

Investigation of Root Morphological and Architectural Traits in Adzuki Bean (*Vigna angularis*) Cultivars Using Imagery Data

Pooja Tripathi¹ and Yoonha Kim^{2,†}

ABSTRACT Roots play important roles in water and nutrient uptake and in response to various environmental stresses. Investigating diversification of cultivars through root phenotyping is important for crop improvement in adzuki beans. Therefore, we analyzed the morphological and architectural root traits of 22 adzuki bean cultivars using 2-dimensional (2D) root imaging. Plants were grown in plastic tubes [6 cm (diameter) × 40 cm (height)] in a greenhouse from July 25th to August 28th. When the plants reached the 2nd or 3rd trifoliolate leaf stage, the roots were removed and washed with tap water to remove soil particles. Clean root samples were scanned, and the scanned images were analyzed using the WinRHIZO Pro software. The cultivars were analyzed based on six root phenotypes [total root length (TRL), surface area (SA), average diameter (AD), and number of tips (NT) were included as root morphological traits (RMT); and link average length (LAL) and link average diameter (LAD) were included as root architectural traits (RAT)]. According to the analysis of variance (ANOVA), a significant difference was observed between the cultivars for all root morphological traits. Distribution analysis demonstrated that all root traits except LAL followed a normally distributed curve. In the correlation test, the most important morphological trait, TRL, showed a strong positive correlation with SA ($r = 0.97^{***}$) and NT ($r = 0.94^{***}$). In comparison, between RMT and RAT, TRL showed a significantly negative correlation with LAL ($r = -0.50^{***}$); however, TRL did not show a correlation with LAD. Based on RMT and RAT, we identified the cultivars that ranked 5% from the top and bottom. In particular, the cultivar “IT 236657” showed the highest TRL, SA, and NT, while the cultivar “IT 236169” showed the lowest values for TRL, SA, and NT. In addition, the coefficient of variance for the six tested root traits ranged from (14.26-40%) which suggested statistical variability in root phenotypes among the 22 adzuki bean varieties. Thus, this study will help to select target root traits for the adzuki bean breeding program in the future, generating climate-resilient adzuki beans, especially for drought stress, and may be useful for developing biotic and abiotic stress-tolerant cultivars based on better root trait attributes.

Keywords : imagery analysis, root architectural trait, root morphological traits, total root length

Adzuki bean (*Vigna angularis*) contains enriched carbohydrates and proteins, so it regards as an important annual legume crop that is widely cultivated in China, Japan, Korea and India (Hori *et al.*, 2006). It is widely used as a functional food due to its various health benefits (Han *et al.*, 2015). It has been studied for its numerous health benefits such as detoxification, antioxidant and anti-obesity properties (Amarowicz *et al.*, 2008; Kitano-Okada *et al.*, 2012).

Due to the consequences of climate change, there have been alterations in the rainfall patterns in Korea, which has increased

the occurrence of drought from spring to summer (Park *et al.*, 2012). Such climatic condition has affected the planting date of upland crops such as soybean, sesame and adzuki bean (Chun *et al.*, 2016). Cultivated adzuki bean is subjected to severe drought periods, and it has been reported that they are sensitive to soil moisture content during early vegetative growth stages (Chun *et al.*, 2021). Adzuki bean is one of the important legume crops in Korea, so it is necessary to understand its morphology and adaptation to water stress (Chun *et al.*, 2021). It has been reported that drought stress in adzuki beans can inhibit root and

¹Master Degree Student, Department of Applied Bioscience, Kyungpook National University, Daegu 41566, Korea

²Assistant Professor, Department of Applied Bioscience, Kyungpook National University, Daegu 41566, Korea

[†]Corresponding author: Yoonha Kim; (Phone) +82-53-950-5710; (E-mail) kyh1229@knu.ac.kr

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shoot growth and physiological and biochemical characteristics such as the potential activity of photosystem II, photochemical efficiency in different adzuki bean cultivars in the seedling stage (Luo *et al.*, 2014).

