gandum-1’, developed by the Plant Genomics and Molecular Breeding (PGMB) Lab, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan was selected for developing mutant population. The seed of this cultivar was exposed to an optimized concentration of 0.8% (v/v) of ethyl methane sulphonate for 2 h at 35 °C. The seed was sterilized by performing three washings with 5% sodium hypochlorite and 70% ethanol to eradicate their residues. The procedure for developing the mutant population was described earlier by Hussain et al. (2018).

Generation advancement and screening/selection of mutant lines

The mutated population was advanced to M4 generation by raising single spike rows of M1 through M3. From planting to harvesting, recommended agronomic practices were applied to each generation. In total, 44 mutant lines were selected on the basis of their yield response and grown in randomized
complete block design (RCBD) in three replicates under two water regimes, i.e. well-watered conditions (W1 regimes), i.e. four irrigations while other set was grown in rainfed conditions (W2 regimes), i.e. ~170 mm rainfall. In total, 24 high yielding mutant lines were selected on the basis of drought tolerance indices and advanced to M5 generation. The drought indices such as tolerance index (TOL), geometric mean productivity (GMP), mean productivity (MP), stress susceptibility index (SSI) and stress tolerance index (STI) are calculated by following formulas:

\[
\begin{align*}
TOL &= YP - YS \\
MP &= (YP + YS)/2 \\
GMP &= \sqrt{YS \times YP} \\
STI &= \frac{(YP \times YS)}{P^2} \\
SSI &= \left\{1 - \left(\frac{YS}{YP}\right)\right\} / \left\{1 - \left(\frac{SP}{P}\right)\right\}
\end{align*}
\]

Here, \(YS\) and \(YP\) represent grain yield under drought stress and normal condition, respectively. Mean yield of all genotypes under normal condition is denoted as \(P\) and \(S\) indicates the mean yield of genotypes under stress.

**Physiological parameters**

These 24 mutant lines along with NN-1 (wild) were tested for physiological parameters such as photosynthetic absorbance rate (PAR) at leaf surface, sub-stomatal conductance, transpiration and photosynthetic rate under both the water regimes (W1 and W2 regime). Data were recorded using LcPro-SD portable Photosynthetic System. A total of five mutant lines (NN1-M-363, NN1-M-506, NN1-M-700, NN1-M-701 and NN1-M-1621) were selected on the basis of physiological parameters.

**Exome capture assay**

These selected mutants and their parent NN-1 (wild) were sown for isolation of a high-quality DNA by adopting the protocol described by (Dvorak et al. 1998) with few amendments as described by (Hussain et al. 2018). After sequencing the exome capture libraries, the sequence of each DNA fragment from start to stop codon was assessed using bioinformatic tools like Fastq software, ‘bwaain’ and ‘bwa sampe’ programs, samtools and bamtools, MAPS (http://comailab.genomecenter.ucdavis.edu/index.php/MAPS) and ‘mpileup’ pipeline (http://comailab.genomecenter.ucdavis.edu/index.php/Mpileup). The real SNPs were differentiated from mutations by applying an additional MAPS feature. The homozygous and heterozygous threshold was setup independently. Using Ensembl Variant Effect Predictor (VEP) release 78 in offline mode, the effect of mutation on gene function was predicted and detected.

**Biochemical assays**

For assessment of drought resistance, selected mutants were further tested for studying their biochemical responses under both the regimes (W1 and W2) for two consecutive normal wheat growing seasons. Various biochemical parameters such as enzymatic and non-enzymatic assays were analysed on M6 and M7 generations.

**Enzymatic assays**

Activity of superoxide dismutase (SOD, \(\mu \text{ min}^{-1} \text{ mg}^{-1}\)) was measured by deploying a protocol demonstrated earlier (Giannopolitis and Ries 1997). The photochemical reduction in nitro blue tetrazolium (NBT) is inhibited by SOD at 560 nm, and this inhibition is used to assay SOD activity. The reaction mixture was prepared by mixing 0.95 cm\(^3\) phosphate buffer (50 mM), 0.5 cm\(^3\) methionine (13 mM), 1 cm\(^3\) NBT (50 μM), 0.5 cm\(^3\) EDTA (75 mM), 0.005 riboflavin, enzyme extract (50 µg/mL) and ionized H\(_2\)O (0.25 ml). The solution was irradiated by a fluorescent lamp (30 V) for 15 min. Afterwards, inhibition in photochemical activity of NBT and non-irradiated reaction were analysed at 560 nm and were used to monitor the SOD activity.

