

DECIPHERING THE CHANGE IN ROOT SYSTEM ARCHITECTURAL TRAITS UNDER DIFFERENT NITROGEN REGIMES IN WHEAT (*Triticum aestivum* L.)

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ABSTRACT

Root system architecture (RSA) plays a critical role in plant adaptation to varying nitrogen (N) availability by influencing soil exploration, nutrient acquisition, and plant growth. To investigate the diversity of RSA responses to contrasting N availability, a diverse panel of 453 wheat genotypes, including landraces, historical cultivars, and modern varieties, was evaluated. Cultivars, exotic cultivars, gene pool accessions and advanced wheat breeding lines were assessed in controlled glasshouse conditions. A completely randomized design with three biological replicates was used to phenotype the RSA at the tillering stage under two N doses N50 (60 kg N ha⁻¹), corresponding to 50% of the recommended N application rate and representing half of the recommended N availability, and N100 (120 kg N ha⁻¹), corresponding to the recommended field N application rate. Broad sense heritability of platform traits ranged from 0.50 for shoot fresh weight to 0.83 for the root volume. The N regimes showed contrasting relationships between biomass and RSA traits in a correlation analysis. The shoot biomass correlated positively with root traits, although not strongly, under N50, indicating a greater tendency to invest in root growth rather than shoot growth under low nitrogen availability. Contrary to this, positive trends between shoot biomass and RSA traits were observed under N100, indicating enhanced functional integration of root and shoot growth at N100 levels. Under N100, total root length had stronger positive correlations with both root surface area and root branching, indicating the more coordinated and efficient root system organization under optimal N availability. The genotypes were categorized as superior, intermediate and poor performing plants based on the RSA Index (RSAI) and Total Plant Biomass (TPB) under N50. Several genotypes like WATAN, CHIRYA-3, 1PFAU, M07_FRET-1, MEHRAN-89 and Fakhr-e-Sarhad remained in the top 20% in all the trials and showed better root architecture and biomass production under N deficient conditions. Genotypes such as Chakwal-50, BORLAUG-16, HUW-234, EXCALIBUR, and CS/TH exhibited vigorous root system under the evaluated nitrogen conditions. The genotype SC consistently ranked among the lowest performers, reflecting poor adaptation to low-N environments. The overall genetic variability for these RSA traits under contrasting N regimes indicated that root architectural traits could be valuable selection criteria for the development of wheat cultivars with improved adaptation and productivity under reduced N inputs.

Key words: Nitrogen treatments, root imaging, rhizovision, wheat, multivariate analysis.

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INTRODUCTION

Global wheat (*Triticum aestivum* L.) production in 2025 is projected at approximately 800.1 million tonnes, representing a marginal 0.3% increase over the previous season, according to FAO (2025). It contributes significantly to global food security by providing carbohydrates, proteins, vitamins, and minerals for human nutrition (Shewry and Hey, 2015). However, increasing global population and climate change are placing enormous pressure on agricultural systems to produce higher yields with limited resources (Lynch, 2021). Among essential nutrients required for crop growth, nitrogen (N) is the most critical and frequently limiting nutrient affecting wheat productivity. Nitrogen fertilizers play a key role in improving crop yield and grain quality, but their inefficient use often leads to environmental problems such as nitrate leaching, soil degradation, and nitrous oxide emissions. More than half of the N applied to fields is lost, causing serious environmental and health problems (Martínez-Dalmau *et al.*, 2021).

Root system architecture (RSA) is the spatial distribution and structure of the root system (Lynch, 1995). The integration of these architectural traits (root length, root diameter, root branching, root angle, root depth) produces a highly complex root system that is challenging to characterize and phenotype, particularly as root-system complexity increases

with plant age. Therefore, the majority of research focuses on a small number of integrative RSA characteristics, including root system biomass or total root length (Im *et al.*, 2026). The RSA traits are highly variable among wheat genotypes and can be reliably measured under controlled conditions, thereby RSA is a robust and reproducible phenotypic trait for genetic and physiological investigations (Rufo *et al.*, 2020). Plant growth is positively correlated with morphological and physiological attributes of roots. The capability of root system to uptake essential nutrients and water from soil depends on root key traits like length, diameter, and weight (Hafsa *et al.*, 2025). Some RSA characteristics, such as main or lateral root length, number of lateral roots, root density, root area, or specific root length, have been phenotyped in some recent research; however, phenotyping capabilities are often restricted to seedlings or young plants under controlled environmental conditions (Adeleke *et al.*, 2020). Understanding how plants grow and develop under diverse environmental conditions is crucial for improving crop productivity. Plants are strongly influenced by their surrounding environment and continuously adjust their growth and development to cope with changing conditions. The nutrients that are most essential for plants include nitrogen (N), which is used to make proteins and contributes greatly to plant growth, yield, and productivity (Rouina *et al.*, 2025). Nitrogen availability significantly affects root development and RSA. There is a high plasticity for root growth of plants under different N supply. In low N availability conditions, plants may develop longer roots, higher root branching and expanded root surface area to explore the soil and improve nutrient acquisition. Under high N conditions, however, the elongation of the roots can be inhibited because of the increased allocation to the shoot (Jia *et al.*, 2021). This adaptive response enables plants to optimise nutrient uptake whilst reducing metabolic costs of root growth. A number of studies in wheat have shown a close relationship between root architectural characteristics and N uptake efficiency and plant performance (Zeng *et al.*, 2026). Deeper and more extensive root systems have been found to more efficiently extract N from the soil, especially from deeper layers where nitrates might be leached out. Root traits such as increased root length density, greater root surface area, and enhanced lateral root development are considered important indicators of improved N acquisition (Lou *et al.*, 2026). Thus, there is a need to identify root traits that will improve N uptake to significantly aid the breeding of wheat cultivars with improved N use efficiency.

Studying root systems in natural soil conditions is often challenging due to the hidden nature of roots and the complexity of soil environments. Root studies are traditionally done in the field and these methods are labor intensive and do not provide information about root architecture (Lynch, 2021). In order to overcome these limitations, controlled experimental systems, such as PVC pipe experiments, have been used extensively for the study of root growth and development under controlled environmental conditions (Shashidhar *et al.*, 2012). PVC pipe systems provide a semi-controlled soil environment, which allows the growth of the roots along soil depth to be followed and root distribution patterns to be better evaluated. These systems are especially valuable for research on root characteristics and nutrient uptake efficiency of cereal plants in deep soil (Feng *et al.*, 2012).

In addition, the recent developments in root phenotyping technologies allow imaging and quantitative analysis of root traits in high throughput. The image-based analysis tools, like RhizoVision Explorer, allow quick measurement of the root morphological properties such as total root length, root diameter, root surface area, root volume and branching patterns from digital root images. The integration of PVC pipe systems with high-throughput imaging improves the efficiency and accuracy of root trait analysis and allows the evaluation of multiple genotypes under different N conditions. High-throughput imaging approach enables rapid, standardized, and non-destructive acquisition of root phenotypic traits across a large number of genotypes. This approach facilitates quantitative assessment of root architectural attributes and improves the efficiency and reproducibility of root phenotyping compared with conventional manual measurements (Weihs *et al.*, 2024). Therefore, the present study was aimed to investigate the RSA of wheat genotypes under different N regimes using a PVC pipe experimental system combined with high-throughput root imaging. The objectives of this study are to (a) investigate how wheat root traits respond to different nitrogen (N) levels and (b) improve our understanding of variation in root architecture among wheat genotypes.

