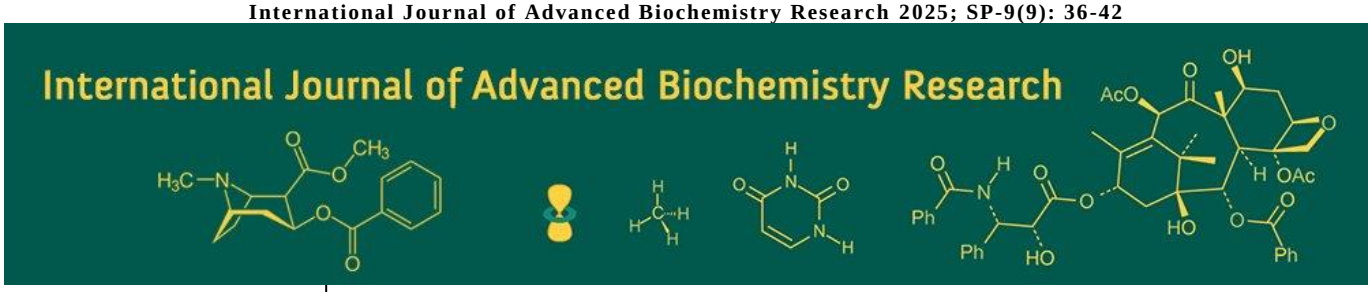

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Multivariate approaches for evaluating phenotypic variability and trait associations in M_4 generation of kodo millet (*Paspalum scrobiculatum* L.) mutants obtained by gamma radiation

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Abstract
The present study assessed phenotypic variability among 250 M_4 generation kodo millet (*Paspalum scrobiculatum* L.) mutants developed through gamma irradiation, along with three checks, using multivariate approaches. The experiment was undertaken at Shaheed Gundadhoor College of Agriculture and Research Station Jagdalpur during *Rabi*-2024 utilizing augmented block design as layout. Principal Component Analysis (PCA) revealed four principal components explaining 71.5% of total variability, with grain yield per plant, number of grains per raceme, biological yield, and flowering traits emerging as major contributors. The first principal component (PC1) explained the largest share of variation (26.9%), mainly contributed by grain yield per plant (0.54), number of grains per raceme (0.492), and biological yield (0.46). Genetic divergence analysis grouped the mutants into ten clusters, with the largest being Cluster VI including 42 lines. The highest intra-cluster distance was observed in Cluster III (2.886), followed by Cluster I (2.671), reflecting the existence of considerable genetic divergence within these groups, while maximum inter-cluster distance was observed between Cluster X and Cluster I, indicating the presence of highly divergent genotypes suitable for future hybridization. Regression analysis highlighted grains per raceme, biological yield, and harvest index as key predictors of grain yield per raceme. The model showed a high coefficient of determination, with grains per raceme (75.47%) and biological yield (71.09%) explaining maximum variability. These findings demonstrate the utility of multivariate approaches in identifying superior mutant lines for breeding programs.

Keywords: Multivariate analysis, principal components, cluster analysis, regression analysis, genetic divergence

Introduction
Millets, often referred to as "minor cereals," rank sixth among the world's most important cereal crops (Das *et al*., 2019; Sarita & Singh, 2016) [3, 18]. They are recognized as "crops of the future" because of their ability to tolerate drought, withstand extreme climatic conditions, and resist many pests and diseases. These small-seeded annual grasses belonging to family Poaceae are cultivated globally for food, fodder, feed and oil, with more than 20 known species. (Das *et al*. 2019) [3]. They are adaptable to both kharif and rabi seasons, have long shelf life, and are nutritionally superior to most staple cereals, thus being promoted as "Nutri-Cereals". Kodo millet (*Paspalum scrobiculatum* L.) an indigenous, drought-tolerant, small-grain cereal, is one of the oldest domesticated crops of India, with evidence of its cultivation dating back nearly 3000 years (Arendt & Dal, 2011) [1]. It is a self-pollinated, tetraploid species (2n = 4x = 40) belonging to the subfamily Panicoideae. Locally, it is known by names such as varagu, kodon, haraka, and arakalu. Besides India, its cultivation extends to countries like China, Russia, Africa, and Japan. In India, it is predominantly grown in Madhya Pradesh, Chhattisgarh, Tamil Nadu, Karnataka, and Gujarat.
India is the largest producer of millets, contributing 38.4% of global production. Among minor millets, Madhya Pradesh and Uttarakhand together account for nearly half the national output. In Chhattisgarh, during 2023-24, small millets covered 48,000 hectares with a production of 19,000 tonnes and productivity of 390 kg/ha (Directorate of Economics & Statistics, 2022-23).

