Agro-morphological evaluation of gamma ray-induced mutant populations and isolation of harder grain mutants in wheat (*Triticum aestivum* L.)

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Abstract

Gamma ray-induced mutations have been widely used to improve existing crop germplasm and create novel genetic variation. In the current study, a multi-year experiment was carried out to induce and isolate mutants with desirable agro-morphological traits and improved grain hardness through evaluation of induced mutant populations generated in soft-textured wheat variety HPW 89 irradiated with gamma ray dose of 250, 300 and 350 Gy. Mutagen sensitivity studies revealed a higher frequency of biological damage and seedling mortality for doses beyond 300 Gy in the M_1 generation. However, the mutagenic treatments in the M_{2-3} populations significantly altered the magnitude of the biometrical traits. Results from the variability and association studies among traits showed that biological yield per plant, 1000-grain weight, spike length, grains per spike and plant height may be prioritized for higher genetic gain and could be used as selection criteria parameters. Multivariate analysis indicated induction of heterogeneity among mutant populations. Overall, 250–300 Gy doses were found ideal for a successful wheat mutation programme and 293 agro-morphologically superior wheat mutants were identified, out of which 108 had semi-hard grain texture based on single kernel characterization system. Among these, nine mutants were found to have the highest grain hardness index due to induced changes in one or both puroindoline genes. Hence, these mutants identified for several traits along with harder grain texture will serve as important genetic resource in future wheat-breeding programmes.

Introduction

Around the world, sustainable wheat production and twin pressures of climate change and population growth are some primary concerns of crop growers and stakeholders. It is estimated that by 2050, a 70% increase in wheat production will be needed to fill the expanding gap in food demand. This can be achieved by increasing yield per unit cultivation area and through developing climate-resilient genotypes using the variability available in the wheat germplasm. Cultivar development and improvement depend upon the deployment of suitable breeding methods when sufficient variation is available in the existing germplasm for a given trait (Cheng *et al.*, 2015). The traditional breeding procedures involving inter- and intra-hybridization are based on the presence of existing natural variation in the germplasm of a crop species and finding desirable recombinants with a precise assortment of traits which creates a critical bottleneck for varietal development under a rapidly changing environment (Shivakumar *et al.*, 2018; Bakshi *et al.*, 2022). Hence, a fast, powerful and proven novel approach such as mutation breeding can assist plant breeders in generating variability and simultaneously allowing selection of mutants with altered phenotypes with greater frequency than the occurring natural variability. Induced mutagenesis involves the improvement of a single character or a line without substantially altering the entire genome and may even result in the development of an entirely novel character different from the parental genotype (Maghuly *et al.*, 2018). In different crop species, about 3432 direct and indirect mutant varieties have been developed through physical and/or chemical mutagenesis which are documented in the IAEA, Mutant Variety Database (IAEA, 2023). Mutation induction with radiation has been the most frequently used method, accounting for 90% of the total mutation-derived varieties (IAEA, 2023). Among all mutagens, gamma rays are the most popular physical mutagen as they can help address various challenges like improving crop productivity, resistance to stresses and enhancing quality for developing nutritious and resilient crops for the future (Riviello-Flores *et al.*, 2022; Bharat *et al.*, 2024). These have been used for the development of 50% of the mutant varieties in different crops (Dhole *et al.*, 2024). These have also been

used as a proven mutagen source for broadening genetic variability for improving grain yield and component traits upon which selection can be operated in the M_2 and subsequent generations (Rana *et al.*, 2024*a*). The success of the induced mutagenesis approach in wheat can be quantified from the official release of 275 mutant varieties by 2023 and among these mutant wheat varieties, around 69 varieties have been improved for one or more quality traits. However, the selection of effective and efficient mutagens and their potent doses are essential for recovering high frequency and spectrum of desirable mutations before initiating any mutation programme (Mangi *et al.*, 2016). In earlier studies, the significance of micro-mutations in crop improvement has been emphasized for identifying mutant genotypes with a desirable set of quantitative traits (OlaOlorun *et al.*, 2021). The genetic variability generated thus can be used for developing resistance to diseases, higher yield and wider adaptability to changing environmental conditions (Ahloowalia *et al.*, 2004; Khursheed *et al.*, 2018).

Efforts to improve wheat quality through gamma irradiation have been made in wheat and traits such as grain number, size, shape and weight, sedimentation value, protein content, gluten content, gluten index and grain hardness (Lai *et al.*, 2014; Balkan, 2018; Cao *et al.*, 2018; Kenzhebayeva *et al.*, 2018, 2019; Rahemi *et al.*, 2018; Abdelaleem and Al-Azab, 2021; Balkan *et al.*, 2021; Korkut *et al.*, 2021) have been improved or modified using mutated alleles. The foremost contributing factors determining end-use quality are endosperm texture, protein content and gluten strength. Among these quality parameters, the texture of the wheat kernel is one of the most critical factors determining the milling properties and end-use quality for the preparation of bread, cookies and pastries (Boehm *et al.*, 2018). Wheat kernel texture is categorized into 'soft' and 'hard' types, which are controlled by two linked *pin* genes located on chromosome 5DS (Morris, 2002; Bhave and Morris, 2008). When both puroindoline genes are in their 'functional' wild state, grain texture is soft and when one of the puroindolines is either absent or both altered by mutation, then the resultant is a semi-hard/hard grain texture. These grain texture types are due to spontaneous mutations in both *Pin* genes and infer that there is a possibility to create additional alleles at this locus. Isolation and identification of mutations at *Pin* locus require precise phenotyping of grains with a modified texture which is evaluated using particle size index (Delwiche, 2000; Osborne *et al.*, 2001), single kernel characterization system (SKCS) (Kosmolak, 1978), pearling value (Chung *et al.*, 1975) and near-infrared reflectance (Wetzel, 1984; Manely *et al.*, 1996). In this study, an elite high-yielding soft-textured wheat variety HPW 89 was selected having a high degree of resistance to yellow rust, and brown rust with wider adaptability to rainfed ecosystems but has limited consumer preference due to chalky grains, poor *chapatti*-making quality and storage problems owing to soft grain texture. Cultivars with soft grains have been found to possess wild-type alleles and single locus genetic control of grain hardness offers an opportunity to isolate elite mutant lines. Moreover, scanty literature is available on wheat mutation induction, especially in hexaploid wheat. Besides, this may be the first or among a few initial reports to induce hardness in soft-textured genotype *via* induced mutagenesis. Hence, the present study was undertaken to induce genetic variability and isolate potential mutants having desirable agro-morphological traits with higher grain hardness for rainfed areas of the Northern Hill Zone.

Materials and methods

Experimental materials

Healthy, dry, uniformly sized and well-filled seeds with a moisture content of 12% of soft-textured bread wheat variety HPW 89 (INTERMEDIO RODI/HD 2248) were procured from Rice and Wheat Research Centre (RWRC), Malan, Himachal Pradesh, India.