In general, root morphological trait (RMT) and root architectural trait (RAT) involves in the productivity of most crops, because these traits are not only important in water uptake but also participate in inorganic nutrient uptake from soil (Manschadi and Christopher, 2006). Therefore, understanding RMT and RAT in various crops are necessarily required for improving water and nutrient uptake as well as enhancing the efficiency of water and nutrients (Prince *et al.*, 2017; Kim *et al.*, 2020; Tripathi *et al.*, 2021b). Among the various traits associated with mitigation of drought stress in crops, RMT and RAT are considered to be the most vital traits that enables plants to uptake water from deeper soil layers (Vadez *et al.*, 2008). Despite the fact that RMT and RAT, both are very important traits for mitigating drought stress through water and nutrient uptake, however, few studies on RMT and RAT analysis have been conducted in cultivated soybean (*Glycine max* L.) and wild soybean (*Glycine soja* Siebold & Zucc.) (Kim *et al.*, 2021a; Kim *et al.*, 2021b; Tripathi *et al.*, 2021a). On the other hand, there was no related research on adzuki beans for detailed RMT and RAT evaluation reported. Only root morphology and spatial distribution depending on different moisture contents in soil were conducted in an adzuki bean cultivar, which was named “Arari” (Chun *et al.*, 2021). It is well known that perhaps the root is the very first plant part to respond to stress under various soil environmental conditions

such as drought, salinity and flooding stress (Kunert *et al.*, 2016). As a result, recently, the improvement of RMT and RAT has been identified as target traits in the breeding program to enhance the resistance to unfavorable soil and weather conditions (Kim *et al.*, 2020). For all these reasons, current breeding efforts in leguminous crops are centered on identifying superior root traits genotypes and then using those genotypes as breeding material for crop improvement (Vadez *et al.*, 2008; Wang *et al.*, 2019). Previously our research group assessed RMT and RAT in cultivated soybean (Kim *et al.*, 2021a; Tripathi *et al.*, 2021a), and wild soybean (Kim *et al.*, 2021b) using imagery data to supply soybean breeding programs for the improvement of root morphological traits. Our research group used this information for developing breeding populations and the identification of QTLs responsible for root traits in cultivated soybean. Despite the fact that adzuki beans are a significant upland crop in South Korea, research into RMT and RAT evaluation in adzuki beans has received little attention. Thus, in this experiment, we evaluated the RMT and RAT among the 22 adzuki bean accessions using well established root imaging analysis technology to uncover new new information about RMT and RAT in adzuki beans.

MATERIALS AND METHODS

Plant materials and growth conditions

Adzuki bean 22 cultivars which were donated from NAC to the Rural Development Administration in South Korea were used in the experiment (Supplementary Table 1). Among 22

Table 1. Analysis of variance of six root morphological traits.

Parameters	Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRL	cultivar	21	1803240	85868.59	3.62	<.0001
	rep	4	143305.8	35826.45	1.51	0.2089
SA	cultivar	21	17931.03	853.8584	3.84	<.0001
	rep	4	1754.267	438.5667	1.97	0.1088
AD	cultivar	21	0.095002	0.004524	5.11	<.0001
	rep	4	0.005913	0.001478	1.67	0.167
NT	cultivar	21	13484540	642120.9	2.69	0.0011
	rep	4	777859.2	194464.8	0.82	0.5194
LAL	cultivar	21	0.013014	0.00062	5.34	<.0001
	rep	4	0.001569	0.000392	3.38	0.0139
LAD	cultivar	21	0.241331	0.011492	6.32	<.0001
	rep	4	0.023153	0.005788	3.19	0.0185

Note: TRL, total root length; SA, surface area; AD, average diameter; NT, number of tips; LAL, link average length; LAD, link average diameter; and rep, replication.