Kodo millet has a relatively long growth duration, requiring 4-6 months to mature. Nutritionally, kodo millet is gaining popularity as a health-promoting cereal and a potential substitute for rice and wheat. Being highly self-pollinated due to cleistogamous flowers (Hariprasanna, 2017) [6], hybridization in kodo millet is challenging. Mutation breeding, therefore, offers an effective alternative to expand genetic variability and improve specific traits. Both physical mutagens (gamma irradiation) and chemical mutagens (e.g., EMS) are employed to induce variability. While M_1 helps evaluate mutagenic effectiveness, subsequent generations (M_2, M_3, and M_4) are crucial for identifying stable, heritable mutations. By the M_4 generation, true-breeding mutant lines are generally fixed, making them valuable for crop improvement programs.

Materials and Methods

The present study was carried out during the *Rabi* season 2024 at Shaheed Gundadhoor College of Agriculture and Research Station, Jagdalpur. The experimental material comprised 250 mutant lines of kodo mill*et al*.ong with 3 check varieties. The experiment followed an Augmented Block Design as layout, and the crop was sown on 15th of October 2024, all recommended agronomic practices were employed to ensure uniform establishment and healthy growth. In the experiment, data were collected by randomly selecting plants from the field at the optimum stage of growth and development. A total of 12 observations were recorded, focusing on quantitative traits such as days to 50% flowering (DAS), days to maturity (DAS), plant height (cm), flag leaf length (cm), flag leaf width (cm), raceme length (cm), test weight (g), number of grains per raceme, grain weight per raceme (g), number of productive tillers, biological yield, and harvest index (%). Principal Component Analysis (PCA) is a widely used multivariate statistical technique that helps in simplifying complex datasets. It helps in identifying a smaller number of components that can explain most of the total variation present in a dataset. Components with eigenvalues greater than one are usually considered significant. The interpretation of PCA is based on eigenvalues, proportion of variance explained, factor loadings (eigenvectors), and their graphical display through biplots. The formation of clusters and the estimation of inter-and intra-cluster divergence form the basis for designing hybridization programs. Mahalanobis D^2 statistics, introduced by Mahalanobis in 1936, is a powerful multivariate method that evaluates multiple traits and their interrelationships at the same time to calculate genetic distances among genotypes. Grouping of genotypes into different clusters was done by using Tocher's method as described by Rao (1952) [11].

Regression analysis is a key statistical method used to examine and predict the relationship between a dependent variable and one or more independent (predictor) variables. The effectiveness and significance of the model are assessed using statistical measures such as the coefficient of determination (R^2), F-statistic, and p-values. A higher R^2 suggests that the model more effectively explains yield variability. The p value ($p<0.05$) indicates statistically significant relation with dependent variable.

Results and Discussion

Principal Component Analysis (PCA) is a powerful statistical tool in modern data analysis because this is a well-known multivariate statistical technique. It reduces the dimensionality of large data set by transforming the original correlated variables, known as principal components while retaining most of the variation present in original data. The observations for twelve quantitative characters were recorded and the multivariate technique, principal component analysis was estimated. The current study identified 4 Principal Components (PCs) with Eigen value greater than 1.00 which accounted for 71.5% of the total variation. The Eigen values, percent variance, percent cumulative variance and factor loading of different characters studied are presented in Table 1 and Table 2. PC1 has the highest eigen value (3.233) followed by PC2 (2.639) PC3 and PC4 had the eigen values of 1.519 and 1.193 respectively.

From the Principal Component Analysis, PC1 accounted for the highest proportion of variance (26.9%), mainly contributed by traits such as grain yield per plant (0.54), number of grains per raceme (0.492), and biological yield (0.46). PC2 explained 22% of the variance, with a cumulative variance of 48.9%, primarily influenced by days to 50% flowering (0.557) and days to maturity (0.551). The third principal component, PC3 contributed 12.7% of the variance, raising the cumulative variance to 61.6%, and was largely associated with harvest index (0.496) and test weight (0.276). PC4 explained 9.9% of the variance, with a cumulative variance of 71.5%, and was mainly related to flag leaf width (0.590) and test weight (0.471).

In the previous findings of Patil *et al*. (2017) [10] genetic diversity in finger millet was examined using principal component analysis and found three principal components showing 98.31 percent of total variation. Suman *et al*. (2019) [15] studied 55 finger millet genotypes using multivariate analysis and revealed that the first four principal components with eigen value of greater than 1.33 contributed about 66.54% of total variability. Shashibhushan *et al*. (2022) [17] studied 40 pearl millets genotypes for eight quantitative characters, found that the first three principal components with eigen value greater than 1 contributed 82.3% percent of total variability.