MATERIALS AND METHODS

Plant materials and experimental conditions: Diverse collections of 453 genotypes (Table S1) were collected from Wheat Research Institute, AARI, Faisalabad and National Agricultural Research Center (NARC), Islamabad. This diverse germplasm includes; wheat landraces (n=50), Pakistan's historical cultivars (rainfed and irrigated areas, n=106), exotic cultivars (n=37), Gene Pool (n=180) and advanced lines (n=80). Experiments were conducted in glasshouse at National Institute for Genomics and Advanced Biotechnology, (NIGAB), NARC, Islamabad (Figure 1). The seeds were germinated in petri dishes, sanitized with 10% sodium hypochlorite, and dipped in 70% ethanol. Ten seeds (healthy) were positioned on damp filter paper and allowed to grow in the dark with temperature 24°C ±2. Five uniform seedlings of each genotype were transferred to each PVC bag, and each bag was considered one independent biological replicate. Three independent bags were established for each genotype × nitrogen treatment combination, resulting in three biological replicates per treatment. At the tillering stage, three seedlings were randomly selected and harvested from each bag for phenotypic and

biomass measurements. The three seedlings sampled from a given bag were treated as subsamples within that biological replicate rather than as independent replicates. Polythene bags filled with sandy loam soil were placed in PVC pipes 152.4 cm in length and 11.43 cm in internal diameter (5 ft long $\times$ 4.5 inches wide), in a glasshouse. The physicochemical properties were determined for the experimental soil before applying the nitrogen treatments to assess its initial fertility status. The soil was analyzed using the ammonium bicarbonate-diethylenetriamine pentaacetic acid (AB-DTPA) extraction method for available nutrients. The initial soil nutrient status indicated nitrate-nitrogen (NO_3^- -N) of $5.88 \pm 0.14 \text{ mg kg}^{-1}$, available phosphorus (PO_4^{2-} -P) of $3.08 \pm 0.18 \text{ mg kg}^{-1}$, and available potassium (K) of $154.51 \pm 4.94 \text{ mg kg}^{-1}$. The glasshouse conditions were kept at 16 hours of daylight at 22–25 °C and 8 hours of darkness at 10–14 °C. Three biological replicates, each consisting of five plants, were used for each genotype under a completely randomized design (CRD) and two treatments of N, i.e. N100 (120 kg ha^{-1}), recommended dose and half of the recommended dose N50 (60 kg ha^{-1}).

At sowing, all the soil columns were fertilized with a mixture of N, P, and K (50, 60, and 40 kg ha^{-1}). For the N100 treatment, the first split (0.15 g N per PVC column/5 kg soil) was applied at sowing, and the second split (0.15 g N per column) was applied at the two-leaf stage, 15 days after sowing. The first split for the N50 treatment was 0.075 g N per column at sowing, and the second split was 0.075 g N per column at 15 days after sowing. The columns were watered using a measuring cylinder to ensure an equal amount of water supply to all the genotypes in both treatments. At the tillering stage (Zadoks 21), 40 days after sowing, three representative seedlings were sampled from each biological replicate for root-system analysis. Three consecutive washes were used to thoroughly clean the roots and separate them from the soil.

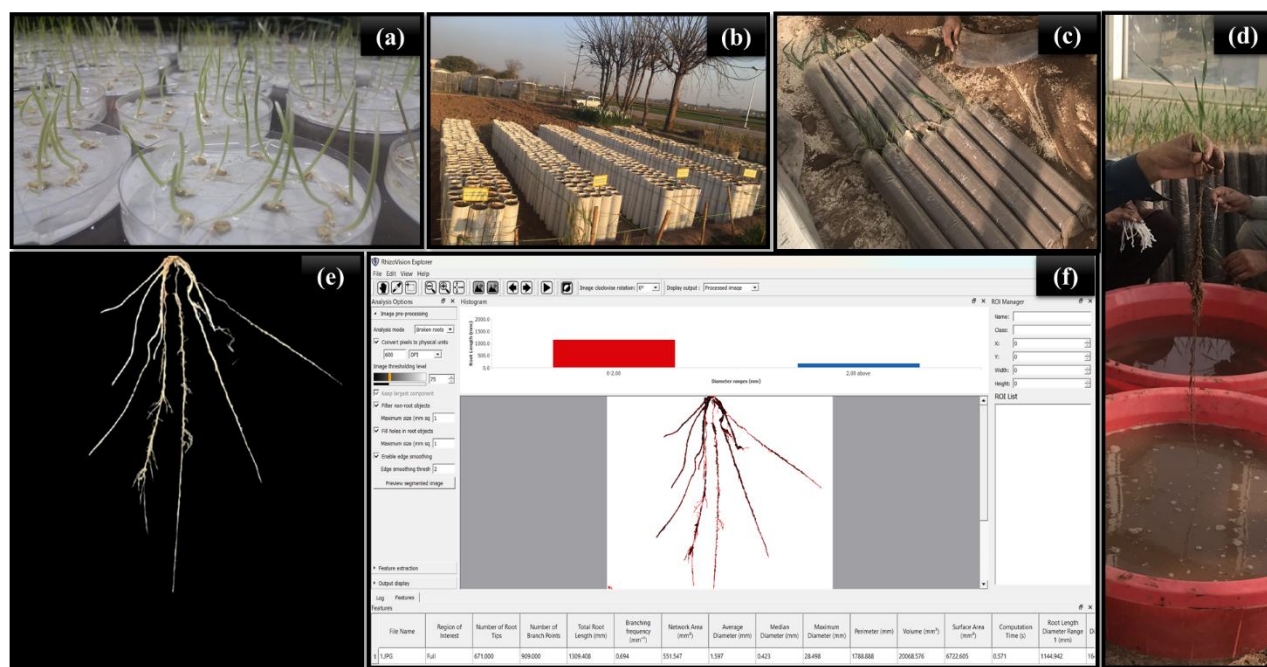


Figure 1: Root phenotyping pipeline. (a) Seedling in petri plates, (b) Experimental setup of wheat seedlings grown in vertically installed PVC pipes, (c) Root acquisition from polythene bag, (d) Washing of wheat seedling roots after extraction from PVC pipes, (e) Root image, (f) Root quantification using RhizoVision software.

High-throughput root imaging; Roots were spread on benchtop and high-quality images were taken by smart cellphone (iPhone 7plus) with resolution 1080 $\times$ 1920 pixels, 16:9 ratio ($\sim$ 401 ppi density). For further study, the root image background was eliminated. Images were analyzed using RhizoVision Explorer version 2.0.2 and algorithms described by (Seethepalli, 2020). A root diameter threshold of 0.3 mm was used to distinguish axial roots from lateral roots. RhizoVision Explorer (version 2.0.2) was employed to import and analyze high-resolution root images for high-throughput quantification of root morphology. This open-source software facilitates rapid and standardized extraction of multiple root traits from scanned or camera-acquired images, enabling robust phenotyping of root systems (Seethepalli *et al.*, 2021). Root images were analyzed using RhizoVision Explorer to extract 12 RSA traits, including total root length (TRL), total root surface area (TRSA), root volume (RV), root diameter (RD), number of root tips (NRT), root branch frequency (RBF), number branch points (NBP), root network area (RNA). Together, these traits define root size, spatial expansion,

and root exploration capacity, and have been commonly applied for evaluating root foraging efficiency and the RSA in functional and breeding research.

Estimation of plant biomass traits: Seedlings were carefully removed from pots and three plants per treatment were gently rinsed with tap water to remove any remaining soil particles. Following the separation of the shoots and roots, an electronic scale was used to quickly record the fresh weight (g plant⁻¹). Shoot and root samples from all plants within a column were combined, oven-dried to constant weight, and weighed. Biomass was then expressed as mean dry weight per plant (g plant⁻¹) by dividing the total dry weight of each column by the number of plants sampled.

