Received: 15 Oct 2025 | Accepted: 29 Nov 2025 | Online: 02 Dec 2025

Electronic Journal of Plant Breeding

Research Article

Root traits profiling for identification of nitrogen efficient wheat Lines

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Abstract
With the goal to mitigate production costs, promote sustainable agriculture and reduce high nitrogenous fertilizer consumption in wheat, nitrogen use efficiency (NUE) need to be strengthened by integrating morphological and traditional approaches. The Root system architecture (RSA) exert a major role in determining the amount of Nitrogen (N) uptake by various genotype under various N regimes. Total 147 germplasm lines of wheat were characterized for root characters at seedling stage grown under optimum (4 mM) and low nitrogen (0.04 mM) condition during 2024-25 at Shandong Agriculture University in Artificial condition. The shoot length, average diameter and shoot biomass significantly decreased at low N, while total root length, the primary root length, total root surface area, total number of root tips, root biomass, total root volume and root to shoot ratio significantly increased in artificial condition. Based on the results of the principal component analysis (PCA), PC1 and PC2 collectively explained 76.45 % and 72.39 % of the variability under optimal N and low N, respectively. Maximum portion of variability was exhibited by total shoot length, shoot biomass, root to shoot ratio, root volume and primary root length in both nitrogen condition. Cluster analysis showed that under low N, cluster I germplasm lines had higher root growth and lower shoot growth while cluster III germplasm lines had reduced root growth. These N-efficient germplasm lines can be utilized for further utilization in breeding programme.

Keywords: Wheat, Nitrogen stress, Root system architecture, PCA

INTRODUCTION

Wheat is one of the oldest and most widely grown cereal crops, domesticated around the same period as rice and in the fertile Crescent of the Near East about 10,000 years ago (Erenstein *et al.,* 2022a). The world's foremost three staple grains are rice, wheat, and maize, and each is grown on about 200 million hectares (M ha) and considered as primary source of nourishment (Erenstein *et al.,* 2022a; Erenstein *et al.,* 2022b). Wheat (*Triticum aestivum* L.) crop provides sustenance for almost half of the world's population and constitutes 20% of the calories consumption by humans (Ulukan, 2024). The primary factor limiting the narrowing of the wheat yield gap in the majority of the world's regions is the availability of soil nutrients, particularly nitrogen (Martre *et al.,* 2024). Implementing efficient N management in crop production and optimizing N use efficiency (NUE) is critical for striking a balance between environmental and food security goals (Zhang *et al*., 2022).

From a physiological aspect, NUE can be defined as the plant biomass produced per unit of available soil N. Components of NUE like absorption, assimilation, photorespiration and relocating nitrogen storage proteins should be focused to strengthen the nitrogen management and enhance grain protein (Beatty *et al.,* 2017). For the purpose of future advancements in breeding and agronomic management, it is essential to comprehend the causes underlying the variability in nitrogen (N) utilization efficiency (NUtE), or grain yield per unit of N uptake at maturity (NupMAT) (de Oliveira *et al*., 2020). While judging wheat crop yield, NUE is a crucial concept as it illustrates the nutrient loss from environment. Particularly with regard to breeding efforts, it aims to find the cultivars with enhanced reliability for assigning growth or nitrogen delivers to the economical portion of plants (Fiaz *et al.,* 2024). In contrast to other cereals, wheat roots are less developed that can be accomplished by breeding varieties with most extensively developed root (Lemaire and Ciampitti, 2020).

Nitrogen use efficiency is an integrated approach of morphological, biological, genetic, agronomic, and developmental factors simultaneously attributed by soil factors, root structure, environmental factors and management practices (Hirel *et al.,* 2011). Root architecture of plant is one of major component to impact nitrogen uptake of plant and study on root traits crucial for comprehending how plants respond to low nitrogen stresses. So, improving nitrogen use efficiency by developing crops and/or varieties that produce more with less nitrogen is an important aim to armor the environment and support more sustainable and productive farming. The inherent mechanisms and the regulation for imparting productivity with respect to of N uptake and utilization could be identified using the controlled environmental conditions as it is difficult to screening in field condition (Nagar *et al.,* 2018, Ranjan *et al.,* 2019).