Factor loadings for twelve quantitative characters

In PC1, all traits showed positive loadings, with the highest contributions from grain yield per raceme (0.541), number of grains per raceme (0.492), biological yield (0.467), productive tillers (0.341), harvest index (0.246), and flag leaf length (0.108). This indicates PC1 mainly captures yield-related variation. Similar trends were reported by Gupta (2022) [4] in pearl millet.

PC2 was dominated by positive loadings for days to 50% flowering (0.557), days to maturity (0.551), and plant height (0.468), while negative loadings were recorded for biological yield (-0.118), number of grains per raceme (-0.065), and grain yield per raceme (-0.034). In the previous findings of Ladumor *et al*. (2021) [7] similar results were noticed where positive loadings for days to 50% flowering, days to maturity, and plant height have shown positive loadings. Thus, PC2 primarily represents growth duration and plant stature, with limited contribution from yield traits.

The third principal component, PC3 showed positive associations with traits such as days to flowering (0.178), days to maturity (0.112), flag leaf width (0.014), harvest index (0.496), test weight (0.276), and grain yield per raceme (0.097). In contrast, negative associations were

observed for plant height (-0.012), flag leaf length (-0.485), raceme length (-0.586), productive tillers (-0.098), number of grains per raceme (-0.021), and biological yield (-0.188). For the fourth principal component (PC4), positive loadings were noted for flag leaf length (0.303), flag leaf width (0.590), raceme length (0.207), harvest index (0.265), test weight (0.471), and grain yield per raceme (0.025), while negative loadings were recorded for days to 50% flowering (-0.194), days to maturity (-0.212), plant height (-0.140), productive tillers (-0.260), number of grains per raceme (-0.202), and biological yield (-0.121). Overall, the results indicate that traits with higher loadings in PC3 and PC4 particularly harvest index, test weight, and flag leaf attributes play a major role in genetic variability and can be prioritized in breeding programs for trait enhancement.

Table 1: Eigen values, variance proportion and cumulative proportion for PC's

Particulars	PC1	PC2	PC3	PC4	PC5
Eigenvalues	3.233	2.639	1.519	1.193	0.928
Proportion	0.269	0.220	0.127	0.099	0.077
Cumulative Proportion	0.269	0.489	0.616	0.715	0.793

Table 2: Factor loadings for 4 principal components

Particulars	PC1	PC2	PC3	PC4
Days to 50% flowering (DAS)	0.034	0.557	0.178	-0.194
Days to maturity (DAS)	0.023	0.551	0.112	-0.212
Plant height (cm)	0.002	0.468	-0.012	-0.140
Flag leaf length (cm)	0.108	0.273	-0.485	0.303
Flag leaf width (cm)	0.039	0.106	0.014	0.590
Length of raceme (cm)	0.101	0.207	-0.586	0.207
Productive tillers	0.341	-0.063	-0.098	-0.260
Number of grains per raceme	0.492	-0.065	-0.021	-0.202
Biological yield per plant(g)	0.467	-0.118	-0.188	-0.121
Harvest index (%)	0.246	0.104	0.496	0.265
Test weight (g)	0.214	0.067	0.276	0.471
Grain yield per raceme (g)	0.541	-0.034	0.097	0.025