The protocol devised by (Chance and Maehly 1995) was used to determine peroxidase activity (POD, \(\text{min}^{-1} \text{ g}^{-1} \text{ FW}\)). The reaction mixture consisted of phosphate buffer (50 mM), guaiacol (20 mM), H\(_2\)O\(_2\) (40 mM) and enzyme extract (0.1 mL). POD activity was calculated by the estimation of the oxidation of guaiacol and peroxidation of H\(_2\)O\(_2\) using an extinction coefficient of 2.47 mM\(^{-1}\) cm\(^{-1}\).

The catalase activity (CAT, \(\text{min}^{-1} \text{ g}^{-1} \text{ FW}\)) was measured by adopting a procedure described earlier (Chance and Maehly 1995). The reaction mixture contained phosphate buffer (50 mM), H\(_2\)O\(_2\) (5.9 mM) and enzyme extract in a volume of 3 mL. The catalase activity was determined by the rate of H\(_2\)O\(_2\) decomposition at 240 nm after every 20 s.

The ascorbate peroxidase (APX, \(g^{-1} \text{ FW} \text{ h}^{-1}\)) was quantified using the methodology proposed by (Cakmak 1994). The reaction mixture comprised of phosphate buffer (50 mM), sodium-EDTA (0.1 mM), H\(_2\)O\(_2\) (12 mM), ascorbic acid (0.25 mM) and sample extract in a total volume of 1 ml. The rate of ascorbate oxidation at 470 nm was monitored after every 20 s to measure APX activity. Finally, an
extinction coefficient of 2.8 mM⁻¹ cm⁻¹ was used to calculate the APX activity.

**Non-enzymatic assays**

For the detection of malondialdehyde contents (MDA, μmol g⁻¹ FW), a published protocol was followed (Cakmak and Horst 1991). In total, 1 g leaf tissue was added into 3 cm³ of trichloroacetic acid solution (0.1% w/v) and centrifuged at 20,000 rpm for 15 min. The cocktail was made by adding 0.5 cm³ supernatant and 3 cm³ thiobarbituric acid (0.5%) prepared in TCA (20%). Then, mixture was incubated for one hour at 95 °C and centrifuged at 10,000 rpm for 10 min. Final concentration of MDA was estimated by measuring the change in optical density of supernatant at 532 nm and 600 nm using extinction coefficient (156 mmol⁻¹ cm⁻¹).

The hydrogen peroxide concentration (H₂O₂, μmol g⁻¹ FW) was measured by adopting a published protocol (Bates et al. 1973; Velikova et al. 2000). In total, 0.2 g leaf tissue was mixed into trichloroacetic acid solution and centrifuged at 10,000 rpm for 15 min. After centrifugation, a volume of 500 ul of each supernatant and potassium phosphate buffer (10 mM) were mixed together and 1 mL of potassium iodide solution (1 M) was added. The absorbance was recorded at 390 nm, and activity of the H₂O₂ was determined by comparing with the standard curve drawn from H₂O₂ (Sigma-Aldrich) via spectrophotometer.

**Statistical analysis**

Analysis of variance (ANOVA) and least significant difference (LSD) for each trait were estimated using Statistix 8.1 and SPSS16 software (Inc 2001). The statistical significance was computed at 5% probability.

**Results**

In the present studies, significant differences were found for yield per unit area among the mutant lines grown under both the water regimes (well-watered, W₁ and rain fed conditions, W₂) at p < 0.001. Wheat yield ranged from 3016.07 to 5500.56 kg ha⁻¹ in W₁ regime. While in W₂ regime, wheat yield fluctuated between 2256.32 and 4965.32 kg ha⁻¹. Highest yield was depicted by NN1-M-451 with 9.73% relative reduction (RR) under W₂ regime, while the lowest yield was determined for NN1-M-320 with 25.19% RR under W₂ regime (Table 1).

In this study, minimum TOL was calculated for NN1-M-363, NN1-M-628, NN1-M-41, NN1-M-700, NN1-M-1621 and NN1-M-506, while for SSI, NN1-M-363, NN1-M-628, NN1-M-41, NN1-M-506, NN1-M-700 and NN1-M-701 depicted lowest values (Table 1). Under normal conditions, these genotypes demonstrated high yield, while under W₂ regime, their yield was reduced. We selected 24 mutant lines which showed high MP and GMP values and minimum values for SSI and TOL as these were drought resistant and high yielding genotypes.