### Population development and experimental site

The mutagenic treatments for irradiation in HPW 89 were first optimized by performing radiosensitivity tests and estimation of median lethal dose (LD_{50}) using seedling mortality *via* probit analysis of gamma irradiation (γ-rays) using 12 doses ranging from 25 to 300 Gy with increments of 25 Gy (Rana *et al.*, 2022). Based on the observed LD_{50} dose, out of the total 9000 seeds irradiated, 3000 seeds were treated with three individual doses viz., 250, 300 and 350 Gy each at Nuclear Agriculture & Biotechnology Division, Bhabha Atomic Research Centre (BARC, Mumbai, India) at the rate of 0.862 kGy/h. Generation-wise methodologies employed in the field experiment are described below.

M_1 generation

A total of 9000 bread wheat seeds of HPW 89 (3000/treatment) were sown in a randomized complete block design with 100 seeds/set and 30 replications under natural field conditions during *rabi* (mid-November) 2019–20. Each treatment consisted of 10 seeds × 10 rows (100 seeds) along with an 11th row as parental control per replication with 10 cm plant-to-plant spacing and 20 cm row-to-row spacing. All recommended packages of practices for raising the crop were followed such as timely irrigation, and fertilizer dose at the rate of 120 kg N/ha, 60 kg P_2O_5/ha and 40 kg K_2O/ha were added respectively, from sowing until harvesting. During mid-May, the main spike from each M_1 plant was individually threshed and stored for raising subsequent generations. Data on per cent germination (G) in each treatment and replication was observed and recorded regularly up to 30 d after sowing. Besides, seedlings that could survive with normal growth till maturity were expressed as a percentage of the number of plants that survived (PS) to the total number of seeds sown. The graphical representation of the effect of induced physical mutagenesis on germination (%) and plant survival (%) is presented in Fig. 1. The experiment was conducted at the experimental farm, RWRC, Malan, Himachal Pradesh, situated at 32°12′N latitude, 76°42′E longitude in the North-Western zone of India at 950 a.m.s.l. elevation. The agro-climatic condition of the location represents the mid-hill zone of Himachal Pradesh (zone II), characterized by a humid sub-temperate climate with high rainfall (2500 mm/annum). The soil is silt clay loam with acidic pH (5.0–5.6). The spikes harvested from 2219 plants that survived under different treatments were individually threshed and further advanced to M_2 generation.

M_2 generation

A total of 2219 M_1 progenies (250 Gy: 1251; 300 Gy: 756; 350 Gy: 212) were sown as individual spike to row (single row of 2.0 m length) along with control after every 10th row. The progenies were space planted with 10 cm plant-to-plant spacing and 20 cm row-to-row spacing in the field at RWRC, Malan during *rabi* (mid-November) 2020–21 to raise M_2 generation. Mean data for 12 quantitative traits was recorded from five–eight
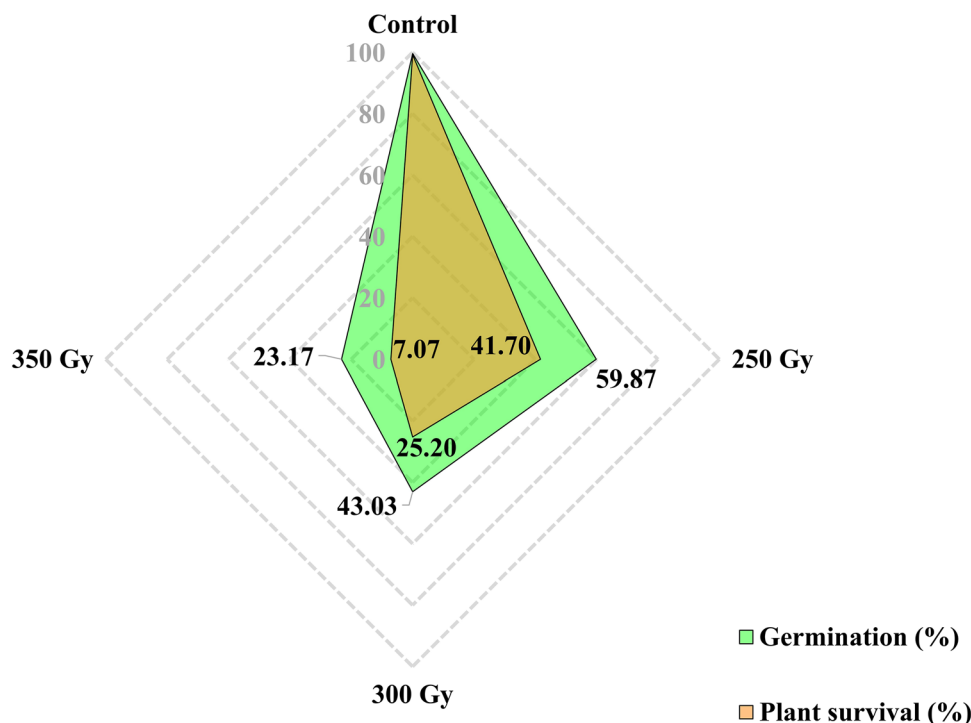
Figure 1. Effect of induced physical mutagenesis on germination (%) and plant survival (%) in M_1 generation of wheat variety HPW 89.

random progenies in each row for days to 50% flowering (DTF), plant height (PH), tillers/plant (NOT), peduncle length (PL), spike length (SL), grains/spike (GPS), grain filling period (GFP), days to 75% maturity (DTM), biological yield/plant (BYP), 1000-grain weight (TGW), harvest index (HI) and grain yield/ plant (GY). Based on the grain yield data, M_2 progenies with higher yield responses from each mutant population were selected, separately harvested and threshed.

M_3 generation

During summer (mid-May) 2021, a total of 1862 selected M_2 progenies (250 Gy: 1164; 300 Gy: 609; 350 Gy: 89) selected in M_2 generation were sown as individual spike to row (two rows of 1.0 m length) along with control after every 10th row with 20 cm row-to-row spacing at the experimental farm, Highland Agricultural Research and Extension Centre (HAREC), Kukumseri, Lahaul and Spiti, for generation advancement as M_3 generation. This location is situated at 32°70′N latitude, 76° 69′E longitude at an elevation of 2772 a.m.s.l. and agro-climatically, it falls under the high hill temperate dry zone of Himachal Pradesh (zone IV). The soil is sandy to sandy loam in texture, neutral in reaction and low-to-medium in fertility. Mean data for 12 quantitative traits were recorded from five progenies in each row. The progenies with higher grain yield statistics were selected and seeds harvested from each selected plant were threshed separately. A total of 293 agro-morphologically superior M_3 progenies were screened (250 Gy: 281; 300 Gy: 8; 350 Gy: 4) along with six checks viz., HS 542 (MILAN/KAUZ//PRINIA/3/ BABAX), HS 562 (OASIS/SKAUZ//4*BCN/3/2*PASTOR), HPW 349 (OASIS/SKAUZ//4*BCN/3/PASTOR/4/KAUZ*2/YACO// KAUZ), HS 507 (KAUZ/MYNA/VUL/BUC/FLK/4/MILAN), HS 490 (HS 364/HPW 114//HS 240/HS 346) and parent variety HPW 89 and tested for grain hardness using SKCS 4100 (Method 55-31, AACC 2000) (Gaines *et al.*, 1996), following

the manufacturer's operation procedure (Perten Instruments North America Inc., Springfield, IL, USA). The mean, standard deviation and distribution of SKCS hardness data were used to classify the tested genotypes into very soft (<10), soft (10–30), semi-hard (30–70), hard (70–90) and very hard (>90).