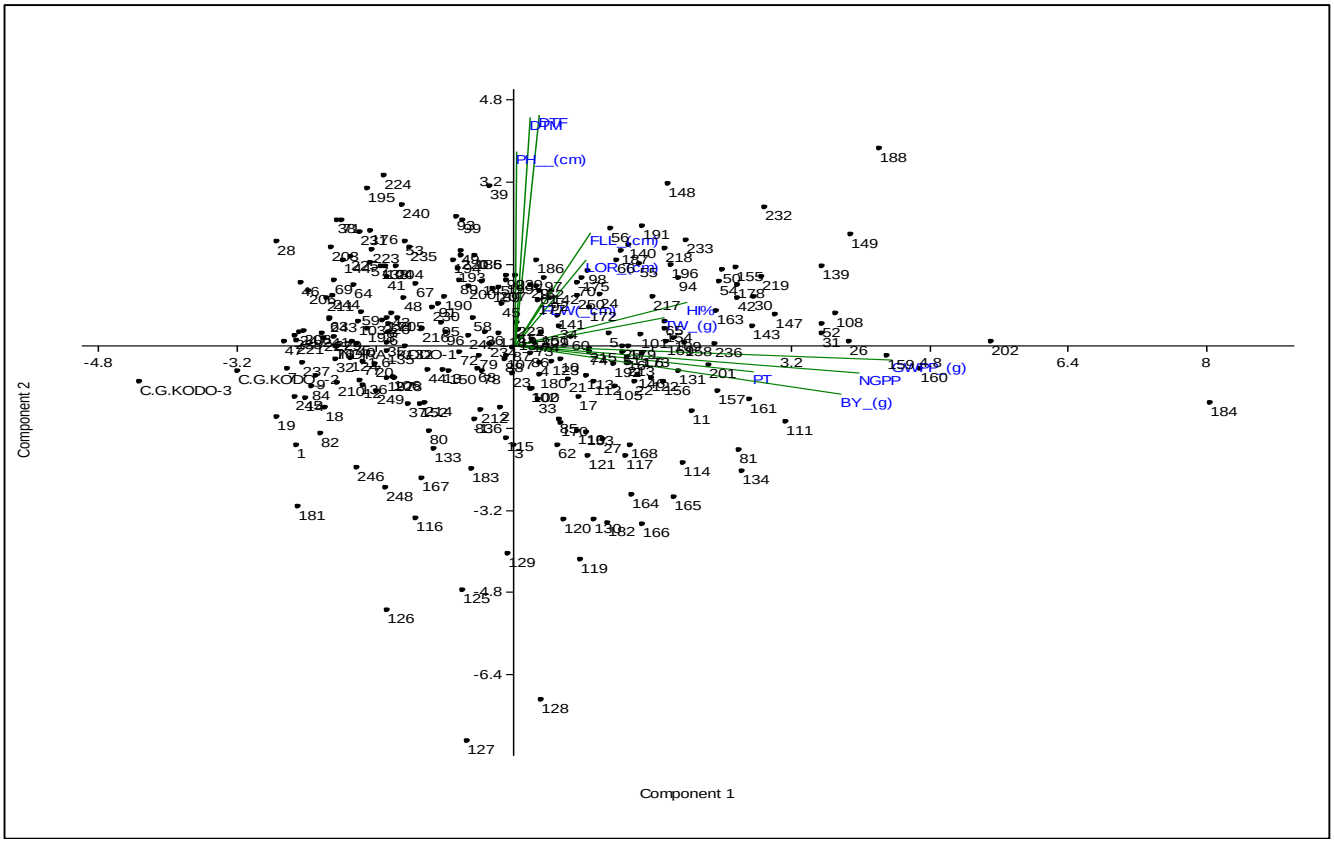


Fig 1: Biplot representation of principal component analysis

Cluster Analysis

Grouping of genotypes into clusters

Cluster formation and the estimation of inter-and intra-cluster divergence provide a basis for designing effective hybridization programs. In the present study, 250 mutant lines were grouped into ten clusters, namely Cluster I, Cluster II, Cluster III, Cluster IV, Cluster V, Cluster VI, Cluster VII, Cluster VIII, Cluster IX, and Cluster X. Among these, Cluster VI contained the highest number of mutants (42 lines), followed by Cluster III with 36 lines, Cluster II with 29 lines, Cluster VII with 25 lines, Cluster V with 24 lines, and Cluster I with 18 lines. Clusters IX and X included the least, with 15 lines each, presented in Table 3. Such clustering reflects the relative genetic proximity among lines. This information is crucial for crop improvement, as it facilitates the selection of genetically

diverse parents and supports transgressive breeding. It is generally recommended to select parents from distinct clusters to maximize heterosis. Previous studies also highlight variability in cluster, Nirubana *et al*. (2017) reported 11 clusters in 103 kodo millet genotypes, while Sahu (2023) [16] used Mahalanobis' D^2 statistics to classify 102 kodo millet mutants into five clusters. Similarly, Nireekshitha *et al*. (2024) [8] identified eight clusters among 38 kodo millet genotypes, whereas Subramanya & Ravikumar (2020) [13] grouped 33 finger millet genotypes into 10 clusters using D^2 analysis. Reddy & Vengadessan (2022) [12] reported 10 clusters from 42 genotypes, and Venkataratnam *et al*. (2025) assessed genetic divergence in 50 little millet genotypes using Mahalanobis' D^2 and also identified 10 clusters.