accessions, 13 accessions were collected in South Korea (KOR) and rest were collected in the Asia region [3 accessions from Afghanistan (UNK), 5 accessions from Japan (JPN), 1 accession from China (CHN)] (Fig. 1). All seeds were subjected to a treatment process (seed scarification) to increase the germination rate by slightly scraping the ends of the seeds using a nail clipper before planting. The experiment was conducted in a greenhouse which was in Kyungpook National University Research Center, Daegu. Two scarified seeds were sown in polyvinyl chloride (PVC) pipes [6 cm (diameter) × 40 cm (height)] containing horticultural soil used for seed germination and plant proper growth (Tobirang, Baekkwang Fertility, South Korea). To use PVC pipe as a pot, one side of the end was covered with

non-woven fabric, then the soil was poured into the other side of the PVC pot. The experiment was carried out approximately for a month (from July 25th, 2021 to August 28th, 2021) and the average temperature and humidity of a day were around 32°C ± 3°C and 67% ± 5%, respectively. All PVC pots were randomly placed at a greenhouse and an experiment was conducted with 5 replications ($n=1$).

Imagery data collection in root

When the plants reached the 2nd or 3rd trifoliate leaves stage, the roots were harvested. To collect root samples, The sieve is prepared, then PVC pots pour into a sieve. We carefully took out plant samples from dumped soil, then removed the shooting part with scissors. After that, root samples were washed with clean tap water to remove soil particles from the root. Washed-root samples were immediately kept in a zip-lock bag containing 10 ml of clean tap water to prevent root drying. During samples collection, a zip-lock bag was kept in an icebox, then all root samples were kept in a refrigerator until root analysis. Root image was captured at scanner (Expression 12000XL, Epson, Japan) coupled with transparent tray (length: 30 cm; width: 20 cm). We carefully poured clean root samples with tap water on the transparent tray, then again removed tiny soil particles or root debris using tweezers to collect a clean image. The acquired image was analyzed via root image analysis software (WinRHIZO Pro Software, Regent Instruments Inc., Canada) (Fig. 2). WinRHIZO Pro classifies roots into various diameter classes. In WinRHIZO software, various RMT and RAT are possible to

Cultivated Variety Origin

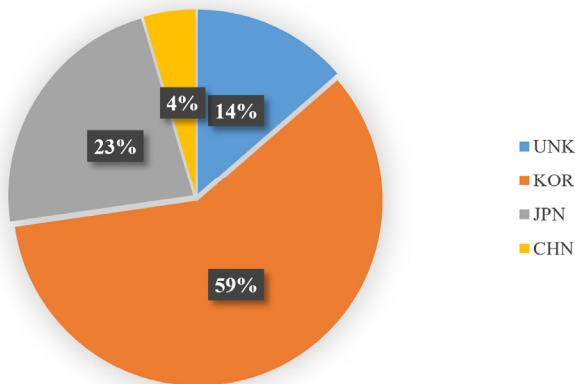


Fig. 1. Variation in seed collection regions among 22 adzuki bean cultivars selected for this study.