Characterizing wheat germplasm lines that use nitrogen efficiently is essential for development of low-N tolerant wheat genotypes. The present study aimed at achieving the following objectives in order to support the identification of wheat varieties that can flourish under constrained N conditions: (1) To assess the genetic variation in root characteristics in 147 wheat germplasm lines with both optimal and insufficient N supply, (2) To identify root characteristics substantially impacting genetic variability, classifying possible germplasm lines with effective root growth under N stress.

MATERIALS AND METHODS

A collection of 147 breeding lines was used in this study. These lines were collected from Shandong Agriculture University, China and examined for root traits in two nitrogen regimes during the year 2024-25. The seeds were surface sterilized with 70% ethanol for 2 min followed by washing with sterile water 4–5 times. Subsequently, the seeds were treated with 0.1% $HgCl_2$ for 10 min followed by washing with sterile water four times and allowed to germinate in an incubator at 25±1°C in the dark. Once the primary roots attained a length of about 1 cm, they were grown in pots (4 inches wide) filled with perlite and vermiculite (1:2 ratio by volume) as described by Sinha *et al*. (2018). The study was arranged in completely randomized design with three biological replicates to evaluate the two factors: nitrogen levels and genotypes. The MS liquid media without any nitrogen source was added with 4 mM and 0.04 mM NO_3- in the nitrogen-rich and nitrogen free media respectively, and these were used as nutrient solutions at regular intervals throughout the growth period. The calcium level in all media was kept the same by changing the amount of $CaCl_2$. The number of seedlings grown was 4 seedlings per pot.

Analysis of Root System Architecture: Images were scanned using a root scanner (EPSON Flatbed Scanner), and they were then analyzed using WinRhizo 2000 Pro software (Regent Instruments Inc., Canada). otal root surface area (TSA, cm2), total root length (TRL, cm), total root volume (RV, cm3), average diameter (AD, mm), and total number of root tips (Tips) were among the root characteristics that were noted. After dividing the plants into shoots and roots, the lengths of the roots and shoots were measured independently. These tissues were dried at 50 °C, and their dry weight was measured. All data were expressed on a per plant basis and three independent seedlings were taken for observations.

Statistical analysis: Three biological replicates were used for the measurements presented in this study, and the MINITABSTAT program was used to statistically analyze the data by performing an analysis of variance (ANOVA) for each parameter independently. The relative values of each character were used for hierarchical cluster analysis by using statistical software ORIGINLAB (version 2024, USA). Silhouette indices and Akaike's Information Criterion (AIC) were calculated to find out the optimum number of clusters. The Euclidean distance, Ward's linkage approach, and dendrogram structure were used to create the framework of the hierarchical cluster. The Kruskal-Wallis test was employed to perform analysis of variance and level of significance between clusters (Kruskal and Wallis, 1953). This statistic test estimate the rank of all observation in the all cluster analyzes the rank sums of clusters of differed sizes to measure the overall difference. Principal component analysis and correlation analysis was done by ORIGINLAB (USA, 2024). Agglomerative Clustering was performed using PYTHON Scikit (version 1.3). Principal component analysis classifies the traits by calculating eigenvectors (directions) and eigenvalues (importance) from the covariance matrix and convert data into new features known as principal components. Biplots were developed which demonstrates how each variable contributes to each principal component, can assist in determining which variables have the most impact.