Intra and Inter cluster distances

Clustering and the assessment of intra-and inter-cluster distances form the basis for identifying genetically diverse parents. In the present investigation, the highest intra-cluster distance was observed in Cluster III (2.886), followed by Cluster I (2.671), Cluster IV (2.517), Cluster IX (2.442), Cluster VII (2.388), Cluster VIII (2.342), Cluster X (3.12), Cluster VI (2.302), and Cluster V (2.170), while the lowest was recorded in Cluster II (2.167). A higher intra-cluster distance reflects greater genetic variability within that cluster, whereas lower values denote closer genetic affinity among its members (Suryanarayana *et al*., 2014) [14].

The maximum inter-cluster divergence was found between Cluster X and Cluster I (5.984) given in Table 4, followed by Cluster IV and I (5.801) and Cluster III and I (5.559), suggesting the presence of wide genetic divergence and their potential use in developing superior segregants. Conversely, the lowest inter-cluster distance was observed between Cluster VI and VII (2.214), followed by Cluster X and V (2.478), indicating a relatively narrow genetic base. Comparable results have been reported earlier by Gautham (2020) recorded maximum inter-cluster distance between Cluster II and I (4.292) among three clusters. Similarly, Niharika (2022) [9] reported maximum divergence between Cluster II and I (4.65), followed by Cluster III and II (3.20) and Cluster III and I (2.85). Sahu (2023) [16] also noted that Cluster IV and II showed the highest inter-cluster distance (3.513), followed by Clusters IV and III (3.412). These findings highlight that genotypes from clusters with higher inter-cluster distances are more promising for heterotic expression and broadening the genetic base through recombination.

Cluster mean values for all quantitative traits

Cluster mean analysis revealed substantial variability across the twelve quantitative traits, providing a useful basis for identifying clusters with superior performance.As depicted in the Table 5, Cluster III which included 36 mutants, recorded the highest means for number of grains per raceme (316.97), productive tillers (9.23), grain yield per raceme (1.85), plant height (90.46), and test weight (6.39). Cluster X exhibited superiority for days to maturity (114.40), days to 50% flowering (76.20), and flag leaf length (45.50). Cluster V showed the highest mean value for raceme length (9.40), while Cluster II excelled in harvest index (36.30), Cluster VII in flag leaf width (0.96), and Cluster VIII in biological yield (4.89). Cluster IV also displayed higher mean values for flag leaf length, grains per panicle, test weight, and grain yield per panicle. On the other hand, Clusters I, VI, and IX did not exhibit superiority for any trait. These findings indicate that certain clusters serve as valuable sources for specific trait improvement, thereby offering scope for targeted selection in breeding programs. Similar observations were made by Charitha (2023) [2], who reported three clusters among 64 finger millet genotypes, with Cluster III containing the maximum genotypes and showing the highest mean values for traits such as days to flowering (69.91), days to maturity (101.3), and plant height (102.59).

Table 3: Clusters representing number of genotypes

Cluster	No of genotypes
I	18
II	29
III	36
IV	15
V	24
VI	42
VII	25
VIII	34
IX	15
X	15

Table 4: Intra and Inter cluster distance between clusters

Clusters	I	II	III	IV	V	VI	VII	VIII	IX	X
I	2.671									
II	4.128	2.167								
III	5.559	3.279	2.886							
IV	5.801	2.737	4.070	2.517						
V	4.833	3.536	4.230	3.562	2.170					
VI	3.960	3.062	5.077	3.327	2.888	2.302				
VII	5.175	3.238	4.957	3.188	2.812	2.214	2.388			
VIII	3.935	2.587	2.831	3.335	2.879	2.767	3.010	2.342		
IX	3.225	2.848	3.477	4.304	3.415	4.091	4.423	3.155	2.442	
X	5.984	4.146	4.032	3.606	2.478	3.283	3.565	3.118	4.780	2.312