Statistical analysis: A two-factor factorial under CRD analysis of variance was conducted in R using the “agricolae” package for 453 genotypes evaluated under two nitrogen regimes, with genotype, nitrogen regime, and genotype × nitrogen specified in the statistical model (Table S2). The appropriate linear model was first fitted, and the ANOVA table was generated to partition the total variability into sources such as treatments and error (Mendiburu, 2015). Every trait's broad-sense heritability (H²) was determined using the formula given by Falconer and Mackay (1996), where σ_G^2 is genotypic variance, σ_P^2 is phenotypic variance $\sigma_{G \times N}^2$ is genotype nitrogen interaction variance and r is number of replication. Broad-sense heritability was classified as low (<0.40), moderate (0.40–0.60), and high (>0.60). Based on these thresholds, H² values of 0.50–0.60 were considered moderate, whereas values greater than 0.60 were considered high.

$$H^2 = \frac{\sigma_G^2}{\sigma_{G \times N}^2 + \frac{\sigma_P^2}{r}}$$

Broad-sense heritability (H²) was estimated jointly across the two nitrogen regimes using variance components obtained from the factorial analysis (Table S2). Root system architecture index (RSAIN) was calculated by standardizing each root trait using a constant value of 20. For each trait, 20 was divided by its maximum observed value to obtain a scaling factor, which was then multiplied by all trait values. The scaled values were summed to obtain RSAIN for each treatment. This method was adopted from (Noor *et al.*, 2024; Sultana *et al.*, 2023).

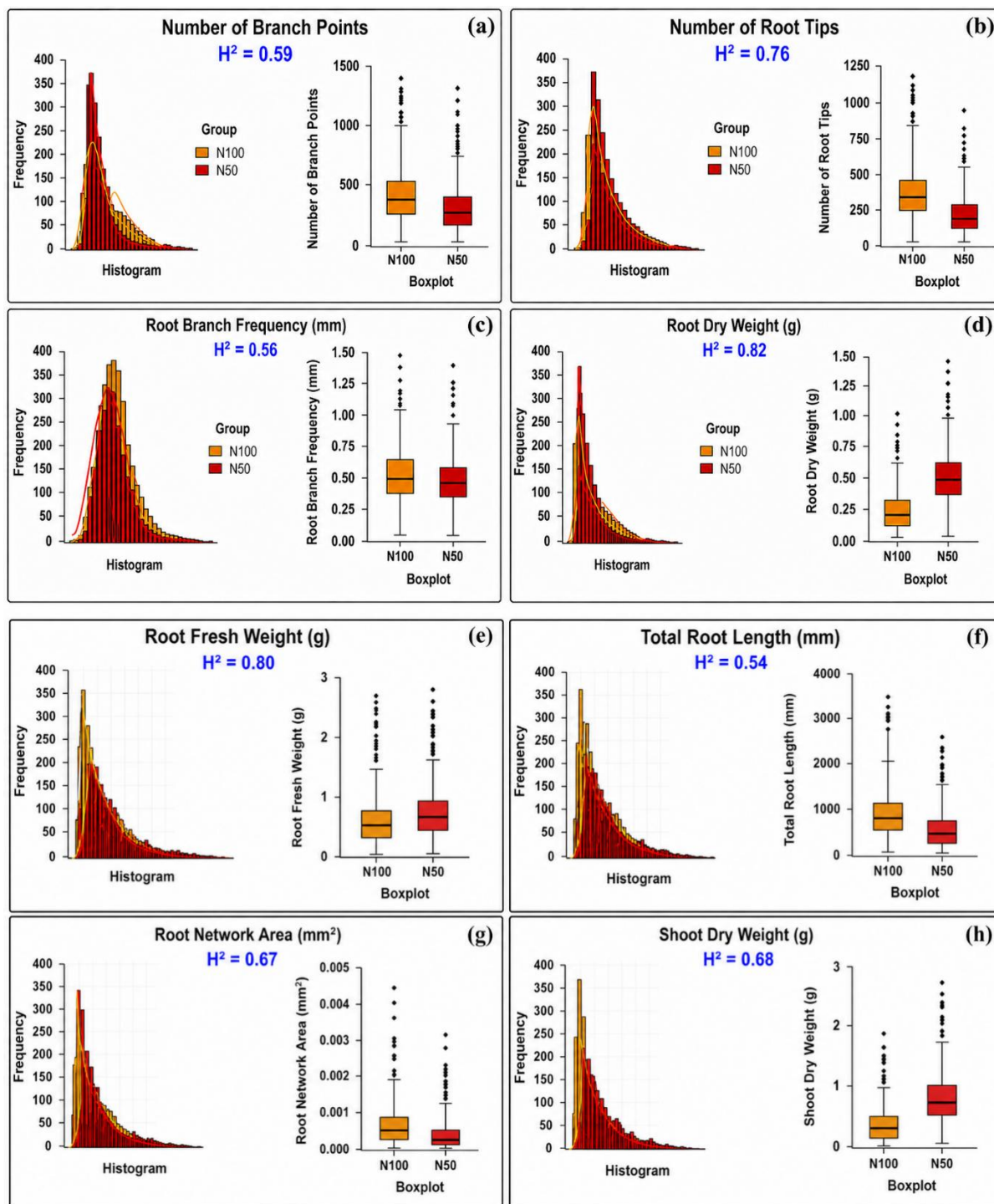
Principal Component Analysis (PCA) was conducted in R software with FactoMineR and factoextra packages. Multivariate variation among genotypes was assessed using standardized values of the measured traits, while the first two principal components were graphically displayed to illustrate the genotype clustering and associations among traits. Pearson's correlation analysis was performed in R using the cor () function, and the resulting correlation matrix was visualized using the corrplot package. Correlation coefficients (r) were interpreted based on their absolute values (r) as weak (0.00–0.39), moderate (0.40–0.59), and strong (0.60–1.00). Positive and negative r values indicated positive and negative associations, respectively. Correlations were considered statistically significant at P ≤ 0.05.

RESULTS

Variation in root architectural traits under contrasting nitrogen regimes: Number of branch points (NBP) ranged from 14–969 under N50 and 19–1493 under N100. NBP were higher in C-288 (969) while minimum NBP was shown by 13348-RFVORB under N50. Under N100 D01_SERI (1493) showed higher NBP and lowest branch points was showed by T3 (19) (Figure 2a). The number of root tips (NRT) exhibited significant variation in response to both N50 and N100 treatments. NRT ranged from 21–77 under N50 and 33–213 under N100, with histograms showing the highest frequency of genotypes (~35%) clustering in the 40–60 range for both regimes. Under N50 T9 (*T. aestivum*) demonstrated the maximum (77) number of root tips, while J19_PR-110 displayed the minimum (21) NRT. Under N100 condition MEHRAN-89 showed higher number of root tips (217) while T2 (*T. durum*) showed minimum NRT (33) (Figure 2b) Root Network Area (RNA) ranged from 5.63–1908 mm² under N50 and 34–1500 mm² under N100. RNA was vigorous of 122526BWP genotype in N50 and A24_1PVN in N100 condition. Lower RNA was showed by H10_IV-1 in N50 and WHEAR/KIRITATI in N100 condition (Figure 2e). Root branch frequency (RBF) ranged from 0.030–0.91 mm under N50 and 0.09–0.94 under N100. RBF was higher in H06_UOS-2 in N50 and in M07_FRET-1 under N100 condition. Lowest RBF was observed in C-273 and BT 2549 in N50 and N100, respectively (Figure 2f).