**Physiological parameters analysis**

In the current investigation, a total of 24 mutant lines having high yield were selected from M₄ population. These mutant lines along with wild type were tested for physiological parameters such as photosynthetically active radiation (PAR) at leaf surface, sub-stomatal conductance (gₛ), transpiration rate (E) and net photosynthetic rate (Pn) under W₁ and W₂ regimes during M₅ generation. Least relative reduction in PAR at leaf surface was observed for NN1-M-700 (4.2%), NN1-M-701 (4.5%), NN1-M-1621 (4.6%) and NN1-M-363(5%) as compared with wild type (5.1%) under W₂ regime (Fig. 1). Regarding sub-stomatal conductance, minimum percentage reduction was recorded for NN1-M-506 (5.5%), NN1-M-1621 (6.5%), NN1-M-363 (7.2%), and NN1-M-701 (7.8%) compared to wild type (7.7%) during water deficient condition. In case of transpiration rate, a decrease of 4.6% (NN1-M-363), 4.7% (NN1-M-506), 5.1% (wild type) and 5.5% (NN1-M-700) was demonstrated. As far as net photosynthetic rate is concerned, the mutants NN1-M-363 (10.93%), NN1-M-701 (11.14%) and NN1-M-700 (11.72%) showed minimum percentage reduction (Table S1).

On the basis of physiological parameters, five mutant lines, i.e. NN1-M-363, NN1-M-506, NN1-M-700, NN1-M-701 and NN1-M-1621 showing minimum reduction in PAR at leaf surface, net photosynthetic rate, sub-stomatal conductance and transpiration rate were selected for conducting exome capturing assays.

**Exome capture assay**

We selected five mutant lines, i.e. NN1-M-363, NN1-M-506, NN1-M-700, NN1-M-701 and NN1-M-1621 on the basis of their response to drought. These mutants were exposed to exome capture assay for the identification of SNPs. In total, 184 SNPs were identified in different genes which are involved in conferring drought tolerance. Overall, 96, 55, 14, 11 and 8 SNPs were detected in NN1-M-701, NN1-M-700, NN1-M-506, NN1-M-1621 and NN1-M-363, respectively (Table 2). The maximum number of SNPs both heterozygous and homozygous was observed in chr-2D (31 SNPs); however, few SNPs were found in chr-5D (7 SNPs) and chr-7B (3 SNPs). These SNPs dispersed on various wheat chromosomes are depicted in a Circos plot (Fig. 1).
Table 1  Yield and drought-related indices of 44 mutant lines along with wild type NN-1 sown under W1 and W2 regime during M4 generation