Table 5: Cluster mean values for all quantitative characters

Cluster		Days to flowering (DAS)	Days to maturity (DAS)	Plant height (cm)	Flag leaf length (cm)	Flag leaf width (cm)	Length of raceme (cm)	Productive tillers	Number of grains per raceme	Biological yield (g)	Harvest index (%)	Test weight (g)	Grain yield per raceme (g)
I	Mean	55.83	92.67	55.56	25.94	0.72	6.97	8.26	265.17	4.88	30.30	5.51	1.46
	SE±	6.33	6.25	10.27	6.33	0.10	0.85	1.53	22.65	0.47	2.09	0.25	0.13
II	Mean	69.41	105.66	69.93	28.99	0.81	7.02	7.87	269.34	4.37	36.30	5.92	1.59
	SE±	3.74	3.98	8.31	5.21	0.13	0.82	1.15	23.40	0.30	1.19	0.20	0.12
III	Mean	73.53	110.17	81.74	36.51	0.80	7.86	9.23	316.97	5.37	34.52	5.99	1.85
	SE±	4.78	4.75	7.82	7.18	0.14	1.28	1.42	29.01	0.39	1.92	0.46	0.17
IV	Mean	74.07	109.20	90.46	31.59	0.81	7.52	8.07	229.87	4.15	35.24	6.49	1.46
	SE±	5.86	5.23	5.78	7.91	0.10	0.74	1.10	27.01	0.35	1.79	0.46	0.13
V	Mean	69.05	106.40	82.69	39.83	0.91	9.40	8.17	250.35	4.29	32.00	5.42	1.37
	SE±	3.90	4.51	5.98	5.97	0.14	0.76	1.00	15.37	0.36	1.75	0.26	0.10
VI	Mean	68.74	105.50	76.13	31.92	0.71	7.07	6.98	229.62	4.02	31.51	5.53	1.26
	SE±	3.33	3.10	10.86	5.25	0.10	0.96	0.95	18.34	0.26	2.14	0.41	0.08
VII	Mean	73.16	109.96	84.67	29.14	0.96	6.96	6.68	238.52	4.05	32.27	5.42	1.30
	SE±	5.14	6.00	7.03	7.54	0.09	0.91	1.01	18.72	0.34	1.92	0.36	0.08
VIII	Mean	70.29	107.94	82.56	28.44	0.75	7.42	8.61	275.76	4.89	31.71	5.53	1.55
	SE±	3.71	4.77	8.39	5.40	0.10	1.16	1.28	13.19	0.38	1.85	0.31	0.10
IX	Mean	59.67	96.47	72.93	37.00	0.91	7.74	8.57	283.13	4.92	33.85	5.82	1.66
	SE±	3.79	4.91	9.58	7.08	0.12	1.03	1.36	17.19	0.36	1.71	0.37	0.08
X	Mean	76.20	114.40	87.56	45.50	0.69	8.94	7.58	254.40	4.49	31.51	5.57	1.42
	SE±	3.23	4.40	5.93	4.92	0.10	1.08	1.14	26.46	0.41	1.47	0.36	0.16