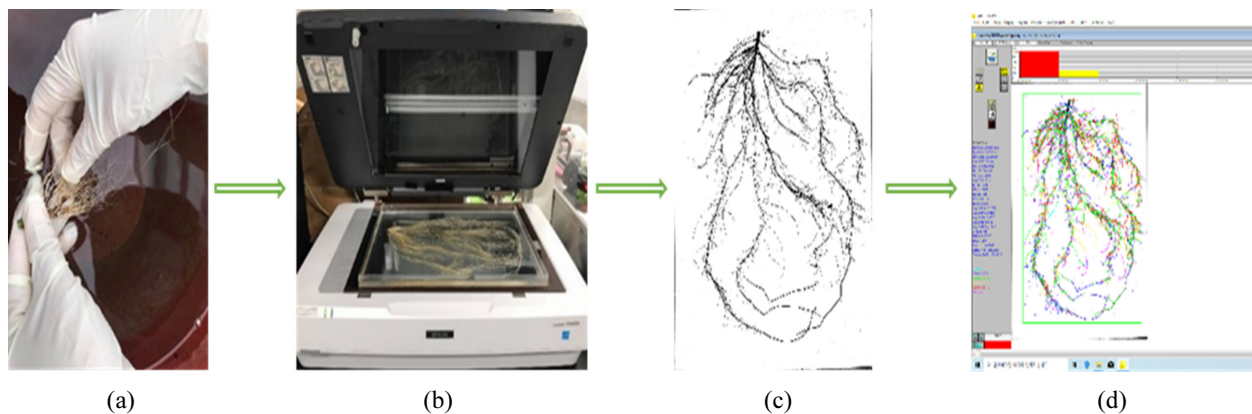


Fig. 2. Scheme of root samples analysis. In this figure, (a) indicated root washing with tap water; (b) showed the process of root sample scanning where clean root samples were placed onto a transparent acrylic tray containing clean tap water; (c) is an image after the scan; and (d) presents the analyzed root morphological and architectural traits by WinRHIZO pro software.

analyze. TRL is the total length of the whole root system that is included in all the diameter classes. Similarly, AD is the average of diameter classes of all root segments. WinRHIZO also has a link analysis function, which is used for the study of morphology and basic connectivity of roots segments. A link is a root segment between a fork and a tip or two tips. For the entire image, this function processes the total number of links, LAL, LAD and other parameters. Among them, TRL, SA, AD and NT was selected to figure out distinguish of RMT among adzuki bean, while link LAL, and LAD was selected to understand the difference of RAT in adzuki beans. Detailed definition of each root trait is described in Table 2.

Statistical Analysis

To determine statistical significance, we conducted an ANOVA test and the correlation analysis was performed with R studio version 1.3.1093 to investigate the relationship among various root phenotypes. Frequency distribution histograms for all root traits among the 22 adzuki bean cultivars and descriptive statistics or inferential statistical analysis were computed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Figures were generated using Microsoft Excel (2013) or R studio version 1.3.1093.

RESULTS AND DISCUSSION

Variability of root morphological traits

The root is an important part of the plant in terms of response to drought stress, it can initiate a response mechanism at a physiological or genetic level (Kim *et al.*, 2020). We reported key root morphological traits of 22 adzuki bean cultivars, such as

TRL, SA, AD, NT, LAL and LAD. The combined Analysis of Variance (ANOVA) demonstrated high significant differences among the cultivars for all the root traits under study (*viz.*, TRL, SA, AD, NT, LAL and LAD) as shown in Table 1. The data of all root morphological traits were normally distributed when checked visually across all the cultivars (Fig. 3). The statistical variation as descriptive statistics in root traits data is depicted in Supplementary Table 2. The RMT, TRL, SA, and AD were observed with a range of 91.46-717.14 cm, 7.74-60.68 cm², and range 0.24-0.36 cm respectively (Supplementary Table 2). The values range (Supplementary Table 2) suggests that there is a significant difference between the highest and lowest cultivars. In addition, among these three traits, SA showed the highest variation with a coefficient of variation (CV) near about 40% followed by SA and AD 38.39% and 10.70% respectively. Similarly, RAT, such as NT, LAL, and LAD observed with a range of 260.50-2036.75 number, 0.06-0.12 cm and 0.25-0.44 cm respectively. NT showed the highest CV with 37.02%, followed by LAL and LAD with 16.12 and 14.26% respectively. As the skewness is in between -1.96 and +1.96, hence the distribution of the data is observed to be univariate and as kurtosis values are less than 3, except for LAL, the dataset is observed to follow a normal distribution and histograms of all root traits excluding for LAL indicate their normal distribution (Supplementary Table 2, Fig. 3).