RESULTS AND DISCUSSION

The analysis of variance was conducted for 10 characters which revealed significant differences in root traits among wheat germplasm lines and between N treatments ($P \leq 0.001$, **Table 1**). The genotype x nitrogen interactions were significant for all the traits which showed that phenotypic performance of germplasm lines varied in different nitrogen regimes. To understand the contribution of genetic factor to the variability, Broad-sense heritability (H^2) was estimated. Heritability (H^2) values varied from moderate to very high for all characters except surface

Table 1. Analysis of variance (ANOVA) and heritability for root traits studied in 147 wheat germplasm lines grown under optimum (4 mM) and low nitrogen (0.04 mM)

S.No.	Traits	F value			Heritability (%)
		Genotype (G)	Nitrogen (N)	G × N	
1	Primary Root Length	68.91***	4343.90***	15.95***	74.71
2	Total Root Length	36.59***	2053.61***	11.88***	64.03
3	Total Surface Area	7.85***	900.95***	4.93***	29.68
4	Average Diameter	91.50***	966.35***	47.35***	47.21
5	Root Volume	65.20***	6382.12***	36.67***	42.43
6	Number of Root Tips	75.72***	6650.85***	22.84***	68.03
7	Root to Shoot Ratio	154.13***	1498.58***	7.48***	93.99
8	Root Biomass	60.91***	2031.56***	12.30***	77.33
9	Shoot Biomass	281.90***	2856.72***	27.36***	89.68
10	Total Shoot Length	288.61***	6581.42***	37.99***	86.25

***Significance level at $p<0.001$ level.

area, which revealed a maximum portion of variance was accounted to genetic factor. The frequency distribution curve also exhibits the level of genotype variation in all root traits (**Fig. 1**).

The mean, standard deviation and ranges of the all traits under low N and optimum N are presented in **Table 2**. All the characters increased under low nitrogen except average diameter, shoot length and shoot biomass. Total surface area increased by 59.45 %, root volume by 58.97 %, root tips by 49.34 %, root to shoot ratio by 30.50 %, total root length by 26.08 %, primary root length by 20.62 % and root biomass by 19.58 % in low nitrogen condition compared to optimum nitrogen.

The current study showed that low N conditions significantly increased the mean of primary root length, mostly due to the axial root growth of plants which show consistency with the results of Tiwari *et al.* (2023). It has been shown nitrogen can influence root development either by altering the external concentration or by altering the plant's internal nutritional status. Wang *et al.* (2022), Odone *et al.* (2023) and Tiwari *et al.* (2023) observed increase in axial root length under low nitrogen in wheat germplasm lines. Additionally, with N deprivation, wheat showed a rather shallow root angle with a larger root number and increased total and lateral root length (Liu *et al.,* 2022). Under low nitrogen availability conditions, plants are able to maximize their N acquisition and use through the architectural response of their roots (Lynch *et al.,* 2019). Surface area, root volume and root tips was increased in all genotypes under nitrogen stress conditions due to spread of roots over a larger region during N-stress which showed similarity with the results of Nagar *et al.* (2018) and Tiwari *et al.* (2023).
The relationships between different root traits were analyzed. The correlation analysis showed significant associations between root traits under both nitrogen condition (**Fig. 2**). As compared to optimum nitrogen, correlation coefficient for majority of root traits was found decrease in low nitrogen. As an instance, the correlation between root biomass and root to shoot ratio ($r = 0.84$) was observed more in low nitrogen than optimum nitrogen ($r = 0.64$). Average diameter of root showed negative association with all traits in both nitrogen condition.

Root length exhibited significant and positive correlation with the surface area, root volume and root tips. The findings align with earlier research that suggested crops will have higher root surface area and a more extensive root system for more effective N uptake and improved adaptation to N scarcity (Gao *et al.,* 2015). Such enhancement in root parameters is commonly believed to be a key strategy to get accessible nutrients like water and nitrate in order to optimize N uptake in N-efficient genotypes.

PCA biplot is deciphering the correlations between the variables. The bi-plot representation exhibited high genetic variability among wheat germplasm lines and simultaneously displays the data from the loading plot and the PC value (**Fig. 3**).Under optimum N, PC1 and PC2 explained 50.88 % and 25.57 % of total variation, while under low N, PC1 and PC2 explained 44.03 % and 28.36 % of total variation respectively, Under both ideal and low N conditions, the contribution of each attribute to genetic variability is shown in **Table 3**. The traits *viz*., total shoot length, shoot biomass, root to shoot ratio, root volume and primary root length described maximum portion of variability in both nitrogen condition.