Statistical analysis

To compare the mutagenic treatments, analysis of variance as per Panse and Sukhatme (1961) was performed to test the hypothesis that mutagens had an impact on the observations recorded. Duncan multiple range test (DMRT) (Duncan, 1955) was used to examine trait differences between the means of the mutagenic treatment and the control. For estimating the extent of variations generated, genotypic coefficient of variation (GCV), heritability (h_{bs}^2) in a broad sense, and expected genetic advance (GA) expressed as a percentage of mean were used as per Burton and De Vane (1953) and Johnson *et al.* (1955). The magnitude of association among traits and their direction were computed using Pearson's correlation coefficients using 'corrplot' (https://cran.r-project.org/package=corrplot), 'ggcorrplot' (https://cran.r-project.org/package=ggcorrplot) and 'PerformanceAnalytics' (https://cran.r-project.org/package=PerformanceAnalytics) packages using RStudio v4.2.2 (PBC, Boston, MA, USA). Multivariate statistical techniques such as principal component analysis (PCA) (Pearson, 1901) and cluster analysis were executed using the International Business Machines Corporation Statistical Package for the Social Sciences (IBM SPSS 20.0) and XLSTAT software (Lumivero, Denver, USA).

Results

Induced variation in quantitative traits

To study the extent of variation generated utilizing induced mutagenesis, a total of 2219 M_1 plant progenies were evaluated in M_2

generation for various parameters altered by induced mutagenesis. Desirable agro-morphological mutants with higher yield statistics from each mutant population were screened, separately harvested and threshed. About 1862 M_2 progenies were further selected for generation advancement in the M_3 generation. Hence, a broad spectrum of variation was observed for most of the traits among mutant lines in the M_2 and M_3 generations of wheat variety HPW 89 at different doses of gamma irradiation (Fig. 2) and the effect of induced physical mutagenesis on these parameters is presented in online Supplementary Tables S1 and S2. Results from the irradiation experiments revealed a significant shift in the magnitude of the 12 parameters studied in both M_2 and M_3 generations from the parental control as per the DMRT test. The mean performance and magnitude of variation determined for all the 12 quantitative traits in the soft-textured HPW 89 wheat variety are described below.

Phenological traits

Three gamma-irradiated populations of soft-textured HPW 89 were evaluated to screen mutants with early flowering, maturity and shorter GFP across M_2–M_3 generation. Early flowering and maturing mutants were isolated from all three mutagenic treatments in both M_2 and M_3 generations. However, mutagenic treatment with the earliest flowering period observed was 121.0 d (300 Gy) versus 130.40 d (control) in M_2 while 92.0 d (350 Gy) versus 101.20 d (control) in M_3 generation. The shortest period for grain filling taken was observed in the mutant population of 300 and 350 Gy in M_2 while 250 and 350 Gy in the M_3 generation. The shortest grain filling period noted was 35.0 d (300 Gy) versus 44.4 d (control) in M_2 and 32.0 d (250 Gy) versus 40.0 d (control) in M_3 generation. Also, mutants in mutagenic treatment i.e. 300 Gy had the earliest maturity period of 161.0 and 131.0 d as compared to 172.8 d in M_2 and 141.2 d (control) in M_3 generation, respectively.

Plant height, tillers per plant and peduncle length

Among the mutagenic treatments, PH showed greater heterogeneity in both mutant generations. The shortest PH was observed in 350 Gy treatment with 78.3 cm plant length versus 98.86 cm (control) in the M_2 generation, whereas 75.7 cm in 250 Gy treatment versus 90.58 cm (control) in the M_3 generation. Also, mutants with higher NOT were recorded in 250 and 300 Gy treatment populations in the M_2 generation while none of the treatments showed higher NOT in the M_3 generation. The highest number of tillers up to 6.00 versus 4.80 (control) was observed in the M_2 generation while 5.00 versus 4.20 in the M_3 generation. Moreover, the plant with the shortest PL was recorded in the 350 Gy treated population of both M_2 generation (6.2 versus 10.60 cm) and M_3 generation (5.1 versus 8.58 cm).
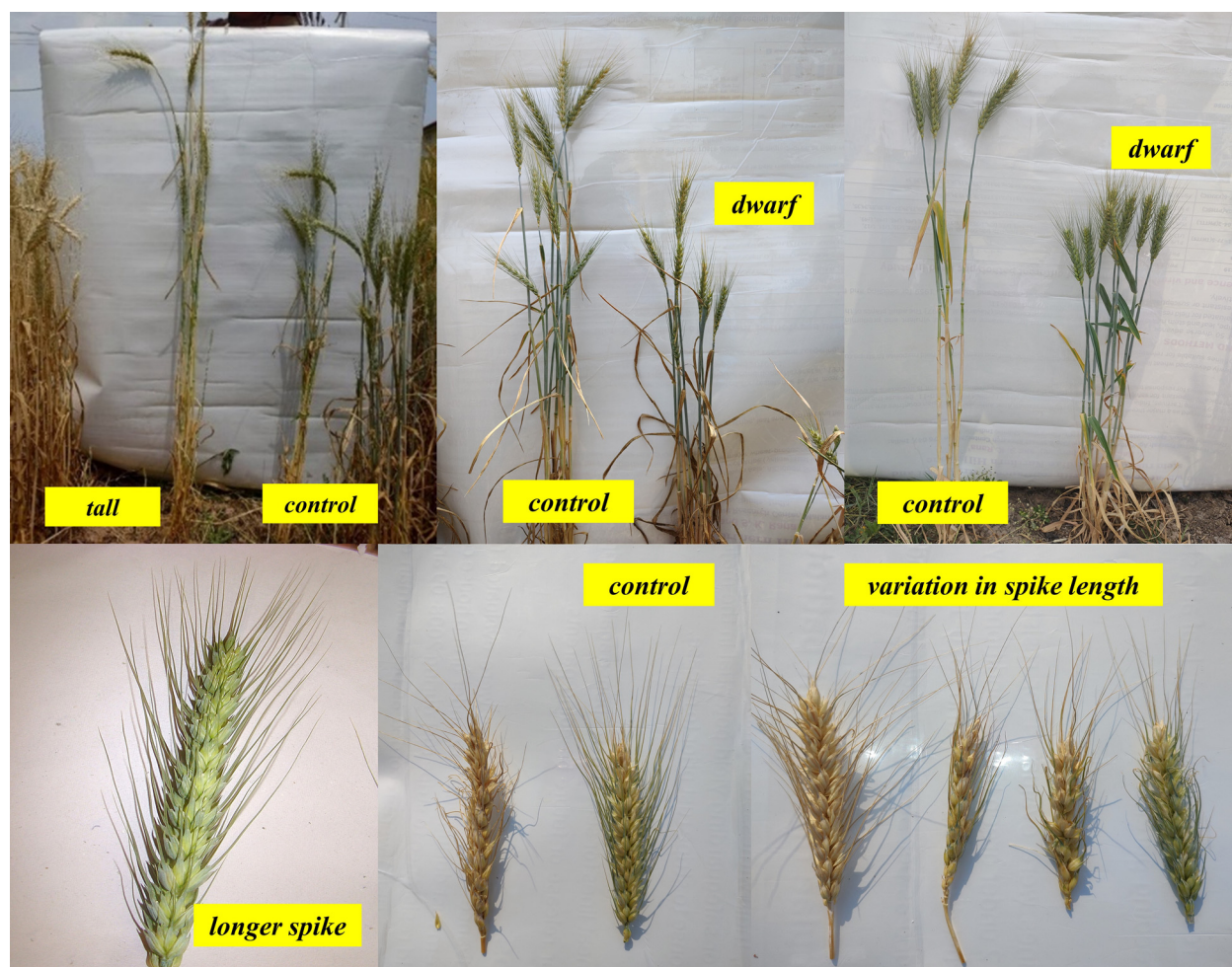
Figure 2. Variation induced in the quantitative traits after gamma irradiation of soft-textured HPW 89 wheat variety in the M_2 generation.