In plants, RMT and RAT are both known to be significant factors for root growth, symbiotic relationships with microflora, and resistance to stresses such as osmotic and nutrient stress. As we mentioned above, investigated six root traits in this experiment were included in RMT and RAT as root phenotyps. The correlation between RMT and RAT was investigated in

Table 2. Description of the root traits analyzed in the study using WinRHIZO software.

Parameter	Description
Total root length	Sum of the length of all root skeleton
Average diameter	Average diameter of roots
Tip	Number of root endings
Surface area	Total surface area is calculated by counting the number of pixels belonging to the root in the root images
Link average length	A link is a root part between two forks or a fork and a tip. Average length of links that belong to the order
Link average diameter	A link is a root part between two forks or a fork and a tip. Average of the average diameters for links that belong to the order

major crops such as soybean (Tripathi *et al.*, 2021a) and maize (Long *et al.*, 2020), but not in Adzuki bean. As a result, we conducted a correlation test among root traits. We used a procedure called PROC FREQ to calculate probabilities of the normality, and *F* distributions by SAS software. In this study,

based on the correlation analysis, we found that root length-related traits like TRL and SA had the highest correlation values when compared to other trait. TRL had a very strong positive phenotypic correlation at 0.05% significance level ($r=0.97$) with SA (Fig. 4). Similarly, we found strong positive correlation