Earlier contribution of root biomass, surface area and root length to genetic variation to N nutrition was observed (Tiwari *et al*., 2023). The root:shoot ratio is an index of

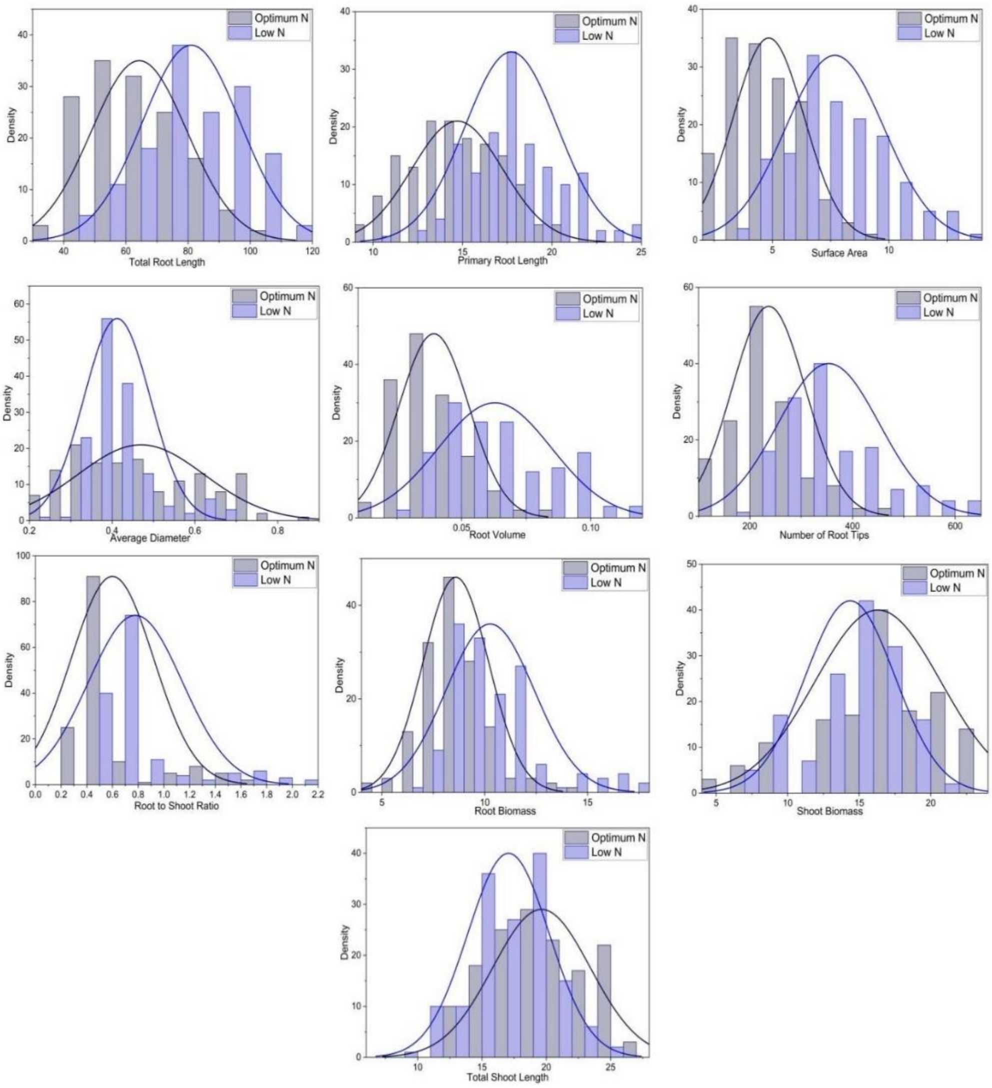

Fig.1. Frequency distribution density graph showing normal distribution for root traits studied in 147 wheat germplasm lines grown under optimum (4 mM) and low nitrogen (0.04 mM)

Table 2. Statistics for various root traits studied in 147 wheat germplasm lines under optimum (4 mM) and low nitrogen (0.04 mM)