| Genotypes          | Yield data and drought tolerance indices |
|--------------------|------------------------------------------|
|                    |                                         |
|                    | Conditions W1 regime | W2 regime | RR  |
|                    |                          |           |     |
|                    | Yield                  | Drought tolerance indices |
|                    | Unit               | Tolerance index | Mean productivity | Geometric mean productivity | Stress tolerance index | Stress index | Stress susceptibility index |
|                    | kg ha⁻¹          | RR       | MP = (YP + YS)/2 | (YP × YS) / (YP)² | (YP - YS) / YP | 1 - (YS/YP) | 1 - (YS/YP)² / (YP/YP)² |
| Local Check-1      | 3639.47           | -18.77  | 683.15              | 3297.895                | 3280.158              | 0.612        | 0.188                   | 2.055       |
| Local Check-2      | 4711.35           | -20.04  | 944.06              | 4239.32                 | 4212.959              | 1.010        | 0.200                   | 2.194       |
| NN1-M-1            | 3760.56           | -5.49   | 206.3                | 3657.41                 | 3655.955              | 0.760        | 0.055                   | 0.601       |
| NN1-M-116          | 4359.29           | -4.53   | 197.34               | 4260.62                 | 4259.477              | 1.032        | 0.045                   | 0.496       |
| NN1-M-118          | 4179.89           | -11.27  | 470.91               | 3944.35                 | 3937.401              | 0.882        | 0.113                   | 1.234       |
| NN1-M-153          | 5224.87           | -6.88   | 359.55               | 5045.095                | 5041.891              | 1.146        | 0.069                   | 0.753       |
| NN1-M-158          | 4280.8            | -17.37  | 743.36               | 3909.12                 | 3891.410              | 0.861        | 0.174                   | 1.901       |
| NN1-M-1621         | 3682.07           | -3.35   | 123.33               | 3620.405                | 3619.880              | 0.745        | 0.033                   | 0.367       |
| NN1-M-205          | 3285.16           | -4.71   | 154.72               | 3207.8                  | 3206.867              | 0.585        | 0.047                   | 0.516       |
| NN1-M-2252         | 4585.77           | -9.45   | 433.42               | 4369.06                 | 4363.682              | 1.083        | 0.095                   | 1.035       |
| NN1-M-236          | 4366.02           | -7.4    | 322.91               | 4204.565                | 4201.464              | 1.004        | 0.074                   | 0.810       |
| NN1-M-252          | 4004.98           | -3.3    | 132.3                | 3938.83                 | 3938.274              | 0.882        | 0.033                   | 0.362       |
| NN1-M-277          | 4466.93           | -13.3   | 594.25               | 4169.805                | 4159.206              | 0.984        | 0.133                   | 1.457       |
| NN1-M-284          | 4058.8            | -4.86   | 197.33               | 3960.135                | 3958.906              | 0.892        | 0.049                   | 0.532       |
| NN1-M-285          | 3426.44           | -12.96  | 444                  | 3204.44                 | 3196.741              | 0.581        | 0.130                   | 1.419       |
| NN1-M-494          | 4314.44           | -17.46  | 753.46               | 3937.71                 | 3919.647              | 0.874        | 0.175                   | 1.912       |
| NN1-M-338          | 4747.23           | -13.7   | 650.31               | 4422.075                | 4410.104              | 1.106        | 0.137                   | 1.500       |
| NN1-M-363          | 4415.35           | -1.07   | 47.09                | 4391.805                | 4391.742              | 1.097        | 0.011                   | 0.117       |
| NN1-M-370          | 3482.5            | -10.26  | 357.18               | 3303.91                 | 3299.080              | 0.619        | 0.103                   | 1.123       |
| NN1-M-41           | 3996.01           | -2.41   | 96.42                | 3947.8                  | 3947.506              | 0.886        | 0.024                   | 0.264       |
| NN1-M-411          | 4594.74           | -3.9    | 179.39               | 4505.045                | 4504.152              | 1.154        | 0.039                   | 0.427       |
| NN1-M-414          | 4500.56           | -5.98   | 269.09               | 4366.015                | 4363.941              | 1.083        | 0.060                   | 0.655       |
| NN1-M-430          | 3825.59           | -3.81   | 145.76               | 3752.71                 | 3752.002              | 0.801        | 0.038                   | 0.417       |
| NN1-M-44           | 4789.84           | -13.87  | 664.52               | 4457.58                 | 4445.180              | 1.124        | 0.139                   | 1.519       |
| NN1-M-451          | 5500.56           | -9.73   | 535.24               | 5232.94                 | 5226.092              | 1.554        | 0.097                   | 1.065       |
| NN1-M-474          | 4588.02           | -17.4   | 798.31               | 4188.865                | 4169.804              | 0.989        | 0.174                   | 1.905       |
| NN1-M-490          | 4419.83           | -7      | 309.45               | 4265.105                | 4262.298              | 1.033        | 0.070                   | 0.767       |
| NN1-M-493          | 4601.47           | -16     | 736.15               | 4233.395                | 4217.363              | 1.012        | 0.160                   | 1.752       |
| NN1-M-320          | 3016.07           | -25.19  | 759.75               | 2636.195                | 2608.681              | 0.387        | 0.252                   | 2.758       |
| NN1-M-504          | 4386.62           | -7.89   | 530.3                | 4121.47                 | 4112.932              | 0.962        | 0.121                   | 1.324       |
| NN1-M-506          | 4612.68           | -2.72   | 125.57               | 4549.895                | 4549.462              | 1.177        | 0.027                   | 0.298       |
| NN1-M-514          | 3330.01           | -11.78  | 392.42               | 3133.8                  | 3127.652              | 0.556        | 0.118                   | 1.290       |
### Table 1 (continued)