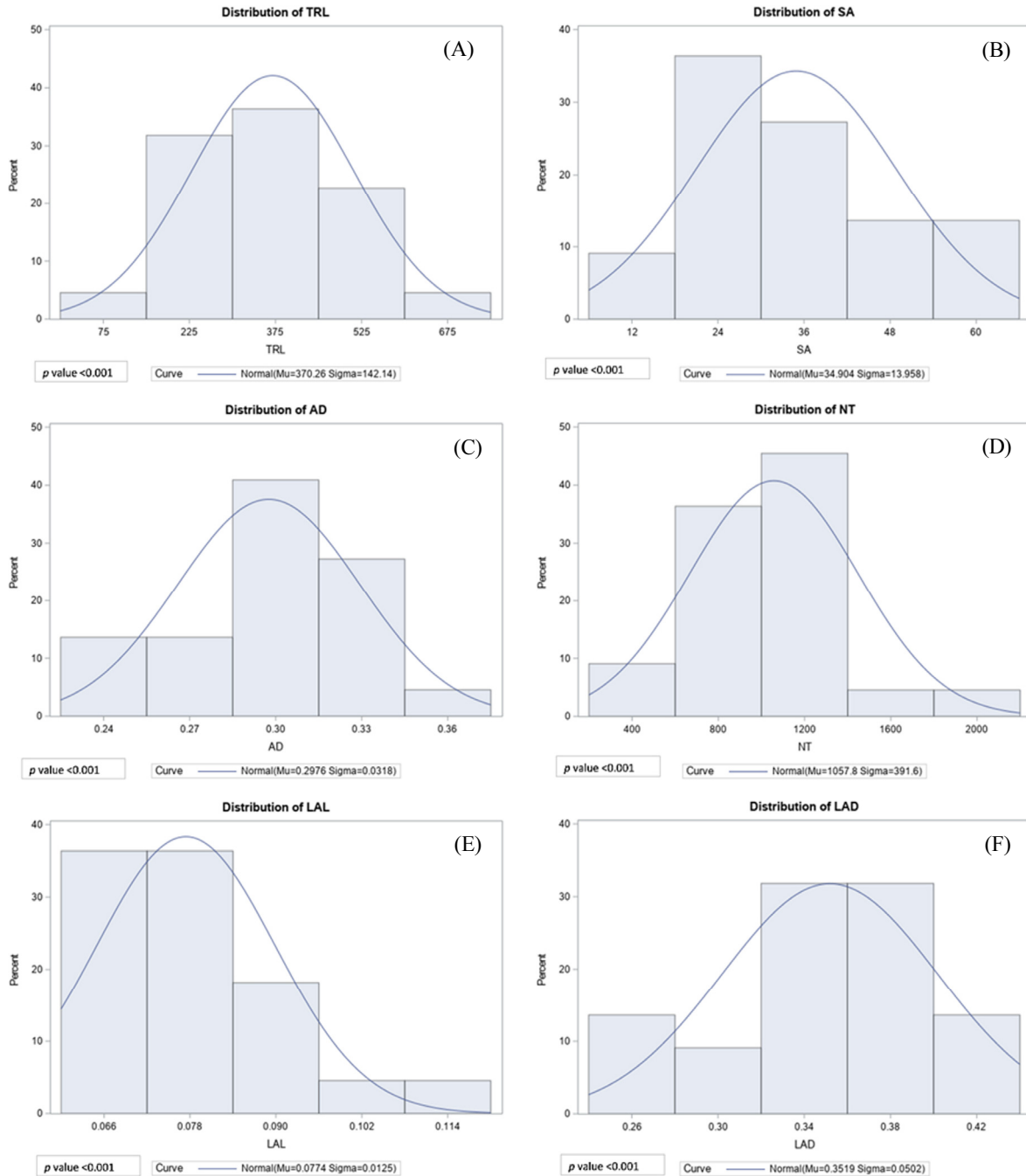


Fig. 3. Histogram of normal distribution curves for root morphological traits. In the figure, abbreviations indicates total root length (TRL), average diameter (AD), surface area (SA), number of tips (NT), link average length (LAL) and link average diameter (LAD).

with TRL ($r=0.94$) and SA ($r=0.89$) (Fig. 4) at 0.05% significance level. On the contrary, LAL showed significant negative correlations with NT ($r=-0.52$), AD ($r=-0.51$), SA ($r=-0.55$) and TRL ($r=-0.50$) (Fig. 3) respectively.

In other legume crops like soybean, root phenotyping of numerous cultivars have been reported (Kim *et al.*, 2021a). Furthermore, during the germination stage, adzuki bean germplasm was evaluated and screened for drought tolerance (Zhu *et al.*, 2019). The novelty of this study is that we used 22 popular and widely grown adzuki bean cultivars to evaluate root morphological and architectural traits in the vegetative stage. As root morphological and architecture traits in plants are considered to be very important traits for crop improvement which is often influenced by environmental conditions as well as some linked traits. To withstand drought stress, a plant must have a strong root system with longer root lengths that can absorb water from deeper soil levels during drought (Turner *et al.*, 2001; Kumar *et al.*, 2012). Thus, we aim to use the existing set of extremely promising cultivars to generate even higher and better root traits genotypes or crop ideotypes like improved RMT and RAT

adzuki beans, which probably withstand extreme environmental conditions greater than popular adzuki bean check cultivars and ultimately contribute to high crop production. Both root elongation and differentiation have been considered important processes in the aspect of how plants respond to external environmental factors that hinder plant growth (Zheng *et al.*, 2016). Numerous highly conserved miRNA profiles were studied from the primary root tips in soybean, which were found to be differentially regulated in response to drought stress (Zheng *et al.*, 2016). This reflects the importance of a number of tips in the roots in response to drought stress, indicating that NT could be a useful trait to indicate the potential for water and nutrient uptake during the response to drought stress. As per our findings, TRL has a very strong positive correlation with NT. As a result, cultivars with high TRL, SA, NT, LAL, and LAD may be considered for breeding traits, particularly to develop drought-tolerant cultivars. Genetic variation in legumes has been reported to affect the number of metaxylems in roots which can alter the water movement throughout the root system and into the shoots, therefore the genes that regulate the root system architecture can be