Spike length and grains per spike

Wide variation for SL in both 250 to 350 Gy mutagenic treatments was observed. A significant increase in spike length in the M$_2$ generation was recorded at 250 and 300 Gy treatments compared to the control with the longest spike of 14.8 cm (250 Gy) versus 9.30 cm (HPW 89). In the M$_3$ generation, significantly longer spikes were found at 250 Gy only with the longest SL of 11.5 versus 8.56 cm (control). Also, GPS observed in the M$_2$ generation showed mutants with a significantly higher number of GPS in all three mutagenic treatments as per the DMRT test and the plant with the highest number of GPS observed was 56.0 as compared to the control (46.2). In the M$_3$ generation, mutants with a higher number of GPS were observed in 300 Gy mutagenic treatment only. Mutants with a higher number of GPS for up to 45.0 were recorded as compared to the control (34.6).

Grain yield and other contributing traits

Seed yield is the most prominent economic trait in all crop improvement and breeding programmes. In this study, the mutant population of 250 Gy had mutants with significantly higher BY (22.5 versus 18.04 g) and GY (11.8 versus 8.72 g), while higher HI was observed at 350 Gy (52.8 versus 48.91%) in mutants observed in the M$_2$ generation. In the M$_3$ generation, mutants with significantly higher BY (20.8 versus 16.42 g), HI (53.7 versus 48.44%) and GY (11.5 versus 8.86 g) were identified in the 250 Gy treated population. For TGW, none of the mutagenic treatments had a significant increase among all the mutants screened. However, mutants with the highest TGW observed were up to 52.7 g versus 48.57 (control) in the M$_2$ generation whereas 49.50 versus 40.80 g in the M$_3$ generation.

Induced agro-morphological variability

Evaluation of variability parameters for these quantitative traits showed induction of significant genetic variations in both M$_2$ and M$_3$ generations (Table 1). In M$_2$ generation, GCV estimates ranged from 0.67 to 8.45% while broad sense heritability (h_{bs}^2) estimates varied from 46.81 to 68.77%. High h_{bs}^2 (>60%) were observed for DTF, GY, DTM and GPS whereas other traits

Table 1. Estimates of variability parameters for 12 quantitative traits in the M$_2$ and M$_3$ generations of soft wheat variety HPW 89

M$_2$ generation						
	DTF	PH	NOT	PL	SL	GPS
Range	121.0–134.0	78.3–108.5	3.0–6.0	6.2–14.1	6.3–14.8	22.0–56.0
Mean ± SE	129.00 ± 0.46	90.99 ± 1.05	4.9 ± 0.22	9.44 ± 0.23	9.74 ± 0.08	48.35 ± 0.72
GCV (%)	0.67	4.26	8.45	7.05	8.43	6.11
h_{bs}^2 (%)	68.77	63.23	59.20	57.90	46.81	59.36
GA (%)	4.11	10.83	7.28	13.67	10.19	8.55
	GFP	DTM	BY	HI	TGW	GY
Range	35.0–50.0	161.0–176.0	10.8–22.5	40.7–52.8	30.2–52.7	4.5–11.8
Mean ± SE	42.15 ± 0.56	171.15 ± 0.61	17.46 ± 0.44	49.84 ± 0.38	48.53 ± 0.64	8.71 ± 0.19
GCV (%)	3.06	0.97	5.66	4.34	2.09	5.01
h_{bs}^2 (%)	65.24	55.24	61.95	58.48	47.82	54.26
GA (%)	6.75	5.65	9.32	7.71	6.89	8.43
M$_3$ generation						
	DTF	PH	NOT	PL	SL	GPS
Range	92.0–105.0	75.7–103.2	3.0–5.0	5.1–10.5	7.1–11.5	29.0–45.0
Mean ± SE	100.35 ± 0.87	88.28 ± 1.67	4.3 ± 0.35	7.93 ± 0.34	10.63 ± 0.24	36.25 ± 1.78
GCV (%)	0.83	5.19	8.94	8.58	9.12	6.38
h_{bs}^2 (%)	62.25	69.52	63.89	71.21	59.52	58.67
GA (%)	4.02	11.55	8.92	11.28	12.93	7.62
	GFP	DTM	BY	HI	TGW	GY
Range	32.0–45.0	131.0–145.0	12.4–20.8	41.7–53.7	32.1–49.5	7.1–11.5
Mean ± SE	38.7 ± 1.07	139.05 ± 0.70	16.88 ± 0.44	48.24 ± 1.16	40.73 ± 0.70	8.17 ± 0.21
GCV (%)	3.92	0.94	6.28	5.96	3.48	6.88
h_{bs}^2 (%)	52.26	61.24	67.85	52.93	56.81	55.19
GA (%)	8.58	13.65	11.27	9.81	7.94	9.57

DTF, days to 50% flowering; PH, plant height; NOT, tillers per plant; PL, peduncle length; SL, spike length; GPS, grains per spike; GFP, grain filling period; DTM, days to 75% maturity; BY, biological yield per plant; HI, harvest index; TGW, 1000-grain weight; GY, grain yield per plant; S.E., standard error; PCV, phenotypic coefficient of variation; GCV, genotypic coefficient of variation; h_{bs}^2, heritability (broad sense); GA, genetic advance as per cent mean.

evaluated showed moderate heritability estimates (30–60%). GA as per cent of mean varied from 4.11 to 13.67%. Among all the traits studied, none of the traits showed high estimates for GA (>20%) while moderate GA estimates (10–20%) were observed for BY, SL and GFP. Besides, moderate h^2_{bs} coupled with moderate GA was noted for PH, NOT, PL, HI and TGW. Variability estimates evaluated in the M₃ generation revealed an increase in GCV, h^2_{bs} and GA for most of the biometrical traits. GCV values for all the quantitative traits ranged between 0.83 and 9.12% whereas h^2_{bs} estimates ranged from 52.26 to 71.21%. High h^2_{bs} values (>60%) were noted for HI, DTF, SL, NOT, BY and GY while the rest of the traits had moderate h^2_{bs} estimates (30–60%). Also, GA varied from 4.02 to 13.65% among all the traits. SL, TGW, GFP, PL and DTF showed moderate levels of GA estimates (10–20%), while the rest of the traits had low estimates of GA. Moreover, high h^2_{bs} coupled with moderate GA was observed for DTF and SL. Besides, moderate h^2_{bs} coupled with moderate GA was noted for PL, GFP and TGW.