S.No.	Traits	Optimum N			Low N		
		Range	Mean	SD	Range	Mean	SD
1	Primary Root Length	8.9-21	14.69	2.61	10.1-24.5	17.72	2.61
2	Total Root Length	35.23-107.45	64.26	15.52	43.21-126.45	81.02	16.86
3	Total Surface Area	1.95-9.45	4.81	1.53	3.48-14.1	7.67	2.12
4	Average Diameter	0.19-0.89	0.47	0.15	0.21-0.71	0.41	0.084
5	Root Volume	0.011-0.094	0.039	0.014	.021-0.126	0.062	0.02
6	Number of Root Tips	105-508	236.75	73.83	167-632	353.58	99.80
7	Root to Shoot Ratio	0.18-1.82	0.59	0.32	0.39-2.11	0.77	0.36
8	Root Biomass	4.34-14.9	8.58	1.64	6.69-18.30	10.26	2.15
9	Shoot Biomass	5.65-23.8	16.30	4.41	6.88-22.9	14.36	3.14
10	Total Shoot Length	11.9-27.8	19.64	3.78	8.1-25.1	17.06	3.17

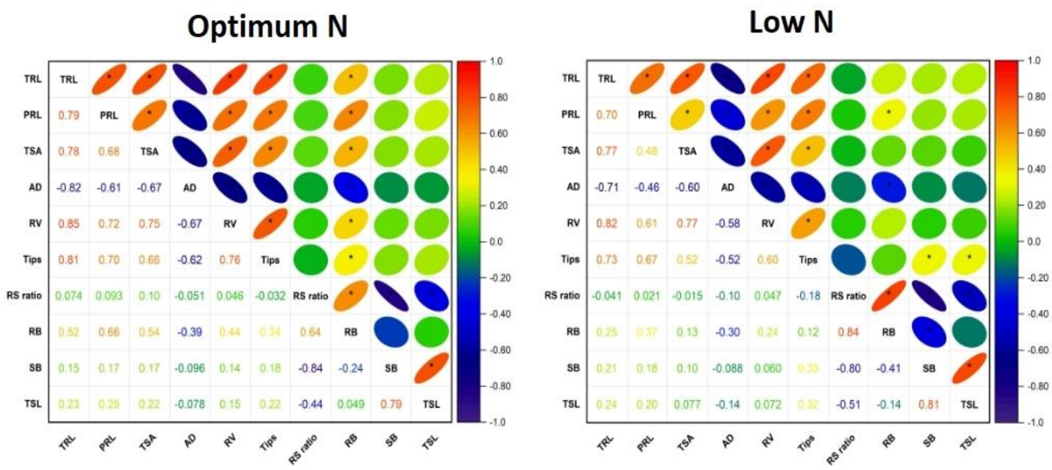

Fig.2. Correlation matrix between all root traits studied in 147 wheat germplasm lines under optimum (4 mM) and low nitrogen (0.04 mM).