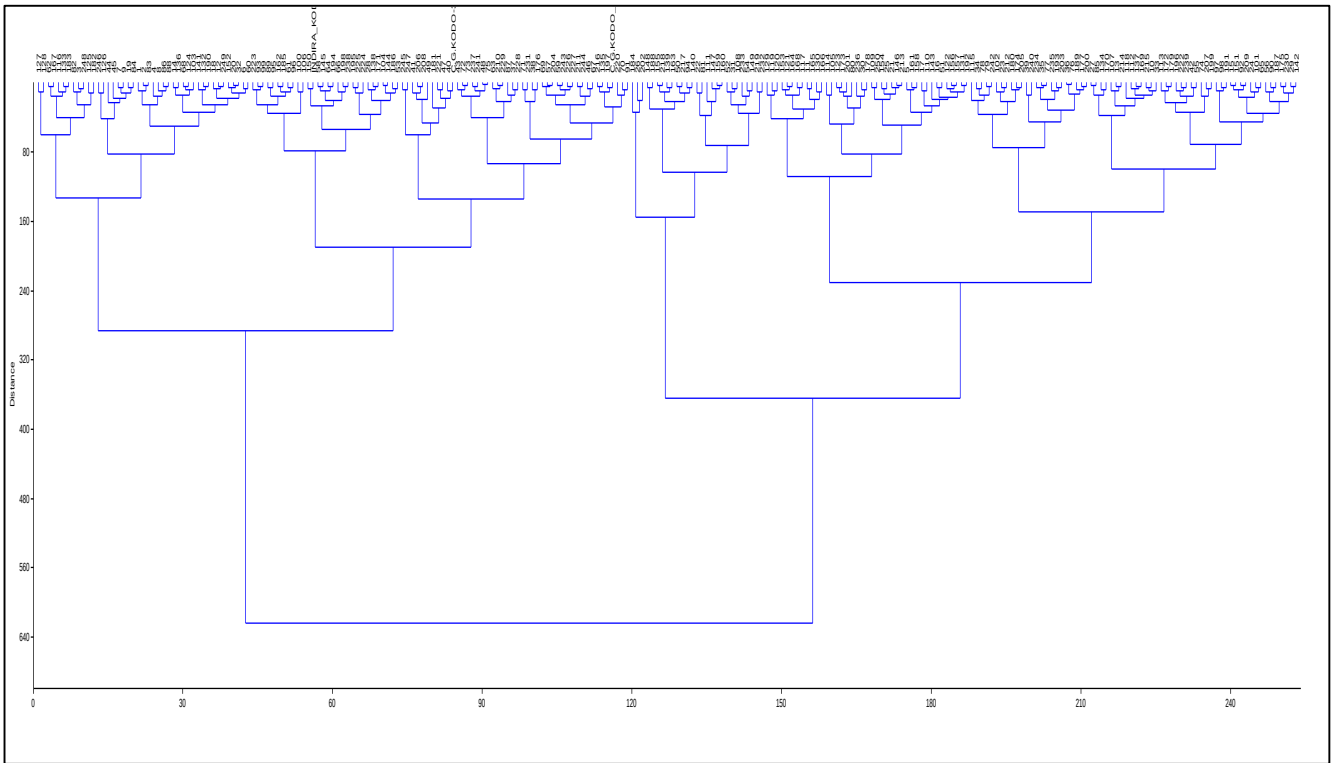


Fig 2: Dendrogram representing clustering pattern of 250 mutant lines.

Regression Analysis

In the current research, multiple linear regression (MLR) was employed as a statistical tool to examine the relationship between a dependent variable (grain yield per raceme) and several independent variables, including days to 50% flowering, days to maturity, plant height, flag leaf length, raceme length, harvest index, number of grains per raceme, test weight, flag leaf width, biological yield, and productive tillers. This approach is widely used in plant breeding to evaluate how different morphological and agronomic traits influence yield, thereby helping in the identification of traits with the greatest impact, prioritizing selection criteria, and understanding both direct and indirect contributions of traits to productivity.

The results revealed that traits such as biological yield ($p = 0.000$), number of grains per raceme ($p = 0.000$), harvest index ($p = 0.000$), and test weight ($p = 0.004$) showed statistically significant positive effects on grain yield per raceme, indicating that these are key yield-contributing traits. The positive significance of these characters suggests that improvement in these traits would lead to corresponding enhancement in grain yield. The regression slopes of all significant characters are presented in the following sections.

Table 6: Parameter estimates table of regression analysis

Parameter	Un-Standardized	Standard Error	t-value	p-value
Const	-1.506	0.038	-39.855	0.000
Days to flowering (DAS)	0.001	0.001	1.222	0.223
Days to maturity (DAS)	0.000	0.001	-0.644	0.520
Plant height (cm)	0.000	0.000	1.650	0.100
Flag leaf length (cm)	0.000	0.000	0.061	0.951
Flag leaf width(cm)	0.004	0.010	0.373	0.709
Length of raceme (cm)	0.000	0.001	0.176	0.860
Productive tillers	0.000	0.001	0.408	0.684
Number of grains per raceme	0.001	0.000	4.734	0.000**
Biological yield per plant (g)	0.300	0.007	43.009	0.000**
Harvest index (%)	0.041	0.001	37.352	0.000**
Test weight (g)	0.016	0.005	2.945	0.004**

Harvest index (%)

Grain yield per raceme exhibited a significant association with harvest index. The positive slope indicates a linear relationship between the two traits. The coefficient of determination (R^2) was 0.234, suggesting that harvest index alone accounted for 23.74% of the variation in grain yield per raceme. This reflects a moderate level of correlation and also implies that other traits contribute to yield variation. In the regression plot, blue dots represent the observed values. A closer clustering of these dots around the regression line denotes a good model fit with lower residual error, whereas a wider dispersion indicates greater variability.

Test weight (g)

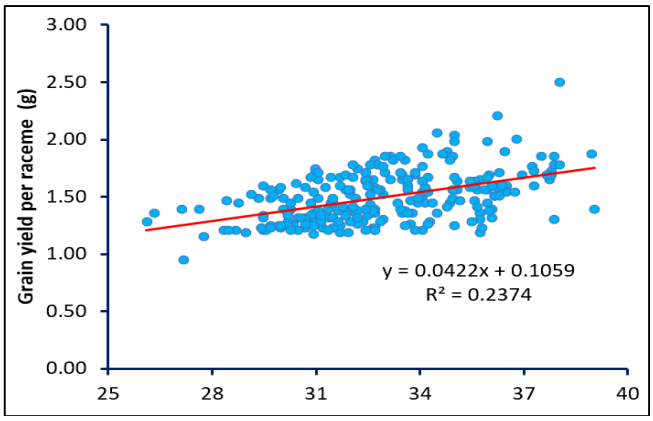
The positive slope confirms a linear association between grain yield and test weight. The R^2 value of 0.1625 indicates that test weight accounts for 16.25% of the variation in grain yield, reflecting a relatively weak correlation. Although both harvest index and test weight show positive linear relationships with grain yield per raceme, harvest index emerges as a slightly stronger predictor based on its higher R^2 value.