The range of root diameter (RD) in N50 was 0.16–9.27 mm and in N100 was 0.16–8.07 mm. RD was higher in PF 70402 under N50 N treatment and T10 (*T. aestivum*) showed weak RD. In N100 N treatment the genotype AZRITW1578 showed higher RD and the genotype PBW343 showed weaker RD (Figure 2g). The range of TRL was 21 to 1289 mm in N50 and 114–1345 mm in N100. TRL was longest (1289 mm) in J02_14151KACHU genotype under N50 N treatment and in N100 N treatment N23_HYPT(P-16) genotype showed longest (1345 mm) TRL. Reduced TRL was shown by H10_IV-1 (21 mm) in N50 and 2PFAU (114 mm) in N100 condition (Figure 2h). Range of total root surface area (TRSA) was 19–1300 under N50 and 130–1624 under N100. TRSA was higher in N50 condition of V-15291KIRIPATI genotype and in N100 of F06_KAUZ genotype. Weakest TRSA was showed by HYT-55-33KACHU genotype in N50 and

C06_T24-*T. aestivum* genotype in N100 (Figure 3 (Figure 2k). Root Volume (RV) ranged from 1.44-386 mm³ under N50 and 8-524 mm³ under N100. RV was vigorous of NR-429NARC genotype in N50 and H10_IV-1 in N100 condition. Lower RV was showed by N23_HYPT(P-16) in N50 and L08_V-14124KACHU-1in N100 condition (Figure 2l). Normal distribution was observed for all studied traits (Figure 2 a-l).



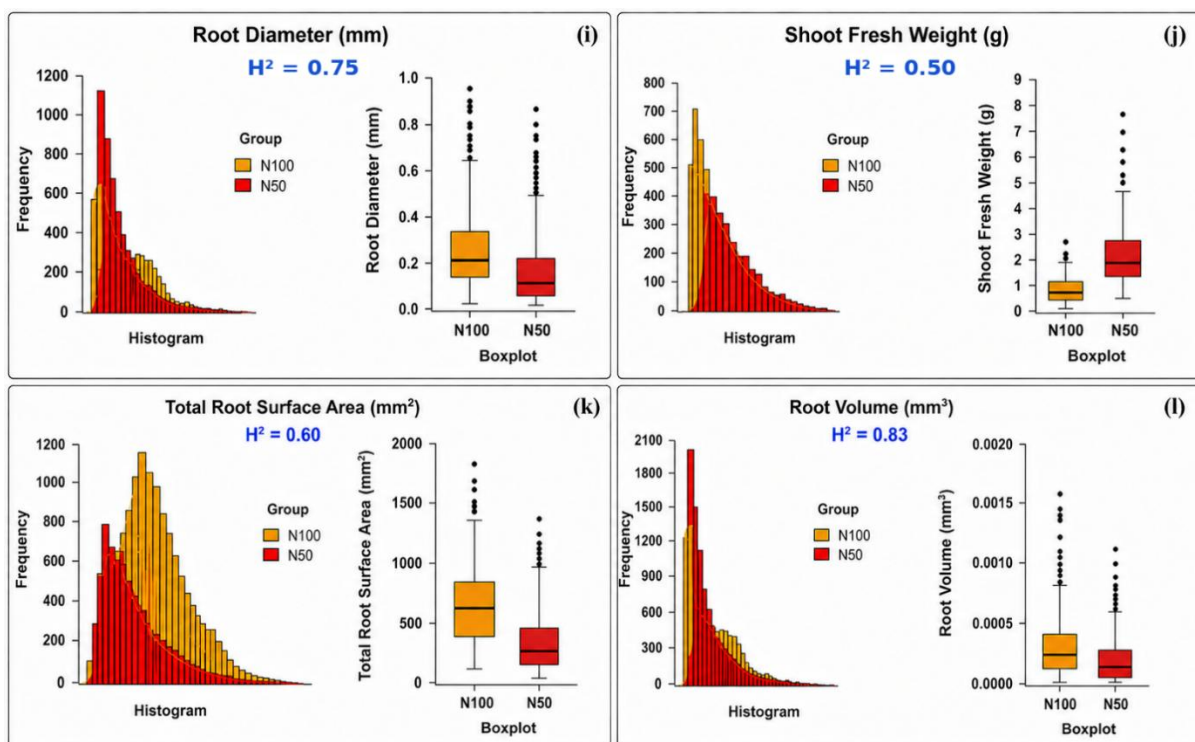


Figure 2: Variability for twelve root traits (a) number of branch points (NBP), (b) number of root tips (NRT), (c) root branch frequency (RBF), (d) root dry weight (RDW), (e) root fresh weight (RFW), (f) total root length (TRL), (g) root network area (RNA), (h) shoot dry weight (SDW), (i) root diameter (RD), (j) shoot fresh weight (SFW), (k) total root surface area (TRSA), (l) root volume (RV). Box-plot showing phenotypic distribution of root traits in N100 and N50 condition. Phenotypic distribution of the investigated traits. Red curves represents N50 and yellow curves representing N100.

Analysis of variance showed that nitrogen regime had significant effect on all the twelve traits ($P < 0.05$ or $P < 0.01$), the highest being for shoot dry weight (SDW: $F = 60882.62^{**}$) and root dry weight (RDW: $F = 20670.96^{**}$), and the lowest for root branching frequency (RBF: $F = 2.31^{*}$) and root network area (RNA: $F = 1.94^{*}$). The differences between the root and shoot architecture were highly significant ($P < 0.01$) among the various genotypes, which showed the genetic diversity of the root and shoot architecture. The only trait where the genotype $\times$ nitrogen interaction was non-significant was root diameter despite significant main effects of nitrogen regime ($F = 19.36^{**}$) and genotype ($F = 1.22^{**}$), that is, RD varied with nitrogen supply, and between genotypes, although there was little difference in the magnitude of the RD response to nitrogen between the two (Table S2).

The heritability (H^2) of the 12 traits evaluated ranged from 0.50 to 0.83, all with values above 0.50. Root volume (RV) had the highest H^2 (0.83), followed by root dry weight (RDW; 0.82) and root fresh weight (RFW; 0.80). Overall, moderate to high heritability was suggested for the traits evaluated and related to root architecture and biomass. High heritability does not, however, prove that the trait is likely to be easily selected under field conditions, but rather that the genetic differences between the plants, under the conditions of the experiment, are responsible for the majority of the variation in the plant's phenotype.

The representative genotypes (Figure 3) were selected based on quantitative variation in phenotypic measurement of the different root traits and confirmed using Principal Component Analysis (PCA). Genotypes with consistently higher or lower mean values on the various root traits were recognized and also were observed to be distinctly separated in the PCA based on their multivariate root-trait profiles (Figure 4-5). Therefore, the selected genotypes were not random, but were chosen to be the contrasting extremes of root phenotypic variation based on the combined evidence of the quantitative trait data and PCA.