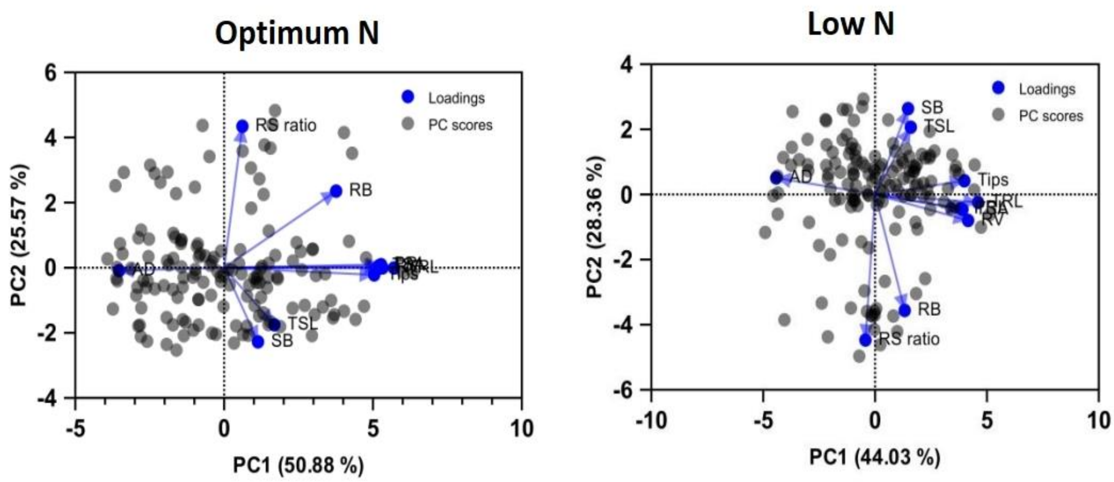

Fig.3. PCA biplot denotes PC1 and PC2 indicating principal components 1 and 2 respectively, for root traits in wheat genotypes under optimum (4 mM) and low nitrogen (0.04 mM) supply.

Abbreviations: TRL - total root length (cm), PRL - primary root length (cm), TSA - total root surface area (cm^2), AD - average diameter (mm), RV - root volume (cm^3), Tips - total number of root tips, RSR - root to shoot ratio, RB - root biomass (mg), SB - shoot biomass (mg) and TSL - total shoot length (cm)

illustrating the extent to which plant biomass is allocated to the roots. In this study root to shoot ratio was found increased in all germplasm lines and exhibited positive and significant correlation with root biomass. Plant responses to N deficiency have been linked with the enhanced photosynthates allocation to roots which is coupled with increased root parameters, increase of root to shoot ratio and reduced shoot parameters. Several researchers reported significant differences in shoot and root biomass between N treatments and genotypes which accounted for the varying root-to-shoot ratio (Tiwari *et al*., 2023; Wang *et al*., 2022).

Using the relative values of different traits, cluster analysis generated three main clusters (**Fig. 4a**). Agglomerative clustering was also performed to find out the optimum number of clusters (**Fig. 4b**). In agglomerative clustering, each cluster is formed using the euclidean distance. Euclidean distance is the measure of distance used to decide how close two points are to one another. Thirty four of the 147 wheat germplasm lines were allocated to cluster I, 53 belonged to cluster II, and 60 belonged to cluster III. Cluster I showed maximum value for majority of traits except average diameter, shoot length and shoot biomass while cluster II and cluster III showed opposite response (**Table 4**). Therefore, cluster I was classified as nitrogen-efficient, cluster III as inefficient, and the

Table 3. Percent contribution of traits in principal components (PC1 and PC2) towards genetic variability in response to optimum (4mM) and low nitrogen (0.04 mM) supply

S.No.	Traits	Optimum N			Low N		
		PC1	PC2	Total	PC1	PC2	Total
1	Primary Root Length	19.28	0.03	19.31	21.13	0.02	21.15
2	Total Root Length	16.14	0.00	16.14	14.88	0.32	15.20
3	Total Surface Area	11.71	0.05	11.76	15.41	0.27	15.68
4	Average Diameter	14.58	0.00	14.58	10.53	0.02	10.55
5	Root Volume	17.06	0.03	17.09	17.41	0.73	18.14
6	Number of Root Tips	15.34	0.24	15.58	15.34	1.05	16.39
7	Root to Shoot Ratio	4.92	21.59	26.51	1.68	27.46	29.14
8	Root Biomass	0.46	15.21	15.67	0.73	10.55	11.28
9	Shoot Biomass	0.51	34.69	35.20	1.36	30.53	31.89
10	Total Shoot Length	0.87	28.17	29.04	1.52	29.05	30.57