Number of grains per raceme

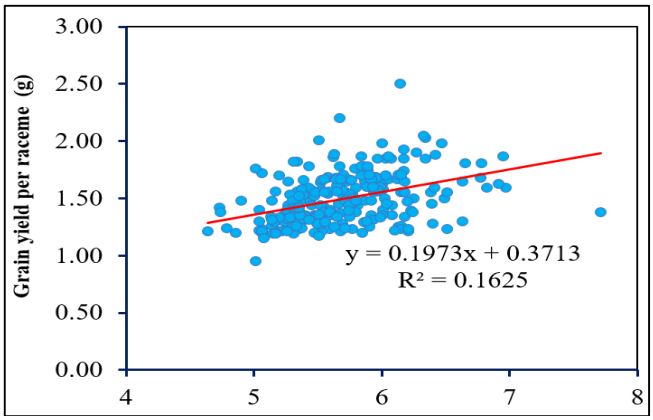
The graph illustrates a positive association between the number of grains per raceme and grain yield per raceme. An increase in grain number corresponds to a rise in yield per raceme. The coefficient of determination (R^2 = 0.7547) indicates that about 75.47% of the variation in grain yield is explained by the number of grains per raceme. This high R^2 value reflects a strong correlation, establishing it as a reliable predictor trait.

Biological yield per plant (g)

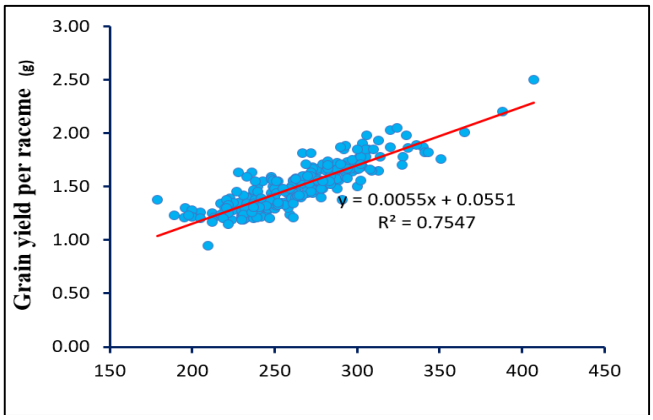
A positive linear relationship was also observed between biological yield and grain yield per raceme. Although the R^2 value was slightly lower than that of number of grains per raceme, it still demonstrated a strong positive association. Biological yield accounted for 71.03% of the variation in grain yield per raceme. The slope further indicated that grain yield per raceme increases at a faster rate with rising biological yield compared to the increase observed with number of grains.



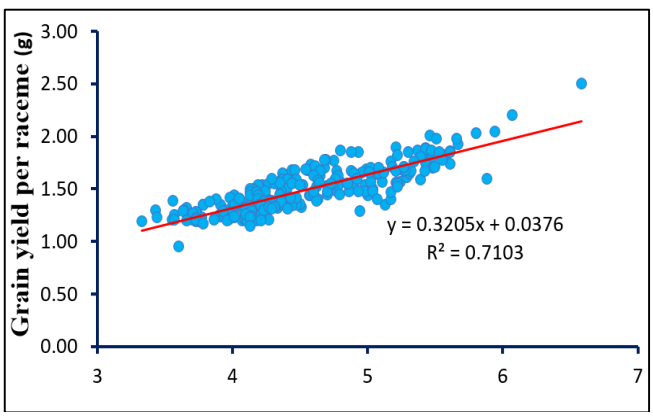
a) Harvest index



b) Test weight



c) Number of grains per raceme



d) Biological yield

Fig 3: Regression lines graph of yield related characters with respect to grain yield.

Conclusion

Principal Component Analysis (PCA) revealed significant variability among 250 mutant lines and has identified four major components explaining 71.5% of the total variation, with grain yield per raceme, number of grains per raceme, biological yield, and days to flowering as major contributors, thereby highlighting these traits as critical selection indices. Multiple linear regression analysis confirmed that traits such number of grains per raceme, biological yield, and harvest index explained a substantial proportion of the variability in grain yield. This analytical confirmation of their predictive value underscores their utility as key selection traits. Cluster analysis has showed high degree of genetic diversity suggesting that genetic makeup of mutant lines falling in this cluster may be entirely different from one another and thus may be utilized for future breeding programmes. Combining PCA, cluster and regression analyses provided a comprehensive understanding of variability, trait relationships and potential breeding value among mutants.

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