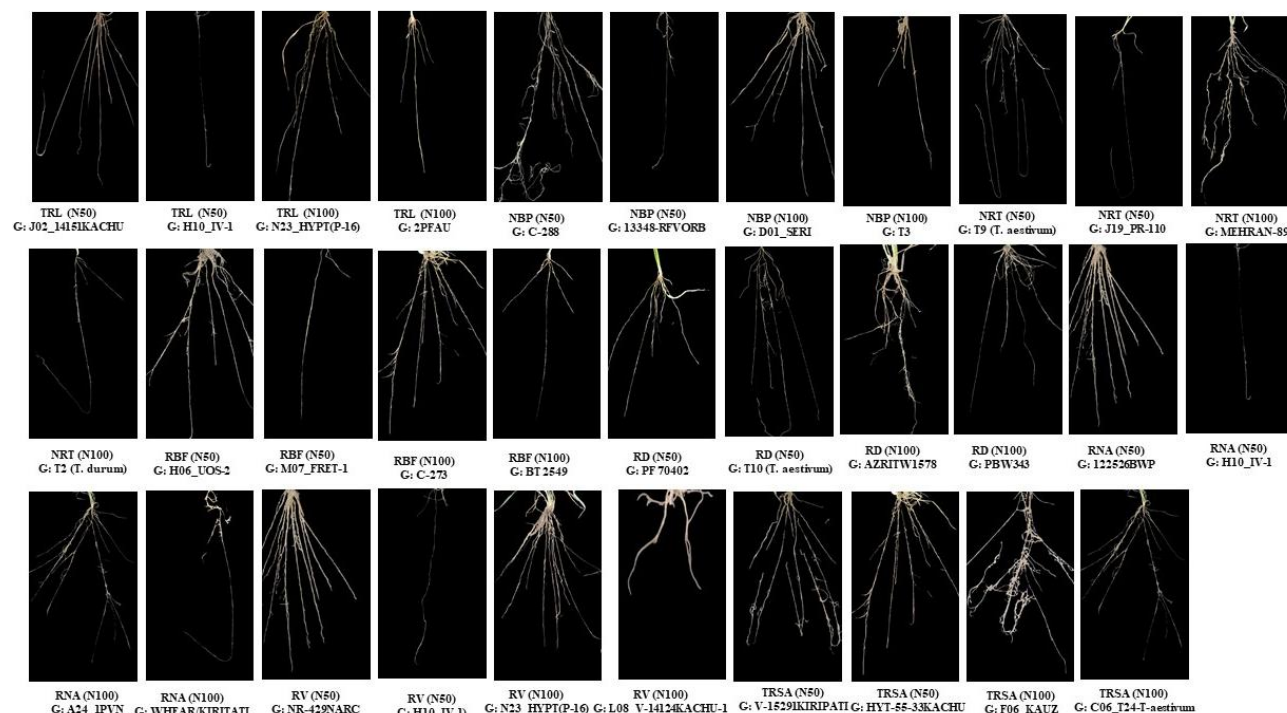


Figure 3: Comparison of root images of wheat genotypes grown till tillering stage under N100 (recommended dose 120 kg h⁻¹) and N50 (half of the recommended dose (60 kg h⁻¹) conditions illustrating roots. Name of the trait Root volume (RV), no. of root tips (NRT), root diameter (RD), total root surface area (TRSA), root network area (RNA), total root length (TRL), number of branch points (NBP), and genotypes under both treatment condition are shown under each of the image.

Principal Component Analysis of Root-System Architecture under N50 Nitrogen: The PCA results demonstrate considerable multivariate variation in root-system architecture among the 453 wheat genotypes. The relatively high contribution of PC1 (42.7%) indicates that the major source of phenotypic differentiation was associated with variation in root architectural characteristics. The separation of specific genotypes from the main population further demonstrates that the wheat germplasm contains substantial diversity in the way root systems are developed and organized.

A particularly important finding was that the genotypes separated in the PCA, including G86 (1PFAU), G290 (J02-14151KACHU), G302 (C-288), G5 (T9 (T. aestivum)), G154 (122526BWP), G75 (NR-429NARC), G58 (WATAN), G347 (M07_FRET-1), G340 (MEHRAN-89), G69 (CHIRYA-3), G405 (14C040), G432 (NR-522), G345 (K13_SA-42), and G451 (J11_Fakhr-e-Sarhad), showed higher values for NRT, NBP, TRL, RV, and TRSA. This combination of traits represents a biologically meaningful root phenotype rather than simply an increase in a single root characteristic. Higher NRT and NBP indicate greater root proliferation, while increased TRL reflects greater longitudinal root development. At the same time, higher RV and TRSA indicate a larger root system with greater root surface development. Together, these characteristics describe an extensive and vigorous root-system architecture (Figure 4).

The simultaneous increase in root number, length, volume, and surface area is particularly relevant under conditions where nutrient availability may restrict plant growth. A larger and more extensively distributed root system can potentially explore a greater volume of the growth medium and provide a larger interface between roots and the surrounding environment. Therefore, the identified genotypes may possess root architectural characteristics that favor greater resource exploration. However, this interpretation should be considered as a potential mechanism rather than direct evidence of improved N acquisition, because PCA and root traits alone cannot establish N efficiency.

Interestingly, the identified genotypes were not necessarily characterized by uniformly high values for all measured traits. Their distinct PCA position was primarily associated with the coordinated expression of NRT, NBP, TRL, RV, and TRSA rather than simply increased root biomass or root diameter. This finding indicates that an effective root phenotype may be achieved through changes in root proliferation, elongation, and spatial development, rather than through increasing all components of root biomass simultaneously. Such trait combinations may reflect different strategies of root-system development among wheat genotypes.

The identification of these contrasting genotypes is therefore important for genetic and physiological studies of root architecture. Genotypes such as G86, G290, G302, G75, G405, G432, and G345, which showed particularly distinct positions in the PCA together with favorable values for multiple root traits, represent promising materials for further investigation. Their performance under contrasting N levels could help determine whether these extensive root architectures are maintained under N limitation or are specifically induced by N availability. Further evaluation using N uptake, biomass accumulation, yield-related traits, or other physiological measurements would be required to establish whether the observed root architecture contributes directly to improved N acquisition or nitrogen-use performance.

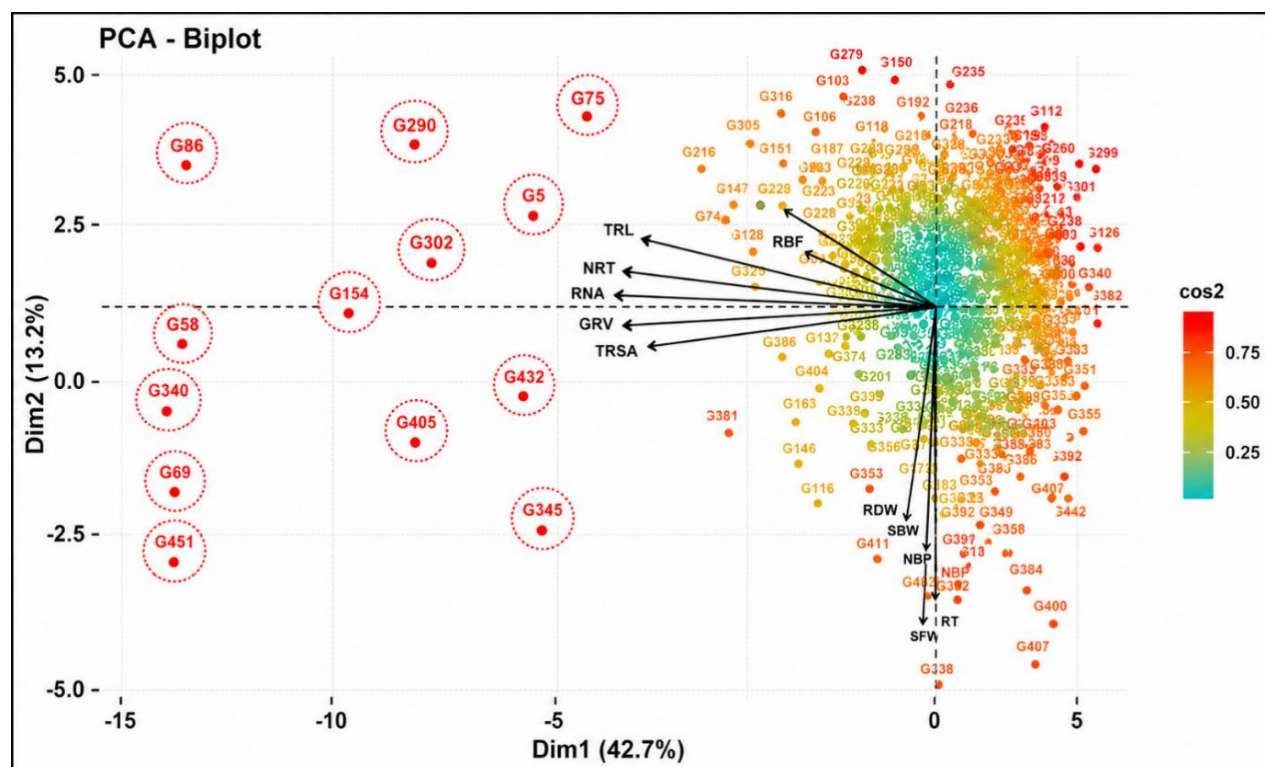


Figure 4. Principal component analysis (PCA) of root-system architectural traits among 453 wheat genotypes under N50 N conditions. The first two principal components (PC1 and PC2) explained 55.8% of the total variation, accounting for 42.7% and 13.2%, respectively. Each point represents an individual wheat genotype, while arrows indicate the contribution and direction of individual root traits to genotype separation. Genotypes highlighted by red circles represent those positioned far from the origin, indicating more distinctive root architectural trait under N50 conditions whereas the remaining genotypes clustered near the origin exhibited relatively average root-trait combinations.