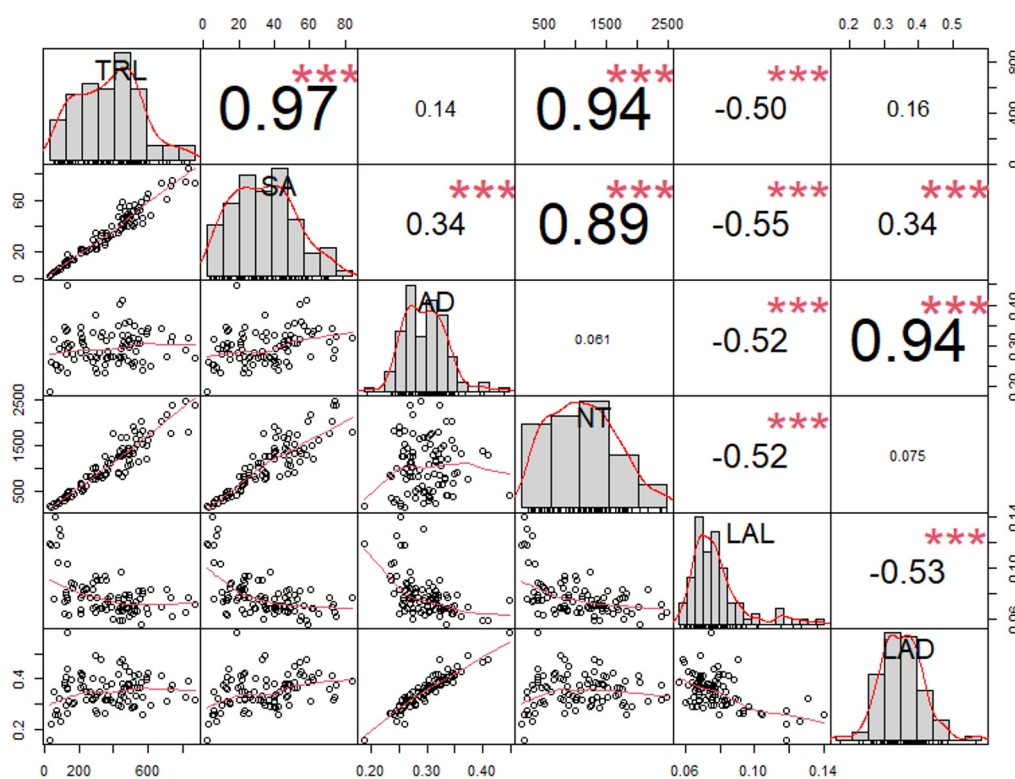


Fig. 4. Correlation analysis between various root morphological traits. In the figure, asterisks (*) represents significance at 0.05 level; $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.0001$ (***).

Table 3. List of top and bottom five cultivars for each root trait.

	TRL (cm)	SA (cm ²)	AD (mm)	NT (number)	LAL (cm)	LAD (mm)
Top 5 cultivars for each root trait	717.13	60.68	0.36	2036.75	0.12	0.43
	IT 236657	IT 236657	IT 236659	IT 236657	IT 236169	IT 209444
	530.47	58.48	0.34	1481.50	0.009	0.43
	IT 236658	IT 236659	IT 209444	IT 236658	IT 236171	IT 236659
	526.54	56.10	0.336	1367.80	0.085	0.42
	IT 236659	IT 236658	IT 236658	IT 236659	IT 270036	IT 154837
	506.65	48.40	0.334	1365.75	0.086	0.39
	IT 229428	IT 229428	IT 154837	IT 238547	IT 294673	IT 236658
	469.05	46.70	0.325	1341.75	0.084	0.37
	IT 216355	IT 216355	IT 221513	IT 229071	IT 229428	IT 216355
Bottoms 5 cultivars for each root trait	91.46	7.74	0.23	260.50	0.064	0.24
	IT 236169	IT 236169	IT 236171	IT 236169	IT 215376	IT 236171
	203.79	17.82	0.24	584.00	0.065	0.277
	IT 294673	IT 236171	IT 240375	IT 294673	IT 236658	IT 240375
	224.70	19.31	0.25	602.00	0.066	0.278
	IT 154837	IT 294673	IT 270036	IT 154837	IT 236659	IT 236169
	226.57	22.38	0.267	642.40	0.067	0.29
	IT 236171	IT 182073	IT 236657	IT 209444	IT 221513	IT 270036
	253.64	24.07	0.268	691.00	0.068	0.31
	IT 182073	IT 154837	IT 236169	IT 236171	IT 238547	IT 236657