Yield data and drought tolerance indices

| Genotypes       | Yield | Drought tolerance indices                                      |
|-----------------|-------|----------------------------------------------------------------|
|                 | Unit  | Tolerance index | Mean productivity | Geometric mean productivity | Stress tolerance index | Stress index | Stress susceptibility index |
| Conditions      | W1 regime | W2 regime | RR | MP = (YP + YS)/2 | GMP = SQRTof (YS × YP) | STI = (YP × YS)/ (P^2) | SI = 1 − (YS/YP) | SSI = 1 − (YS/YP)/(1 − (S/P)) |
| NN1-M-535       | 4330.14 | 3774.01 | −12.84 | 556.13 | 4052.075 | 4042.523 | 0.930 | 0.128 | 1.406 |
| NN1-M-537       | 4610.44 | 3861.47 | −16.25 | 748.97 | 4235.955 | 4219.369 | 1.013 | 0.162 | 1.779 |
| NN1-M-60        | 3648.44 | 3392.8  | −7.01  | 255.64 | 3520.62  | 3518.299 | 0.704 | 0.070 | 0.767 |
| NN1-M-61        | 3569.95 | 3415.22 | −4.33  | 154.73 | 3492.585 | 3491.728 | 0.694 | 0.043 | 0.475 |
| NN1-M-628       | 4321.17 | 4231.47 | −2.08  | 89.7  | 4276.32  | 4276.085 | 1.040 | 0.021 | 0.227 |
| NN1-M-700       | 4224.74 | 4105.89 | −2.81  | 118.85 | 4165.315 | 4164.891 | 0.987 | 0.028 | 0.308 |
| NN1-M-701       | 4704.62 | 4566.86 | −2.93  | 137.76 | 4635.74  | 4635.228 | 1.222 | 0.029 | 0.321 |
| NN1-M-764       | 3993.77 | 3823.35 | −4.27  | 170.42 | 3908.56  | 3907.631 | 0.869 | 0.043 | 0.467 |
| NN1-M-827       | 4608.2  | 4361.53 | −5.35  | 246.67 | 4484.865 | 4483.169 | 1.143 | 0.054 | 0.586 |
| NN1-M-83        | 4027.41 | 3836.74 | −4.73  | 190.67 | 3932.075 | 3930.919 | 0.879 | 0.047 | 0.518 |
| NN1-M-85        | 4099.17 | 3832.32 | −6.51  | 266.85 | 3965.745 | 3963.500 | 0.894 | 0.065 | 0.713 |
| NN1-M-852       | 4253.89 | 3836.8  | −9.8   | 417.09 | 4045.345 | 4039.966 | 0.928 | 0.098 | 1.074 |
| NN1-M-88        | 4191.11 | 3256.32 | −22.3  | 934.79 | 3723.715 | 3694.265 | 0.776 | 0.223 | 2.442 |
| NN1-M-882       | 3269.47 | 2865.32 | −12.36 | 404.15 | 3067.395 | 3060.732 | 0.533 | 0.124 | 1.354 |
| NN1-M-parent    | 4249.41 | 4102.32 | −3.46  | 147.09 | 4175.865 | 4175.217 | 0.992 | 0.035 | 0.379 |
| Mean            | 4192.68 | 3809.77 | −9.13  | 387.2  | 4003.3   | 3996.5   | 0.9    | 0.1   | 1.0  |
| Max             | 5500.56 | 4965.32 | −9.73  | 944.1  | 5232.9   | 5226.1   | 1.6    | 0.3   | 2.8  |
| Min             | 3016.07 | 2256.32 | −25.19 | 47.1   | 2636.2   | 2608.7   | 0.4    | 0.0   | 0.1  |
| SD              | 514.26  | 530.17  | 3.09   | 253.390| 507.070  | 508.701  | 0.227  | 0.061 | 0.664 |
| SE              | 75.074  | 77.398  | 3.09   | 36.961 | 73.964   | 74.202   | 0.033  | 0.009 | 0.097 |
Lines in third circle indicate the distribution of SNPs on five wheat mutants, i.e. Blue; NN1-M-363, Green; NN1-M-506, Yellow; NN1-M-700, Red; NN1-M-701 and Light blue; NN1-M-1621.

The maximum number of mutations was detected in chr.2D. However, minimum mutation density was identified in chr.6A, chr.5A, chr.7B and chr.7D. The mean mutation rate was 0.04 mutations/Mb (Table S2).

In the present studies, out of the 184 SNPs, 31 were induced in genes closely related to abiotic stresses particularly drought such as *ABC transporter type 1, aspartic peptidase, cytochrome P450, transmembrane domain, heavy metal-associated domain, HMA, NAC domain, NAD(P)-binding domain, s-type anion channel, ubiquitin-conjugating enzyme E2, and UDP-glucuronosyl/UDP-glucosyltransferase* (Table S3).

One mutation was observed in each of the *ABC transporter type 1, transmembrane domain, Aspartic peptidase, heavy metal-associated domain, HMA, NAC domain, S-type*