Principal Component Analysis of Root-System Architecture under N100 Nitrogen: PCA of the 453 wheat genotypes under N100 N revealed substantial variation in root-system architecture. PC1 and PC2 explained 46.1% and 13.8% of the total variation, respectively, accounting for 59.9% of the overall variation. Most genotypes were distributed around the center of the PCA plot, whereas several genotypes, including G217 (HYT-20-19HAHN), G253 (V-17189NADICMSS06B00734T), G308 (Marvi-2000), G98 (KINGBIRD#2), G435 (K15_15FJ03), G241 (V-16164PRL), G252 (V-17183SERI), G267 (NING MAI), G186 (ATTILA*2), G447 (Bakhtawar-92), and G6 (BORLAUG-16) were positioned away from the main population, indicating distinct root-trait combinations. PC1 was mainly associated with RV, TRSA, and RNA, while NBP contributed strongly toward the positive PC1 and negative PC2 direction. Genotypes located toward the positive PC1 region therefore showed contrasting root characteristics associated with greater root number, root surface area, and root volume. These results demonstrate considerable phenotypic diversity among the wheat genotypes and identify several contrasting genotypes for further evaluation of root-system architecture under N100 N (Figure 5).

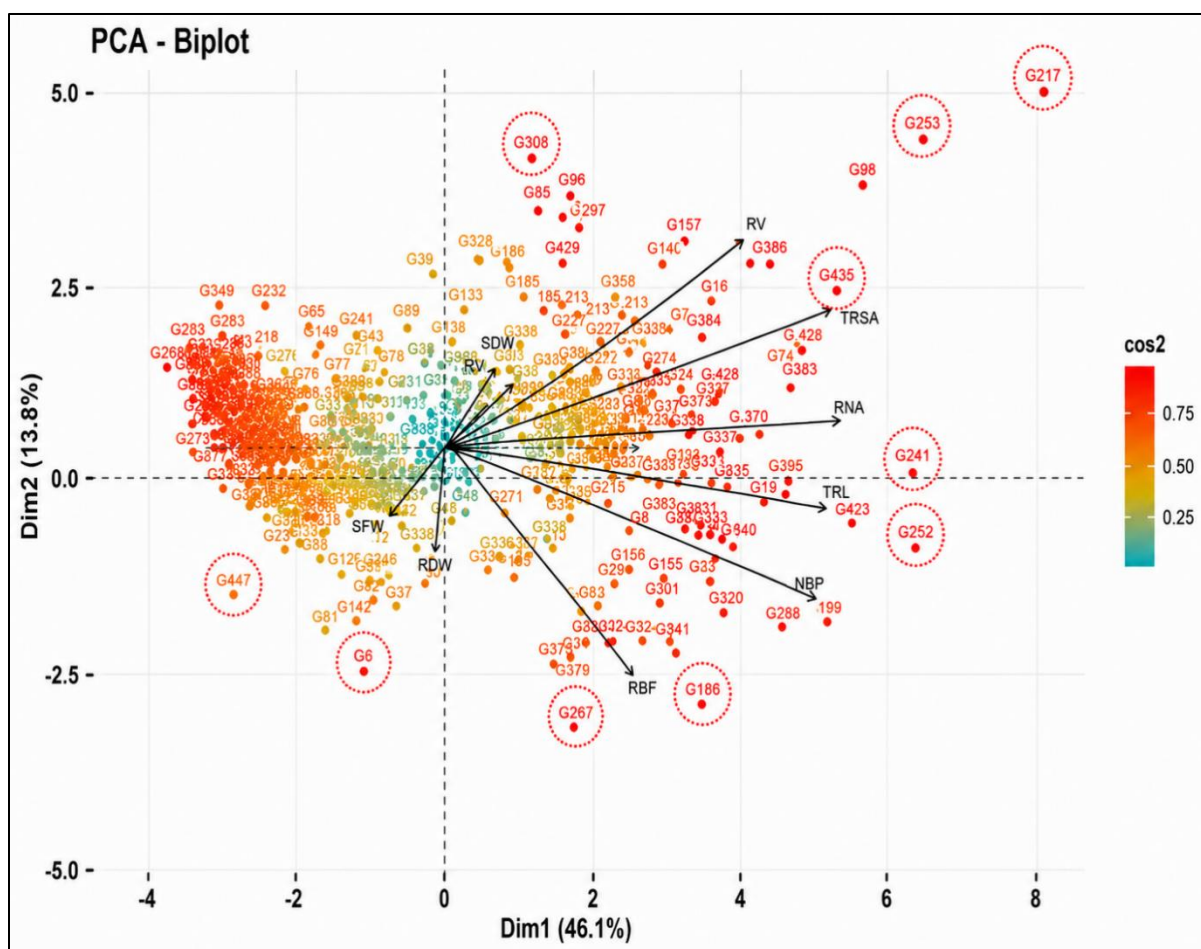


Figure 5. Principal component analysis (PCA) of root-system architecture among 453 wheat genotypes under N100 N conditions. The PCA plot shows the distribution of genotypes based on their multivariate root traits. PC1 and PC2 explained 46.1% and 13.8% of the total variation, respectively, accounting for 59.9% of the overall variation. Arrows indicate the contribution and direction of individual root traits, while genotypes highlighted by red circles represent those positioned far from the origin, indicating more distinctive root architectural trait under N100 conditions, whereas the remaining genotypes clustered near the origin and exhibited relatively average root-trait combinations.

Correlation analysis: The correlation analysis of biomass and root architectural traits differed significantly between N50 (Figure 6a) and N100 (Figure 6b) conditions. Shoot biomass traits (shoot fresh weight and shoot dry weight) showed weak to moderate positive correlations with root variables under N50. Plants prioritize root exploration and nutrient-foraging above shoot growth, resulting in more extensive or deeper root systems without increases in shoot biomass in stress condition. On the other hand, higher positive correlations between shoot biomass and root traits were observed under N100 conditions, showing enhanced functional integration between shoot production and root growth when N is not limiting. This indicates that root architecture characteristics play more favorably into biomass development under recommended N supply. The interactions were different in the various N regimes for total root length, a critical for soil exploration and nutrient uptake. The total root length exhibited moderate positive correlations with root surface area and root number under N50, indicating a synchronized but limited root system response to N deficiency. Conversely, N100 exhibited positive trends with these traits in the case of total root length, suggesting stronger integrated and efficient organization of the root system.