Note: TRL: total root length; SA: surface area; AD: average diameter; NT: number of tips; LAL: link average length; LAD: link average diameter.

prioritized for cultivar improvement and stress tolerance in adzuki bean (Prince *et al.*, 2017; Xiong *et al.*, 2021).

From the total of 22 adzuki bean cultivars, we listed the top and bottom 5 cultivars for every root morphological trait under consideration in this study (Table 3). According to the results, 'IT 236657' demonstrated the highest TRL, SA and NT (Table 3). Likewise, 'IT 236169' showed the lowest value for TRL, SA and NT (Table 3). Similarly, 'IT 236171' showed the lowest AD and LAD (Fig. 4). The root morphological traits evaluation of adzuki beans have not yet been fully reported. Therefore, our results would be useful in a breeding program for developing root reinforced-adzuki bean.

CONCLUSION

We found a significant difference between the cultivars for most of the measured root morphological traits. The highest variation was observed for the SA, followed by the TRL and NT among the tested cultivars. Furthermore, a strong positive correlation was found between TRL and SA. Whereas, LAL

showed significant negative correlations with NT, AD, SA and TRL. This research study would be extremely useful in identifying target root traits for the adzuki bean breeding approach in the future for developing climate-resilient adzuki bean, particularly for drought stress. In addition, it would be desirable to find in further study how these root traits affect the adzuki bean yield and other biotic and abiotic stress tolerance under greenhouse as well as field conditions.

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Supplementary Table 1. List of adzuki bean cultivars.

IT number	Country of origin	Scientific name
154837	UNK	<i>Vigna angularis</i> var. <i>angularis</i>
182073	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
182092	UNK	<i>Vigna angularis</i> var. <i>angularis</i>
189445	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
209444	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
215376	JPN	<i>Vigna angularis</i> var. <i>angularis</i>
216355	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
216356	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
221513	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
229071	JPN	<i>Vigna angularis</i> var. <i>angularis</i>
229428	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
229429	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
236167	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
236169	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
236171	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
236657	KOR	<i>Vigna angularis</i> var. <i>angularis</i>
236658	UNK	<i>Vigna angularis</i> var. <i>angularis</i>
236659	CHN	<i>Vigna angularis</i> var. <i>angularis</i>
238547	JPN	<i>Vigna angularis</i> var. <i>angularis</i>
240375	KOR	<i>Vigna angularis</i>
270036	JPN	<i>Vigna angularis</i>
294673	JPN	<i>Vigna angularis</i>

Supplementary Table 2. Descriptive statistics table for six root traits in 22 cultivars.

Traits	Range	Mean	SD ^a	CV (%) ^b	Skewness	Kurtosis	Pr value
TRL	91.46–717.14	370.26	142.14	38.39	0.33	0.33	0.001
SA	7.74–60.68	34.90	13.96	39.99	0.19	-0.52	0.001
AD	0.24–0.36	0.30	0.03	10.70	-0.01	-0.50	0.001
NT	260.50–2036.75	1,057.76	391.60	37.02	0.23	0.70	0.001
LAL	0.06–0.12	0.08	0.01	16.12	1.92	5.12	0.001
LAD	0.25–0.44	0.35	0.05	14.26	-0.27	-0.24	0.001

^aStandard deviation; ^bCoefficient of variation in percentage.