Association among quantitative traits

The degree of associations among 12 agro-morphological traits as per Pearson correlation coefficients in the M₂ and M₃ generations of HPW 89 are presented in Fig. 3. Results showed a significantly high positive correlation coefficient ($P \leq 0.05$) of GY with BY (0.94) followed by TGW (0.73) and PH (0.50) in the M₂ generation whereas significant positive associations were found of GY with SL (0.69), GPS (0.66), BY (0.59) and NOT (0.52) in the M₃ generation. Significant associations among other quantitative traits were observed such as DTF showed a positive correlation with DTM and PH in the M₂ generation. PH observed in both generations had a positive association with PL, DTM and BY except for DTF, GFP and GY in the M₃ generation. NOT observed in these populations showed significant positive associations with SL, GPS, BY and GY in the M₃ generation. Significant correlations of PL were observed with other traits such as PH, GFP, DTM, BY and HI in the M₂ generation and SL, GPS, BY and GY in the M₃

generation. SL observed in both mutant populations showed a positive association with GPS and TGW in the M₂ generation whereas NOT, PL, GPS, BY and GY in the M₃ generation. Also, negative associations were observed among traits such as DTF with NOT and SL, PH with HI, DTM and BY with HI in the M₂ generation, and GFP with DTF in the M₃ generation. A significant positive correlation of GPS was also found with SL (M₂ generation) and NOT, SL, BY and GY (M₃ generation) in the population of both generations. For the phenological trait i.e. GFP, significant positive associations were found with PH and SL in the M₂ generation and DTM in the M₃ generation. Also, DTM showed similar significant positive associations in the M₂ generation with DTF, PH, PL, GFP, BY and GY whereas PH, PL and GFP in the M₃ generation. BY recorded in both populations had significant positive correlations with PH, PL, GFP, DTM, TGW and GY in M₂ and PH, NOT, SL, GPS and GY in M₃ generation. Significant positive association of TGW was also observed with SL, BY and GY in the M₂ generation.

Multivariate analysis

In order to reduce the dimensionality of the large data obtained in the gamma ray-induced mutant population, multivariate techniques i.e. PCA and cluster analysis were used. PCA results revealed that overall three major principal components (PCs) in the M₂ generation and four PCs in the M₃ generation were extracted which explained >80% of the total variation from the original data having latent roots greater than 1 (online Supplementary Fig. S1 and Table 2). In M₂ generation, PC1 explained 41.6% of the total variation and is significantly contributed by PH, DTM, BY, PL, GFP and HI. The second PC (PC2) is mainly contributed through SL, GPS and TGW, thus explaining 24.5% of the total variation, while the third PC (PC3) explained 14.2% of the total variation through NOT, GY, DTF, GFP and HI. Among the four PCs in M₃ generation, PC1, PC2, PC3 and PC4 explained 37.0, 19.8, 13.2 and 10.2% of the total variability, respectively. The traits that contributed most to each PC included SL, GPS,
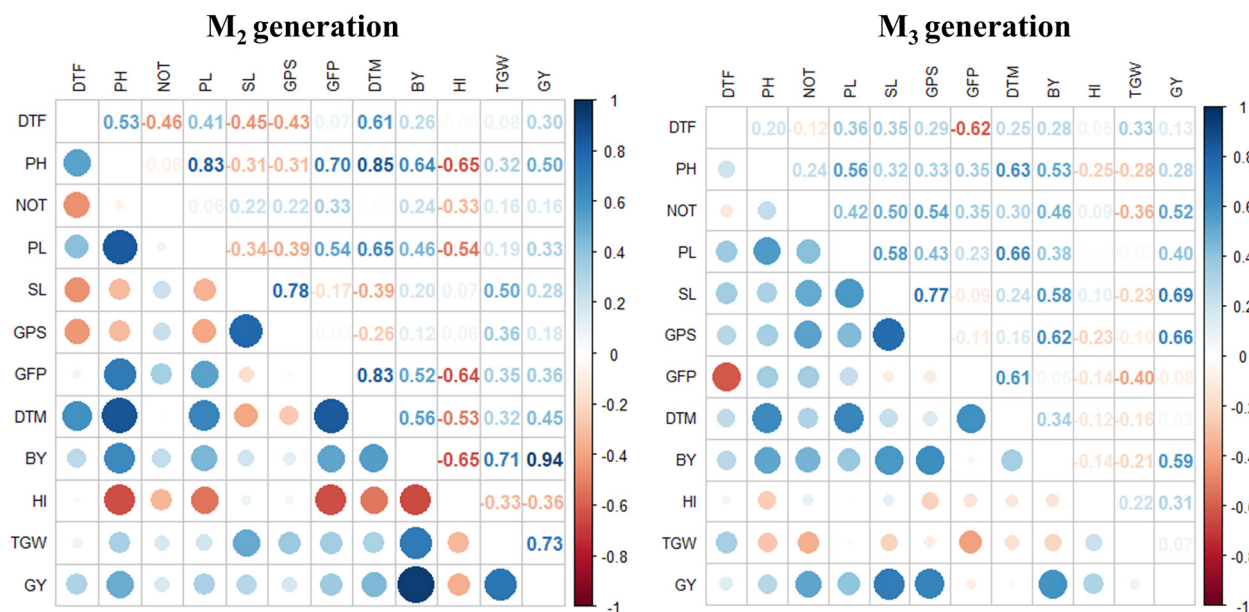

Figure 3. Correlation coefficients between different agro-morphological and yield-attributing traits in M₂ and M₃ generations of HPW 89.