### Table 2 - Detail of SNPs detected in the selected wheat mutant lines

| Chromosome | Genome | NN1-M-701 | NN1-M-700 | NN1-M-506 | NN1-M-1621 | NN1-M-363 | # of SNPs |
|------------|--------|-----------|-----------|-----------|------------|-----------|-----------|
| 1          | A      | 7         | 2         | 1         | 2          | 0         | 12        |
|            | B      | 2         | 1         | 1         | 1          | 1         | 6         |
|            | D      | 1         | 2         | 4         | 1          | 0         | 8         |
| 2          | A      | 4         | 14        | 0         | 2          | 1         | 21        |
|            | B      | 9         | 3         | 0         | 1          | 4         | 17        |
|            | D      | 20        | 10        | 1         | 0          | 0         | 31        |
| 3          | A      | 2         | 0         | 2         | 0          | 0         | 4         |
|            | B      | 2         | 8         | 1         | 0          | 0         | 11        |
|            | D      | 2         | 3         | 2         | 0          | 0         | 7         |
| 4          | A      | 6         | 1         | 0         | 1          | 0         | 8         |
|            | B      | 4         | 1         | 0         | 0          | 0         | 5         |
|            | D      | 3         | 2         | 0         | 0          | 0         | 5         |
| 5          | A      | 4         | 0         | 0         | 0          | 0         | 4         |
|            | B      | 9         | 1         | 0         | 0          | 0         | 10        |
|            | D      | 5         | 0         | 0         | 1          | 1         | 7         |
| 6          | A      | 0         | 1         | 0         | 1          | 1         | 3         |
|            | B      | 4         | 0         | 0         | 1          | 0         | 5         |
|            | D      | 7         | 1         | 0         | 0          | 0         | 8         |
| 7          | A      | 1         | 3         | 1         | 0          | 0         | 5         |
|            | B      | 1         | 2         | 0         | 0          | 0         | 3         |
|            | D      | 3         | 0         | 1         | 0          | 0         | 4         |
| Total      |        | 96        | 55        | 14        | 11         | 8         | 184       |
anion channel and Ubiquitin-conjugating enzyme E2 gene located on chr-2D of NN1-M-701 (114.5 Mb position), chr-2B of NN1-M-1621 (225.91 Mb position), chr-1A of NN1-M-700 (3.28 Mb position), chr-7B of NN1-M-700 (250.85 Mb position), chr-1B of NN1-M-701 (99.74 Mb position) and chr-1A of NN1-M-701 (3.88 Mb position) (Table 2). Similarly, four mutations were observed in Cytochrome P450 gene in NN1-M-700 and one in NN1-M-701. Likewise, four mutations in NN1-M-700, six in NN1-M-701, and one each in NN1-M-1621, and NN1-M-363 were observed in NAD(P)-binding domain gene. A total of six mutations in NN1-M-701 and two in NN1-M-700 were identified in UDP-glucuronosyl/UDP-glucosyltransferase gene (Table S4).

3D protein structure

The SNP identified in chimeric allele (heavy metal-associated domain, HMA) of a drought-resistant mutant (NN1-M-700) was located in HMA domain of chr.1A at 3.28 Mb position. Through computational analysis, it was demonstrated that this SNP causes a substitution of valine with methionine, resulting in a predicted altered protein structure (Fig. 2). This mutation, therefore, is a candidate for contributing to the resistance phenotype in the mutant line.

Biochemical assays

Enzymatic assays

The current results showed an increase in SOD activity in wheat mutants under rainfed conditions. In NN1-M-506, an increase in 35% in SOD activity was estimated during water stress in M6 generation, while in M7, the activity was 41% (Table S5). However, an increase of 21% and 20% in CAT activity was observed in NN1-M-506 and NN1-M-701, respectively, under rainfed conditions in M6 generation. The same trend was observed in M7 for both the genotypes, confirming that these mutants could be useful source for studying drought tolerance mechanism. In case of APX activity, 40% increase was observed in NN1-M-701 in M7 generation while during M6 generation, 23% increase was recorded. Furthermore, NN1-M-506 and NN1-M-wild also revealed improvement in APX activity. 77% increase in peroxidase activity under limited water conditions was observed in NN1-M-701 during M7 generation. Owing to the rise in specific peroxidase activity during drought condition, the most affected mutant was NN1-M-700 depicting 31% increase in peroxidase activity. The NN1-M-701and NN1-M-506 were observed as highly reactive showing elevated peroxidase activity during drought stress; however, NN1-M-700 showed minimum increase in both generations (Table S5).

Non-enzymatic assays

In this experiment, enhancement in MDA content in all mutant genotypes was recorded (Table S5). Maximum increase (44%) was noticed in NN1-M-701 likewise genotype NN1-M-506 also demonstrated significant increase (40%) under drought stress. These findings have shown that under water deficit, the oxidative damage in leaves of NN1-M-701 and NN1-M-506 was greater than that of NN1-M-363, NN1-M-700 and NN1-M-1621. The mutant NN1-M-701 depicted the maximum increase 58% and 47% of H2O2 contents in M6 and M7 generations, respectively. Furthermore, in M4 generation, NN1-wild and NN1-M-506 demonstrated 49% and 53% increase in H2O2, respectively. Likewise, in M7 generation, all the mutant genotypes displayed positive response.

Discussion

Wheat is dominantly a drought loving plant and mitigates the water limited conditions by bringing changes in its morphological, physiological and biochemical properties.