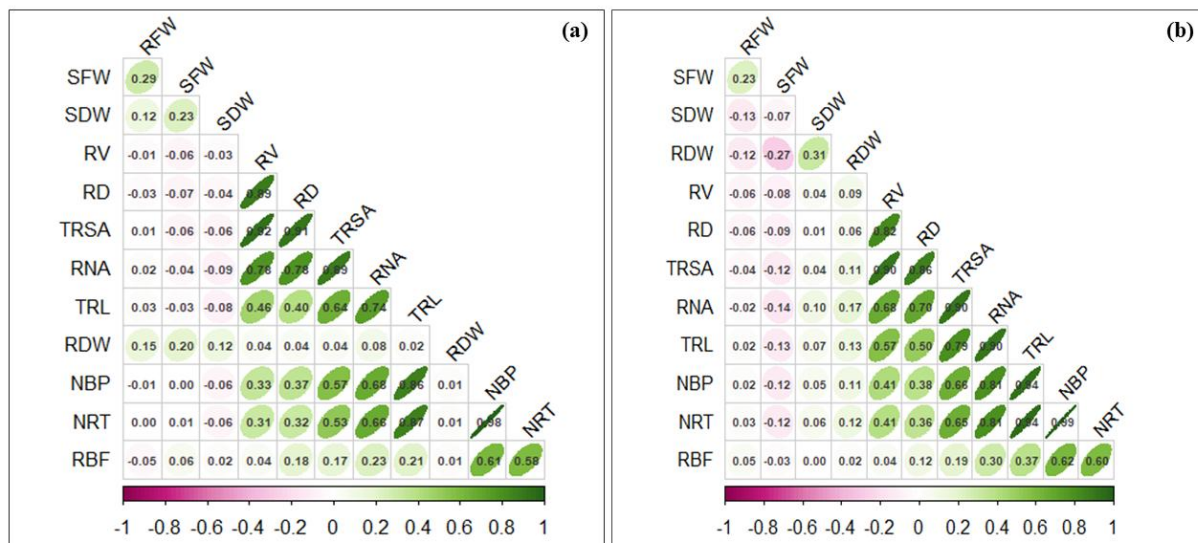


Figure 6: Pearson correlation coefficient for twelve root traits at tillering stage evaluated in PVC pipes under two N levels N50 and N100. **(a)** Root traits under N50 condition **(b)** Root traits under N100 condition. The strength of correlation are indicated by color gradients, ranging from negative (pink) to positive (green) correlations, with values displayed within each cell. Root volume (RV), no. of root tips (NRT), root diameter (RD), total root surface area (TRSA), root network area (RNA), total root length (TRL), number of branch points (NBP), root network area (RNA), shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW).

Genotypic Ranking for RSAI and TPB under N50: To assess genotypic response under the N50 treatment, the genotypes were classified into three groups based on RSAI_N50 and TPB_N50: the upper 20% representing the highest values, the lowest 20% representing the poorest values, and the remaining 60% as intermediate performers. Under N50 conditions, the same set of genotypes, WATAN, CHIRYA-3, 1PFAU, 4V-04179, 6V-04179, VOROBAY, 13BT034V89A038, HYT 60-7KACHU/2, HYT-55-40SAUAL, NARCNR-529, T16 (*T. aestivum*), C-271, SITE, NELOKI, I03_MEHRAN-89, K13_SA-42, M07_FRET-1, O11_CHIRYA-I, M16_V-15306MUTUS_2, B19_SERI, N01_PR-106, N11_HYPT(P-403), F22_14C040, I19_NR-522, E16_C-591, J11_Fakhr-e-Sarhad and L15_Durum-97, consistently fell within the upper 20% group for both RSAI and TPB, indicating superior RSA and tillering performance under limited N supply. In contrast, T20 (*T. aestivum*), INQALAB91*2, NRL-1130, 122526BWP, Chakwal-50, V-16164PRL, FRET2/WBLL1, N02_15C42, V-12266ATTILA, 13348-RFVORB, SERI.1B*2, HUW 234, SAAR, RARI16-1154, BORLAUG-16, INQ-91*2/KHVAKI, SAUAL/MUTUS*2, EXCALIBUR, HYT-30-2WAXWING and CS/TH. SC were consistently grouped in the lowest 20% for both traits, reflecting weaker performance in root architecture and biomass under the same N regime. From these results, it was found that the highest ranking genotypes based on their N50 had better ability to maintain their RSAI and TPB despite being limited in their N availability, an important factor for assessing N efficiency. Their consistent ranking in the upper 20 percent for RSAI and TPB may show a good adaptation to low-N, thereby allowing them to acquire more N as opposed to the poor performers. Those genotypes that were placed consistently in the lowest 20 percent performed poorly in N50. Overall, the contrasting performance classes highlight substantial genetic variation for low-N adaptation and identify the upper-performing genotypes as promising candidates for breeding programs targeting nitrogen-use efficiency.

DISCUSSION

The availability of N has a significant impact on root growth and development such as growth, branching, surface area, angle (Awika *et al.*, 2021) and RSA is of central importance in N stress tolerance, low N supply can stimulate deeper, more extensive root system to improve nutrient acquisition as demonstrated in rice (*Oryza sativa* L.) (Liu *et al.*, 2023). This study took a composite Root System Architecture Index (RSAI) to dissect genetic variation for N efficiency in a diverse wheat panel, identifying the importance of root traits in capturing N resources under suboptimal N supply (Sinha *et al.*, 2020).

The moderate to high heritability values of the RSA traits (range 0.50 to 0.83) found here, similar to the values reported for root angle ($H^2=0.73$) and root number ($H^2=0.67$) in wheat (Canè *et al.*, 2014), meant that the genetic control of these traits is relatively platform-independent and that PVC-pipe-based screening can be a reliable early screening tool. The high heritability of RV (0.83) is similar to other root volume and biomass related traits in maize (*Zea mays* L.) (0.87) (Muhammad and Qayyum, 2013), rice (0.91) (Pariyar *et al.*, 2021), and sorghum (*Sorghum bicolor* L.) (0.81) (Kebede *et al.*, 2025), which further reinforces the use of RV as a low-risk, early generation selection target. Shoot fresh weight is more environment sensitive and less reliable as a stand-alone selection criteria as it has heritability close to 0.50.

The high correlations among RSA traits under both N conditions suggest coordinated trait development as previously reported in other cereals (Lynch, 2013) and with N-mediated regulation, which is the case of nitrate sensing through NRT1.1 that regulates auxin accumulation in the pericycle cells and thus lateral root initiation under low N (Jia *et al.*, 2021). Negatively correlated root fresh weight with RV, RD, NBP and RBF for N50, reflects the adaptation of reorganizing trait coordination from nutrient stress, as evidenced by least metabolic cost of nutrient exploration in the soil under stress (Hermans *et al.*, 2006; Lopez *et al.*, 2023; Duque and Villodon, 2019). This means, that if selection is based on RSA, it should be based on the N regime to be applied for the desired production.

The interaction between genotype and N was significant for most important traits indicating the high genotypic variation in N responsiveness, particularly for biomass related traits such as RDW and SDW ($F = 134.11$ and 92.54 , respectively). This indicates that the ability to utilize extra nitrogen for root and shoot production varies not just in growth rate, but also in the ability to utilize the extra nitrogen, a difference that is key to selection of N-efficient genotypes for breeding. The lack of significant genotype * nitrogen interaction for root diameter indicates that this trait may be less plastic when responding to variation in nitrogen levels than the other architectural traits measured and that variation may be more a function of the nitrogen regime and genotype than of the genotype-specific plasticity. This corresponds to the fact that the growth of root diameter is less canalized, presumably because it is more influenced by the anatomical structure of the root (cortical cell number and size of the stelar tissue) which are fixed in the early developmental stages and are less affected by nutrient availability than elongation, branching and biomass production. These results strongly suggest that the traits which showed genotype x environment interactions (root volume, root surface area, root dry weight) should be given greater importance for selection based on nitrogen responsive root architecture while traits like root diameter might better be used as genotype-defining traits, not influenced by nitrogen supply. The difference in root traits observed among wheat genotypes may be explained by genetic differences in root developmental strategies and their plastic response to nitrogen availability. Wheat has high level of genetic diversity in root length, branching, diameter, and root biomass and these traits are highly responsive to N supply as well (Freschet *et al.*, 2021). This study has confirmed moderate to high heritability values (0.50 to 0.83) which further reflect a high genetic contribution to variation in RSA. Some of the root traits were related to morphological performance, with weak to moderate correlations between root parameters and shoot biomass when grown under N50 conditions and more significant ones under N100 conditions. In wheat, similar relationships between the root system architecture and agronomic performance, biomass, and N uptake were observed (Cormier *et al.*, 2016).