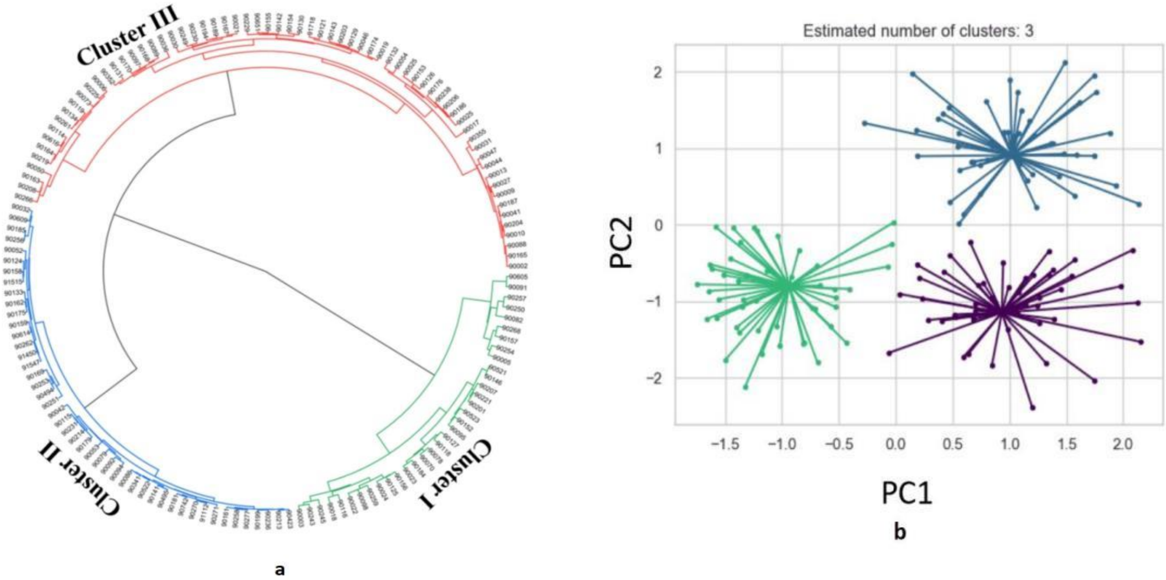

Fig.4. (a) The hierarchical cluster tree was made using Ward's method, on the basis of relative values of traits of wheat germplasm lines. Major clades present different cluster. Cluster I and III showed N efficient and N inefficient lines respectively, while intermediate response showed by cluster II (b) Agglomerative clustering

cluster II demonstrated a moderate response. Graphic visualization of different clusters exhibited relatedness of different characters and its association with cluster (**Fig. 5**). Shorter distance between traits showed their relatedness while more distance show that they are distantly related with each other (**Fig. 5**).

Clustering effectively identifies efficient wheat genotypes by grouping them, which enables a more targeted selection process for breeders (Singh *et al*., 2025). In the present investigation, N-inefficient germplasm lines showed a more significant decrease in shoot length and shoot biomass and increased in root biomass when exposed to nitrogen stress. Some of germplasm lines *viz*., 90605, 90091, 90257, 90250 and 90003, which belong to Cluster I and exhibited significantly high root growth under low nitrogen, are valuable targets for breeders developing nutrient-efficient varieties. This findings indicate that under N deficient conditions, wheat genotypes also undergo the probable biomass adjustment with varying degrees of plasticity. N efficiency in seedlings, however, did not significantly correlate with shoot biomass. This likely means that improvement in N-efficiency of wheat plants grown under N deficit was not directly limited by shoot biomass and length. Similar decrease in shoot dry weight and shoot length in response to low nitrogen observed

Table 4. Analysis of variance across three main clusters using the Kruskal-Wallis test (H value) and cluster means of root characteristics showing the percentage change at low (0.04 mM) N supply compared with optimal (4 mM) N supply

S.No.	Traits	Cluster I	Cluster II	Cluster III	H value
1	Primary Root Length	143.45	123.30	109.56	65.45***
2	Total Root Length	169.04	133.99	105.26	99.41***
3	Total Surface Area	257.91	166.94	124.76	95.92***
4	Average Diameter	80.06	87.45	112.83	47.76***
5	Root Volume	291.70	155.70	122.36	92.22***
6	Number of Root Tips	183.75	167.04	130.57	42.77***
7	Root to Shoot Ratio	147.18	136.21	111.66	34.17***
8	Root Biomass	124.80	116.65	103.36	36.93***
9	Shoot Biomass	89.60	87.22	95.10	10.23*
10	Total Shoot Length	86.04	88.49	87.63	0.19NS