Table 2. Eigen vector values of 12 quantitative traits for first three principal components in the M_2 generation and first four principal components in the M_3 generation of HPW 89

	Eigenvectors						
	M_2 generation			M_3 generation			
Variable	PC1	PC2	PC3	PC1	PC2	PC3	PC4
DTF	0.23	−0.28	−0.45	0.15	0.40	−0.50	−0.03
PH	0.42	−0.11	0.002	0.32	−0.21	−0.31	−0.06
NOT	0.05	0.26	0.53	0.33	−0.11	0.38	0.13
PL	0.35	−0.15	0.11	0.36	−0.03	−0.27	0.28
SL	−0.11	0.51	−0.10	0.39	0.20	0.15	−0.04
GPS	−0.10	0.48	−0.03	0.37	0.19	0.14	−0.31
GFP	0.35	0.05	0.33	0.10	−0.58	0.08	0.24
DTM	0.40	−0.12	0.02	0.28	−0.31	−0.41	0.27
BY	0.36	0.27	−0.12	0.37	0.06	0.03	−0.22
HI	−0.32	−0.10	−0.33	−0.03	0.23	0.24	0.72
TGW	0.23	0.40	−0.21	−0.13	0.37	−0.27	0.30
GY	0.24	0.26	−0.46	0.33	0.26	0.31	0.16
Eigenvalue	4.99	2.93	1.70	4.44	2.37	1.59	1.23
Variation (%)	41.6	24.5	14.2	37.0	19.8	13.2	10.2
Cumulative (%)	41.6	66.0	80.2	37.0	56.7	70.0	80.2

DTF, days to 50% flowering; PH, plant height; NOT, tillers per plant; PL, peduncle length; SL, spike length; GPS, grains per spike; GFP, grain filling period; DTM, days to 75% maturity; BY, biological yield per plant; HI, harvest index; TGW, 1000-grain weight; GY, grain yield per plant.

BY, PL, GY, NOT and PH to PC1, GFP, DTF and TGW to PC2, DTF, DTM and NOT to PC3 and HI to PC4. The magnitude of 12 agro-morphological traits in different mutagenic treatments is represented in Fig. 4. Cos2 value shows the quality and magnitude of the representation of the traits on the PCs. In the M_2 generation, eight traits viz., PH, SL, DTM, BY, GPS, TGW, GFP and PL showed the highest contribution and higher quality representation to the PCs while GY, DTF and HI exhibited moderate contribution and lower representation to the PCs. Also, NOT exhibit the lowest contribution and representation among all the quantitative traits, whereas in the M_3 generation, almost all the traits exhibited the highest contribution to the PCs except TGW (moderate) and HI (low).

To further investigate the structure and overall variability induced in the mutagenized population, cluster analysis is applied to group treatments either in the same or different clusters. A
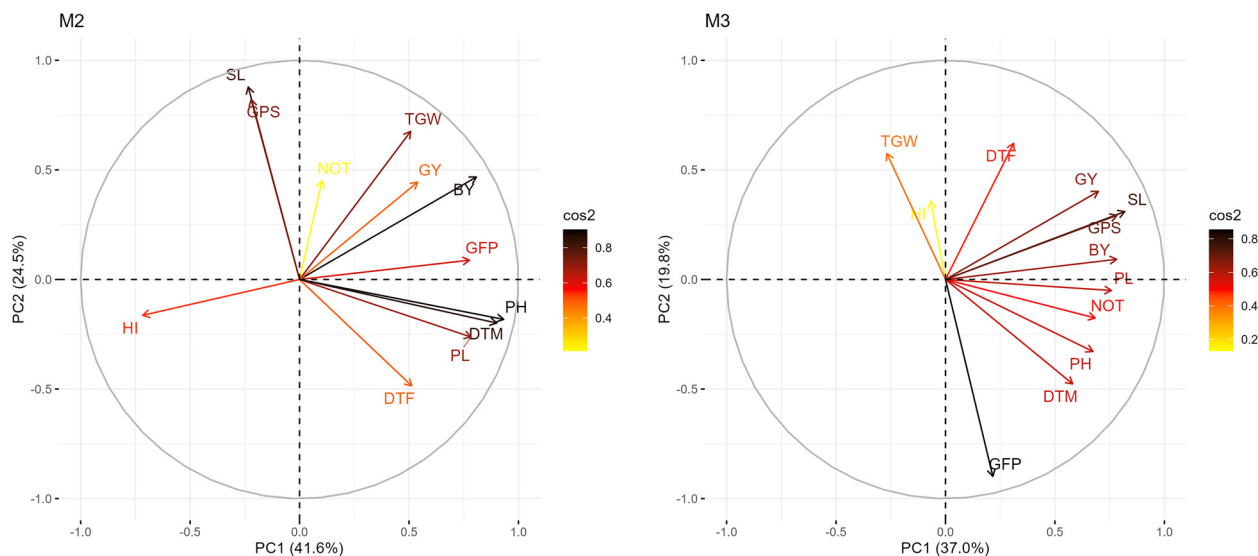
Figure 4. Quality of representation (cos2) of different traits in M_2 and M_3 generations of HPW 89; low cos2 values are coloured in 'yellow', mid cos2 values in 'red' and high cos2 values with 'black' colour.

dendrogram was generated based on squared Euclidean distance as per Ward's method using 12 agro-morphological traits observed in three mutagenic treatments and control in the M_2 and M_3 populations (Fig. 5). Despite the variation among mutagenized treatments along with respective control for the traits evaluated, the hierarchical clustering divided all treatments into two main clusters. Control treatment formed a separate cluster in both generations while similar clustering of the treatments further confirmed the results from PCA. Mutagenic treatments 250 and 300 Gy were in the same sub-cluster while 350 Gy formed another sub-cluster with higher dissimilarity in the M_2 generation whereas 300 and 350 Gy formed separate sub-cluster and 250 Gy made different sub-cluster in the M_3 generation. The formation of these clusters in this study suggested the induction of high variation among mutagenic treatments for all the traits studied in comparison to the parent variety. Besides, the results from the inter-population dissimilarity (Table 3) matrix in M_2 generation revealed that the highest squared Euclidean distance (bold) was between the control (parent) and the mutant population 350 Gy i.e. 57.30 while the lowest distance (italic) was between 250 and 300 Gy treatment (0.06). In the M_3 generation, the highest squared Euclidean distance was found between the control and 250 Gy treatment and the lowest distance between 300 and 350 Gy mutant populations.