Productivity traits data

Increased grain yield of wheat is primary goal in drought affected areas. The use of drought-resistant genotypes with higher yield is an effective approach to reduce harmful effects of drought (Haque and Chowdhury 2020). Under drought stress conditions, significant variations for yield reduction were in accordance with Khakwani et al. (2012). To identify high yielding drought-tolerant genotypes under the normal and drought conditions, drought tolerance indices were used as screening criteria (Mohammed and Kadhem 2017). In previous studies, geometric mean productivity (GMP), mean productivity (MP) and stress tolerance index (STI) were found to be the most appropriate indexes for the identification of drought-tolerant cultivars (Mohammadi 2016). It was reported earlier that genotypes having minimum tolerance index (TOL) and stress susceptibility index (SSI) values were least sensitive to drought and selection exclusively based upon these indices directs high yielding genotypes under drought conditions (Dorostkar et al. 2015). The importance of the SSI was explained by (Fischer and Maurer 1978) and demonstrated that genotypes with less than one SSI value were tolerant to drought. We selected 24 mutant lines which showed high MP and GMP values and minimum values for SSI and TOL as these were drought resistant and high yielding genotypes.
Physiological parameters analysis

Photosynthesis, a primary metabolic process which determines crop production, is affected by drought stress (Chaves et al. 2009). Significant variation was found for all the physiological parameters among all the mutant lines under both water regimes at $p < 0.001$. Water stress caused prominent decrease in net photosynthetic rate ($Pn$), sub-stomatal conductance ($gs$) and transpiration rate ($E$) in wheat and different crops (Zhao et al. 2020). Under drought stress, the plant immediately closes stomata to maintain cellular moisture level (Osakabe et al. 2014). Moreover, diffusion of $CO_2$ from outside atmosphere to the sub-stomatal cavity is reduced, resulting in decrease in the stomatal conductance ($g_s$), and is the major reason of reduction in the photosynthetic rate ($Pn$) during drought (Flexas et al. 2009). Some other studies have also reported reduction in net photosynthetic rate and sub-stomatal conductance in important crops during drought condition, such as in rice (Wang et al. 2018) and wheat (Marček et al. 2019). Similar findings of drought-induced reduction were observed in stomatal conductance (Yan et al. 2016). In case of transpiration rate, a decrease of 4.6% (NN1-M-363), 4.7% (NN1-M-506), 5.1% (wild type) and 5.5% (NN1-M-700) was demonstrated. Reduction in transpiration rate was also reported in some other studies (Zhao et al. 2020). Reduced inhibition of net photosynthetic rate under limited water conditions is of great importance for drought tolerance (Liu et al. 2016).

Exome capture assay

The hexaploid wheat has relatively gigantic genome size of about 17.6 GB, and it is difficult to identify mutations in such a large genome through using whole genome sequencing but exome capturing is one of the most suitable assays which can be exploited to detect mutations induced by EMS. By deploying exome sequencing, mutations can be detected in coding regions of a gene (exon) only which can alter the gene function and expression. The exome sequencing can be exploited for searching mutations in expressed part of the genes using multiple software including MAPS, VEP, etc. It is well known that fluctuations in genomes size are contributed by the non-exonic portions of the genome, thus exome size remains about the same in all plant species. Consequently, exome capture can be practised in various crops regardless of the genome size and cost involved in conducting exome capture assay. This assay is extremely handy and economical for studying the genome of those species where reference genome assembly has not been constructed yet. We selected five mutant lines, i.e. NN1-M-363, NN1-M-506, NN1-M-700, NN1-M-701 and NN1-M-1621 on the basis of their response to drought. These mutants were exposed to exome capture assay for the identification of SNPs. However, chemical mutagenesis is a random process and many mutations are functionally silent. Also, the frequency of mutations in each site of the genome fluctuates substantially. Certainly, it was proved that if concentration of the mutagen remains the same, the rate of mutation ranges from 1 to 20 mutations per Mb in diverse entities (Henry et al. 2014). Yet, the total number of base pairs involved in the analysis varies by chromosome, followed by measuring the density of mutations.

The main reasons of these fluctuations in mutation density are due to the variations in penetrability of EMS within seeds and the differential ability of the cell to repair the damaged DNA which was also observed in previous studies (Hussain et al. 2018). In several other studies, variation in normal density of mutation such as 19.6 mutations per Mb, 20.1 mutations per Mb, 23 mutations per Mb and 33 mutations per Mb was noticed in wheat (Krasileva et al. 2017). In total, 121 mutations were reported in waxy genes in wheat, including silent, missense together with knockout by studying 2348 EMS-mutated $M_2$ plants (Dong et al. 2009). Similarly, in four important genes, i.e. $LBP$, $COMIT1$, $HCT2$, and $4CL1$, SNPs were recognized. In TILLING population of polyploid wheat, the mutation frequency was one mutation per 17.6 kb–34.4 kb. However, in case of $T. monococcum$, only one mutation per 90 kb was identified in waxy genes (Rawat et al. 2012).