*** Significant at 0.001 level, NS - non-significant

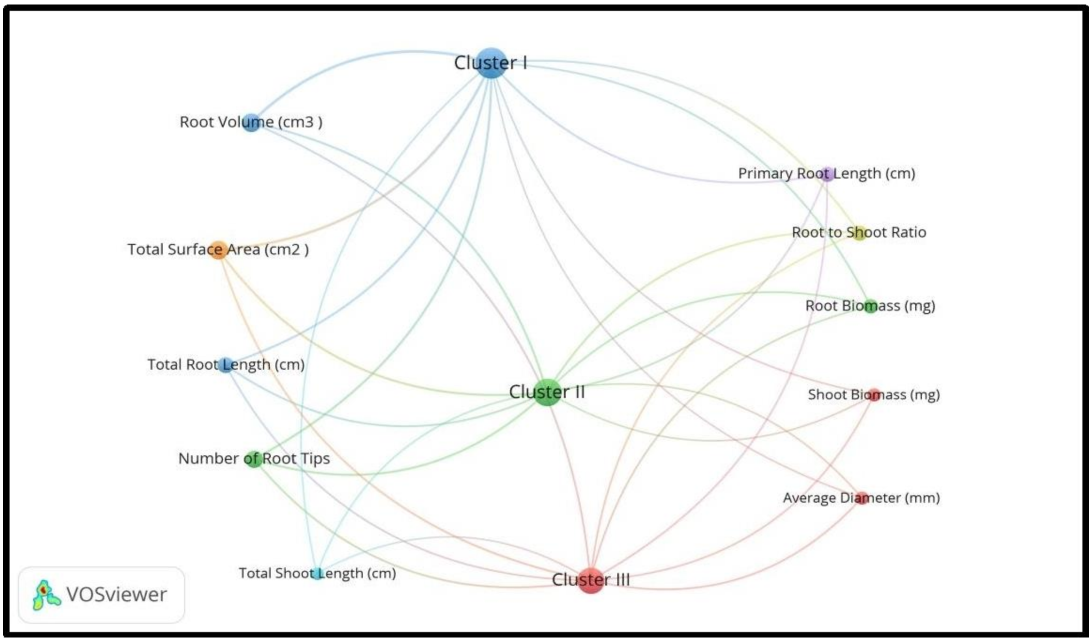

Fig.5. Graphic visualization and cluster visualization of different clusters

by Wang *et al.* (2022) in maize and Tiwari *et al.* (2023) in wheat. Root proportion is very dynamic and most often increase when soil temperature, nutrient availability, and water availability are low. Genetic variation is frequently observed in roots response to nutrient treatments, and the potential of roots to react to soil characteristics presents intriguing opportunities for future crop yield increases (Fageria and Moreira, 2011).

For the identification of elite germplasm lines under a range of environmental conditions, from optimal to stressful, selection indices and multivariate techniques are powerful tools (Yadav *et al.*, 2024). Gaining insight into the growth and framework of roots, this study could provide opportunities for the modification of root traits to maximize agricultural productivity and enhance crop production. The excessive consumption of N fertilizer impacts the ecosystem and depletes the soil of several nutrients, which ultimately reduces wheat production. It becomes imperative to develop wheat cultivars having a high efficiency of usage of N in order to decrease the usage of these fertilizers diminishing yield (Sagwal *et al.,* 2022).

The significant impact of roots in facilitating a plant's uptake of water and nutrients is essential for its existence in nutrient stress condition. Wheat root system architecture features alter in response to low N availability, facilitating higher root growth for better N acquisition. Enhancements have been observed in total root length, the primary root length, total root surface area, total number of root tips, root biomass, total root volume and root to shoot ratio. Thus, these germplasm lines can be exploited as possible donors for improvement of nitrogen use efficiency of high yielding wheat genotypes of low nitrogen use efficiency. Current research on root system architecture traits may serve as the foundation for identifying key genetic elements of root system architecture traits under nutrient limitation in the future.

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