Screening for grain hardness

During M_3 generation, a total of 293 agro-morphologically superior M_3 progenies were screened for grain texture. The M_3 progenies comprised of 281 (250 Gy), 8 (300 Gy) and 4 (350 Gy) from the different mutagenic treatments. These mutants along with six checks viz., HS 542, HS 562, HPW 349, HS 507, HS 490 and parent variety HPW 89 were tested for grain hardness using SKCS. Based on the distribution of SKCS hardness data of 293 superior mutants (online Supplementary Table S3), a total of 17 mutants, all derived from 250 Gy (100%) gamma-ray treatment along with parent variety HPW 89 were classified as very soft grain texture (<10). In the soft texture category, 61 mutants had SKCS scores from 10 to 20 and 107 mutants with

Table 3. Interpopulation proximity matrix based on squared Euclidean distance among different mutant populations in M_2 (below diagonal) and M_3 generation (above diagonal) of HPW 89

		Control	250 Gy	300 Gy	350 Gy	
M_2	Control	**0**	**45.50**	31.12	29.77	M_3
	250 Gy	38.17	**0**	29.10	27.39	
	300 Gy	35.93	*0.06*	**0**	*0.03*	
	350 Gy	**57.30**	29.69	27.65	**0**	

20 to 30 along with check i.e. HS 490. Out of these mutants, 162 mutants were derived from the 250 Gy (96.4%) treated population, five mutants from the 300 Gy (2.98%) irradiated population and one mutant from the 350 Gy (0.60%) population. Also, a total of 108 semi-hard mutants were classified in the range from 30 to 40 (99), 40 to 50 (8) and 50 to 60 (1) along with three checks viz., HPW 349, HS 542 and HS 562 and 60 to 70 (one check variety HS 507). Among these 108 mutants, 102 mutants were derived from 250 Gy (94.44%) irradiated population while three mutants each were derived from 300 (2.78%) and 350 Gy (2.78%) treated population. However, hard mutants were classified in the range of 70–80% and 80–90%, but none of the mutants observed were of hard or very hard (>90) grain texture. Among the 108 semi-hard mutants, highest level of grain hardness was observed in Su-M-195 (50.74) followed by Su-M-241 (44.90), Su-M-112 (44.34), Su-M-4 (42.44), Su-M-62 (42.31), Su-M-106 (41.18), Su-M-161 (40.81), Su-M-222 (40.24) and Su-M-2 (40.17) as compared to parent variety HPW 89 (7.39).

Discussion

Genetic advancement in plant breeding is primarily dependent on the extent of variability found in the existing germplasm. Mutation breeding is one such technique, which generates random mutations, resulting in the improvement of one or a few traits. In mutation breeding, the choice and dose of mutagen for inducing mutations is one of the important steps before
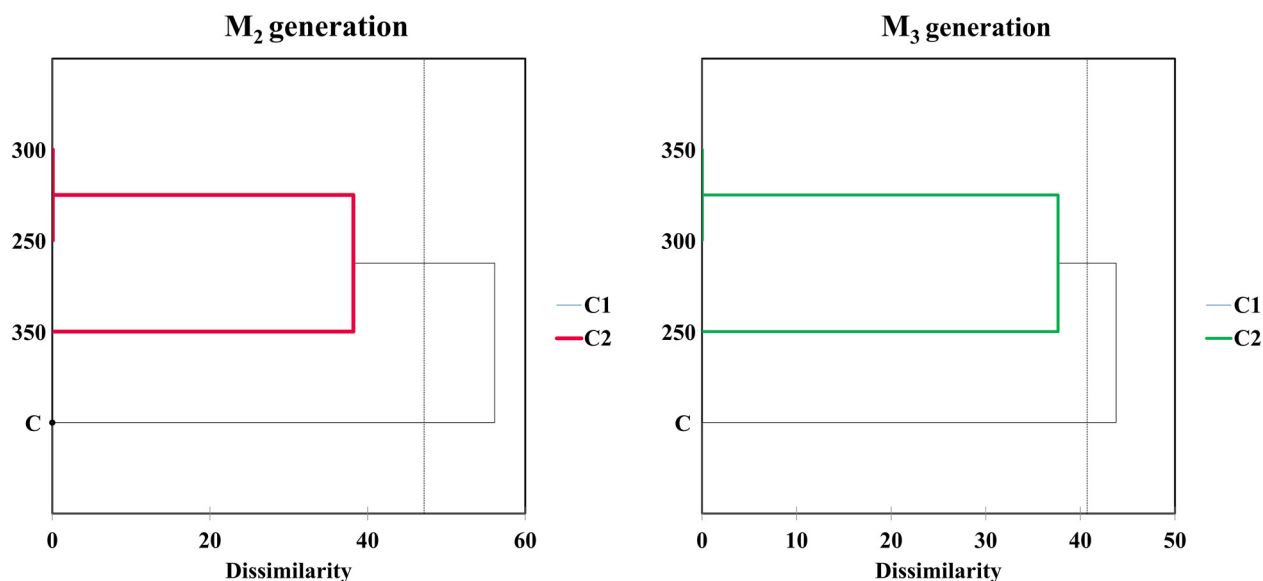
Figure 5. Dendrogram of mutant populations and clustering based on 12 quantitative traits in M_2 and M_3 generations of HPW 89.

initiating large-scale mutagenesis. Hence, the selection of gamma rays and their effective doses were optimized *via* probit analysis using 12 doses (25–300 Gy) based on our earlier study (Rana *et al.*, 2022). During irradiation exposure, gamma rays distort the structure of DNA, causing chemical bonds to dissolve and the production of reactive oxygen species, ionized water (H_2O^+), hydroxyl radicals (OH) and other reactive nitrogen species. These free radicals damage the DNA's building blocks, resulting in changes including substitutions, deletions and chromosomal abnormalities (Reisz *et al.*, 2014). Hence, soft-textured wheat variety HPW 89 was irradiated with three different gamma-ray doses to generate genetic variability and develop mutagenic populations for identification of potential mutants having desirable agro-morphological characteristics along with improved grain hardness. The results from an earlier study by Suliansyah *et al.* (2015) with doses 100–1000 Gy confirmed the effective doses for gamma radiation from 200 to 300 Gy. Radiosensitivity tests of the soft-textured genotype HPW 89 in the M_1 generation showed a higher frequency of biological damage caused to the seedlings as the dose of the gamma irradiation increased. Moreover, some plants even though survived until maturity had sterile spikes with either a few small abnormal seeds or almost no seed formation in higher treatments (350 Gy). Similar reduction/delayed response in germination percentage and plant survival with an increase in the dose of the gamma irradiation was observed in wheat by Irfaq and Nawab (2001) in three cultivars irradiated with 100–400 Gy, Verma *et al.* (2015) with 50–400 Gy, Ahumada-Flores *et al.* (2021) with 100–300 Gy, Chakraborty *et al.* (2023) with 200–450 Gy and Rana *et al.* (2022, 2024*a*) with doses ranging from 25 to 300 Gy.

Inducing micro-mutations for agro-morphologically important traits contributing to yield is the prime objective of any crop improvement mutation programme (Louali *et al.*, 2015; Goyal *et al.*, 2021). However, induction of micro-mutations requires large-scale mutagenesis and thorough screening of large mutagenic populations at the field level to screen mutants with one or few desirable traits. It was observed in the $M_{2–3}$ populations that the magnitude of the quantitative traits was significantly altered by induced mutagenesis. The dose-wise per cent response of different agro-morphological traits among different mutagenic treatments is presented in online Supplementary Fig. S2. This shift was observed in both positive and negative directions, resulting in a wide range of phenotypic variations. The induced mutagenesis altered the expression levels of genes responsible for these traits, leading to notable changes in the overall phenotype. Hence, the effectiveness of mutagenic treatments is not consistent across different traits and it is important to further investigate the factors that influence this variability to optimize the use of few effective mutagenic treatments for desired trait improvements. However, it is also important to consider that different traits may have varying sensitivities to mutagenic treatments, which can explain the observed variations in the performance of the agro-morphological traits. The complex interplay between genes, mutagens and genetic background highlights the intricate nature of mutation induction and its impact on the evolution of the overall species. Similar significant differences for different traits due to induced mutagenesis in wheat genotypes had been reported by Gad *et al.* (2023), Shabani *et al.* (2022) and Rana *et al.* (2024*a*).