Biochemical assays

Enzymatic assays

The SOD protects from the activity of ROS in plants (Noctor et al. 2018). It has the ability of affecting superoxide radicals, catalyses and converts $O_2^-$ to $O_2$ and $H_2O_2$ (Sharma et al. 2012). The SOD activity was increased under drought stress in all mutants, in previous experiments, an increase in SOD activity was also observed in plants facing abiotic stresses, like water deficiency and toxic metal effects (Mishra et al. 2011). The SOD activity could be used as potential selection strategy for screening drought-resistant plants (Zaefyzadeh et al. 2009). Thus, NN1-M-506 could be used as a drought-tolerant genotype in future wheat breeding experiments. Catalase enzymes have an essential role in modulation of ROS in plant cells by deactivation of hydrogen peroxide ($H_2O_2$) (Mailloux 2018). During water limited conditions, CAT regulates harmful levels of endogenous $H_2O_2$ by catalysing a redox reaction within cell peroxisomes (Liu et al. 2014). An increase in catalase activities was noticed in wheat leaves when exposed to extreme water stress, particularly more insusceptible varieties (Simova-Stoilova et al. 2010).
The ascorbate peroxidases are the vital enzymes in plant cells that scavenge H$_2$O$_2$ in different organelle of plant cells as in chloroplast and cytosol to protect against oxidative damage (Strukul 2013). The APX activity was increased significantly; likewise, an increase in APX activity during drought stress was reported by (Abid et al. 2018). Hence, reactive oxygen species production in water deficient cells results in cell damage ultimately leads towards cell death (Qiu et al. 2019). Various antioxidant systems through multiple adaptive mechanisms regulate oxidative stress, one of them is peroxidase enzyme whose activity was increased under moderate level of water deficit (Manuchehri and Salehi 2014). The enhanced expression of peroxidase in a cell is linked with more water retention and thus rewarding tolerance to drought as it was demonstrated in Nicotiana tabacum. Like many other crop species, peroxidase activity was increased in wheat under water limited conditions (Devi et al. 2012). The POD is present in cytosol, vacuoles, extracellular spaces and cell walls. This enzyme is well thought stress indicator which has a wide range of selectivity for phenolic substrates and more attraction for H$_2$O$_2$ than that of catalase. It has the ability to utilize H$_2$O$_2$ in order to generate phenoxy compounds which ultimately polymerizes lignin (cell wall component) (Reddy et al. 2005). Increased POD activity revealed in the present study can be associated with the release of peroxidase localized in the cell walls (Liu et al. 2014).

Non-enzymatic assays

The malondialdehyde production in plants is stimulated by free radicals (Dubey and Pandey 2011). Membrane lipid peroxidation was evaluated with the production of MDA content that indicates the degree of membrane damage under stress. A positive relationship was found among the amount of MDA and demolition of biological membranes. This suggests that increase in MDA contents results in more lipid peroxidation and higher cell deterioration (Sreekanth et al. 2013). In some other findings, higher MDA contents under water stress conditions were demonstrated; however, drought-susceptible and drought-tolerant genotypes expressed differential responses (Kaur et al. 2014). Moreover, the drought-tolerant wheat genotype demonstrated reduction in lipid peroxidation and greater membrane stability (Nadia and Naqvi 2010). The H$_2$O$_2$ is a stress predictor of water deficit conditions in plants. It regulates respiratory pathways (Hasanuzzaman and Fujita 2011). Similarly, wheat plant protects leaves from oxidative stress by activating the antioxidant defence system. Multiple factors such as plant species, stress intensity and plant growth decide the H$_2$O$_2$ detoxification by an antioxidant enzyme (Noreen et al. 2009). During drought stress, H$_2$O$_2$ scavenges the ROS by activating enzymatic antioxidant defence mechanisms in wheat (Hussain et al. 2011). These findings are in close conformity that H$_2$O$_2$ is a stress marker that regulates the antioxidants to decrease the damage under oxidative stress (He and Gao 2009).

Conclusions

In the present study, it was shown that genes relevant to agronomically significant traits can be improved via induced mutations. Also, the mutations induced in functionally important part of the gene can be identified using NGS-based exome capturing assay, a strategy to save time and cost without compromising the significant mutations. This work also highlighted the distribution pattern, variation in frequency of mutation in different mutant lines of wheat. Based on the current investigations, it can be suggested that mutant wheat population NN-Gandum-1 is suitable for exploring the genetic circuits of several genetic mechanisms using forward and reverse-genetic approaches. The mutants were also explored using various biochemical and physiological assays and showed significant variations under rainfed conditions. Hence, this population can be used by the Int wheat community for designing new strategies for mitigating drought stress.

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