The analysis under both N50 and N100 indicated that a significant proportion of the phenotypic variation could be explained by a few composite axes of variation ($PC1 = 42.7\%$ under N50; $PC1+PC2 = 59.9\%$ under N100). It confirms the development of the root system being not regulated independently from one another, but is under the control of synchronized developmental programs (Wang *et al.*, 2021). Genotypes that clustered together along PC1 under N50, such as WATAN, CHIRYA-3, 1PFAU, MEHRAN-89, M07_FRET-1, and Fakhr-e-Sarhad, exhibited elevated NRT, NBP, TRL, RV, and TRSA, with several, The PCA further revealed differential responses of wheat genotypes to N availability. Under N50, genotypes such as 1PAFU (G86), J02-14151KACHU (G290), C-288 (G302), NR-429NARC (G216), WATAN (G58), M07_FRET-1 (G347), MEHRAN-89 (G340), CHIRYA-3 (G69), NR-522 (G432), K13_SA-42 (G345), and Fakhr-e-Sarhad (G451) were distinctly separated from the main population and also in the top 20% for RSAI and total biomass under N50 showed favorable combinations of NRT, NBP, TRL, RV, and TRSA, suggesting that the genotypes were not only superior in N efficiency under N50, but also in the ability to explore soil efficiently under N50, as proposed by (Zeng *et al.*, 2026). In contrast, under N100, several different genotypes, including HYT-20-19HAHN (G217), V-17189NADICMSS06B00734T (G253), Marvi-2000 (G308), KINGBIRD#2 (G98), K15_15FJ03 (G435), V-16164PRL (G241), V-17183SERI (G252), NING MAI (G267), ATTLA*2 (G186), Bakhtawar-92 (G447), and BORLAUG-16 (G6), were positioned farther from the origin, indicating distinct root-trait combinations under adequate N. The majority of genotypes were grouped around the centre suggesting relatively average root-trait combinations, while the different root-trait positions with different N periods suggest genotype-specific N responsiveness and root plasticity. Genotypes that are more distinct under N50 might have higher abilities to sustain root growth in N limitation, whereas those that are more different under N100 might be more sensitive to enhanced N availability. The significant genotype × nitrogen interaction for root growth and branching traits indicated differential responses of genotypes to nitrogen availability, consistent with phenotypic plasticity in root development (Schmidt *et al.*, 2022). The PCA therefore forms different root developmental

strategies amongst the different genotypes and not necessarily a single universal best root phenotype. This favorable phenotype was not only a coordinated growth of the root system but also showed surface expansion and elongation, which were previously reported as separate developmental strategies and not simple variations in root size (Atkinson *et al.*, 2022), indicating that breeding for N acquisition should consider the root system's exploration efficiency rather than indiscriminately increasing root biomass (Wang *et al.*, 2026). The limited overlap in the genotype expression of N50 and N100 indicates that part of the architectural expression of roots is N induced and not constitutive (Zaman *et al.*, 2024). Although PCA indicates some potential genotypes exhibiting response to N nutrition, however it alone cannot determine nitrogen-use efficiency and suggests further characterization, such as direct measurement of N uptake and yield under field stress (Danakumara *et al.*, 2021).

Phenotyping pipeline was developed and combined the plant root growth with smartphone-based imaging for RhizoVision Explorer (Chanumolu *et al.*, 2024) which allowed extraction of multiple root traits from a single image of a plant over 453 genotypes with significant advantages compared to manual measurement. RhizoVision Explorer has also validated with WinRhizo™ software and has been demonstrated to give more accurate estimates of root volume in comparative tests, where WinRhizo™ significantly underestimated the volume (Seethepalli *et al.*, 2021); this is due to the availability of a smartphone camera, which makes it more accessible for large germplasm panels without a dedicated scanner. However, the 0.3 mm diameter threshold for distinguishing axial from lateral roots was applied and may have missed the smallest root hairs, a limitation shared by planar imaging methods (Bereswill, 2025), leading to a focus on macro-architectural features of the roots. Rooting in a limited volume of PVC is also not completely representative of the same conditions in the field, which means that absolute trait values may not be necessarily applicable to the field, but relative genotype rankings should be more accurate. With these limitations, this pipeline is a viable compromise in resolving and throughput for the scale of genetic screening.

Genotypes exhibiting higher RSAI and total biomass were identified as superior for early root and shoot growth under N50. The genotypic ranking based on RSAI and total biomass of plants showed that WATAN, CHIRYA-3, 1PFAU, M07_FRET-1, MEHRAN-89, Fakhr-e-Sarhad were consistently identified as top performers under N50. The correlation between the architecture-based index and whole-plant biomass adds to their potential as parental lines for breeding for nitrogen-use efficiency, because the architecture-based advantage in the index seems to translate into a real growth advantage. Given the high heritability and high correlation with other traits, RV may be a good efficient and low cost pre-screening tool for large germplasm collections. This panel and the quantitative RSA dataset generated here will allow for genome wide association studies to identify candidate genes for N-responsive root plasticity which can be used to speed the breeding process using marker-assisted or genomic selection beyond phenotypic selection. Genotypes with a distinct origin were clearly distinguished under each regime, with 57% of genotypes with semi-arid origin under the N50 regime, and 29% of CIMMYT/Mexico-origin under the N50 regime. This indicates a selection of germplasm with distinctively high rooting ability and root growth under nitrogen limitation, which is of breeding importance for low N conditions.

Through this research, the image-based RSA phenotyping pipeline developed here is shown to be a rapid, low cost tool for screening large germplasm collections for root traits associated with N-deficiency tolerance in wheat, and the genotypes WATAN, CHIRYA-3 and Fakhr-e-Sarhad were identified as promising candidates for N-deficiency tolerance under low N conditions. Future research should confirm best performing genotypes in multi-environment field tests, use this data to combine with GWAS for candidate genes to use in marker-assisted selection for N-use efficiency breeding in wheat, and add this imaging pipeline to an existing, higher-resolution imaging technique like X-ray computed tomography to increase confidence in image-based investigation of NUE breeding in wheat.

Conclusion: This study shows that under different nitrogen regimes (N50 and N100), root system architecture (RSA) in wheat is greatly influenced by N availability, leading to significant diversity in root morphological and biomass-related traits across genotypes. The strong genetic influence is evidenced by the moderate to high heritability values observed for the traits related to RSA, indicating their potential application in breeding. It was possible to discriminate genotypes with different RSA behavior in low nitrogen conditions through principal component analysis, while coordination among root traits was revealed by correlation and multivariate analyses, implying common developmental regulation of roots. In general, the study reveals some promising genotypes (J02-14151KACHU, C-288, T9 (*T. aestivum*), 122526BWP, NR-429NARC, 14C040, NR-522, G345 K13_SA-42, WATAN, CHIRYA-3, 1PFAU, M07_FRET-1, MEHRAN-89, and Fakhr-e-Sarhad) with resilient and flexible root systems under low nitrogen conditions and provides valuable pre-breeding information for the breeding of nitrogen efficient cultivars of wheat. Moreover, the diverse germplasm and phenotypic variation evaluated in the present study provide a useful foundation for future genomic research, including GWAS, to further investigate the genetic basis of wheat root system architecture (RSA) and nitrogen-use efficiency.

Conflict of interest: The authors declare no conflict of interest.

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Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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