Evaluation of variability parameters revealed that the mutagenic treatments induced significant genetic variations in both M_2 and M_3 generations, leading to increased genetic variability for the studied biometrical traits. Traits with higher estimates of heritability and GA are more likely to be passed on to future generations and preferred for the selection of mutants. Moreover, the presence of newly mutated genes may exhibit a pleiotropic effect and may contribute to higher estimates of these parameters. Results from our study showed that high heritability estimates for PH, DTF, SL, GFP, DTM and BY suggested the predominance of additive genes, and selection based on these traits may facilitate maximum genetic gain. However, heritability estimates alone may not provide a complete understanding of the genetic basis of these traits and by considering GA alongside heritability estimates, we can gain a more comprehensive understanding of how much of a trait's variation is due to genetics and the degree of improvement can be possible through breeding efforts. Both heritability and GA exhibited almost independent relationships in the current study, and higher values of both of these parameters could not always be predicted. Hence, traits such as DTF, SL, GFP, BY and GY could be used for selection criteria in wheat-breeding programmes. It may also be suggested that the selection of mutants may be more effective in the M_3 generation for higher genetic gain and increased efficiency in breeding programmes. This is because the M_3 generation is expected to have undergone further genetic recombination and selection, resulting in a greater likelihood of identifying desirable traits. Similar findings were reported by Sharma *et al.* (2015), Awaad *et al.* (2018), Ahmed and Ahmed (2020), Korkut *et al.* (2021), Al-Ebaidy *et al.* (2022) and Kumar *et al.* (2022) in wheat. Furthermore, traits with high/moderate heritability and high/moderate genetic progress could be exploited as prospective traits for selection (Balkan, 2018). In plant breeding, by understanding relationships between traits, breeders can prioritize the selection of traits that have an overall positive influence and cumulative increase in the yield function allowing targeted breeding efforts to maximize the overall productivity of crops. Associations studied among various agro-morphological traits showed that GY is significantly correlated with BY > TGW > SL > GPS > PH > NOT in HPW 89. A high correlation of GY with BY and TGW was also reported in earlier studies in wheat by Rana and Sethi (1998), GY with TGW and PH by Zulfiqar *et al.* (2021), GY with TGW by Fadli *et al.* (2022) and GY with BY and SL by Rana *et al.* (2024*a*). Therefore, selecting traits with stronger associations can aid in the identification of high-yielding mutants with desirable traits.

Complex big-data analysis involving several agro-morphological traits and treatments becomes a time-consuming and error-prone process. Multivariate analysis assists breeders in reducing the dimensions of the enormous data sets into useful measures for effective selection with higher precision. It allows a comprehensive assessment of the gamma ray-induced genetic changes in the mutant population by considering multiple traits simultaneously. Multivariate techniques, such as PCA and cluster analysis, aid breeders in identifying patterns and relationships within the dataset that may not be apparent through simple univariate analysis (Afuape *et al.*, 2011). In the present study, the PCA enabled to define PCs, which accounted for about 80% of the variability induced in both mutant populations. Likewise, three foremost PCs were reported by Ndou *et al.* (2015) and Rahim *et al.* (2020) in mutagenized wheat populations accounting for 57 and 68%, respectively, of the total variations among the various agro-morphological traits. Besides, genetic divergence and overall induced variability were most influenced by biometrical traits such as PH, GPS, BY, PL, GFP and TGW. Additionally, all mutagenic treatments were widely placed away from the

control representing the divergence of the induced mutants. The mutagenic treatments of 250 and 300 Gy were in close vicinity to each other indicating their close effect *via* induced mutagenesis. Moreover, higher effectiveness and efficiency of 250–300 Gy doses of gamma irradiation in inducing mutations is evident in an earlier study by Rana *et al.* (2024*b*). Further demonstrating the heterogeneity brought about by these treatments, hierarchical clustering based on squared Euclidean distance separated the treatments into two major clusters where control formed separate cluster while mutagenic treatments in other cluster indicating the creation of a significant amount of genetic variability by the mutagenic treatments. It has been revealed in several reports that agro-morphological trait-based clustering provides a good measure of genetic divergence and is used to examine genetic linkages among mutant populations (Awaad *et al.*, 2018; Zulfiqar *et al.*, 2021). Hence, gamma irradiation doses ranging from 250 to 350 Gy were however found effective for screening effective mutagenic treatments with higher inter-population distance among clusters along with high mean value of the agro-morphological traits.

Improvement of grain hardness over parent variety (HPW 89) on either side of the index had an impact on biscuits, *chapatti* and bread-making quality. Grain hardness measurement based on the SKCS in our study revealed efficient improvement in the grain hardness of the mutant isolated of soft-textured variety HPW 89. SKCS-based grouping of 293 agro-morphological superior mutants into different grain hardness categories showed that six mutants had very soft grain texture (GH score lower than HPW 89) and a total of 287 mutants with higher grain hardness than the parent variety HPW 89. Also, a higher frequency of harder grain texture mutants was obtained from the gamma rays doses in the order i.e. 250 Gy > 300 Gy ≥ 350 Gy. Grain hardness-based grouping of wheat varieties and landraces of India by Sharma *et al.* (2012) provided information about the end-use quality of these groups. Also, it was found that 108 semi-hard (30–60 GHI) mutants had higher grain hardness which suggested that either the *pina* and/or *pinb* genes controlling grain hardness were completely/partially knocked out in these gamma-irradiated mutants, allelic differences for either one or both genes or other marker trait associations. Among these semi-hard mutants, nine mutants were found to have significantly higher grain hardness index ranging from 40 to 51 as compared to their parent variety HPW 89 (7–8 GHI). The details of the mutagenic treatment and quantitative characteristics observed in these mutants are mentioned in online Supplementary Tables S4.1 and S4.2.

Therefore, to improve one or few traits of an otherwise well-adapted variety and create genotypes with high-yielding potential, especially under changing climate conditions, mutation breeding is an essential tool for producing variability for quantitative traits of economic value. This study demonstrated the role of induced mutagenesis in improving the texture of wheat grain along with agro-morphological traits and has the potential to enhance diversity in mutant populations. Hence, mutagenic treatments with higher inter-cluster distance and higher trait values can be used for the selection of potential mutants whereas 250–300 Gy doses were found ideal for a successful wheat mutation programme. A total of 293 mutants were identified as having superior agro-morphological performance and out of which, 108 mutants had semi-hard grain texture. Therefore, these mutants will serve as important genetic resource and viable substitute for outdated cultivars and pique consumer interest, particularly in the Northwest Himalayan area. The summarized overview of the present study is presented in online Supplementary Fig. S3.

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