

Which common bean root phenotyping method is best? This depends on the phenological growth stage

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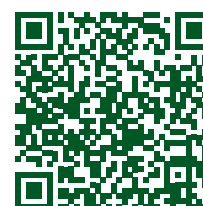
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Abstract: To assess the impact of different phenotyping methods and crop phenological stages on the differentiation of parent versus progeny performance for root traits in common bean breeding programs, a field experiment was carried out in the 2021/22 growing season. In a lattice design, 36 field treatments were tested, based on three genotypes and two root phenotyping methods (Shovelomics and WinRHIZO) applied at six phenological stages ($V_{4-4'}$; $V_{4-8'}$; R_5 ; R_6 ; R_7 and R_8). Eight response variables of the root system were evaluated. The Shovelomics method significantly influenced parent-progeny distinction at the $V_{4-4'}$ (vegetative) and the WinRHIZO method at the R_8 (reproductive) stage. Mean heritability estimates of 0.35 and 0.30 were calculated. As the heritability values were moderate to low, it is worth emphasizing that root phenotyping can be performed in more advanced inbreeding generations, due to the strong environmental influence on root trait expression.

Keywords: *Phaseolus vulgaris* L., Andean and Mesoamerican gene pool, WinRHIZO, Shovelomics

INTRODUCTION

Due to the need to increase yields across a wide range of food crops historically, plant breeding efforts have prioritized the improvement of above-ground traits. Accordingly, the current common bean ideotype was developed to prioritize high grain productivity, leading to significant gains in grain yield over time. Yet, as climate changes have been reducing the availability of water and mineral resources in most bean-producing regions, attention has shifted toward plant traits hidden in the ground (Haus et al. 2020, Maqbool et al. 2022). The expression and quantification of root traits have become a key focus of studies on the development of agronomically efficient plants (Ambachew et al. and Blair 2023). Improved root systems in genotypes are now seen as a valuable outcome of the green revolution (Atkinson et al. 2019). Nonetheless, the genetic regulation of root traits in common bean remains insufficiently investigated. Their inheritance is complex, governed by numerous genes with small individual effects. Recent studies have identified markers linked to quantitative trait loci (QTLs) that determine total root length, fine root length, thicker root length, root length distribution with depth, and root biomass across bean populations (Castiano et al. 2023).



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Despite the relevance of the root system described above, the processes of evaluation and quantification are still rather complicated. This is mainly due to loss of material when roots are collected in the field (Fried et al. 2019). Field measurements of roots are arduous, complex and time-consuming (Marshall et al. 2016, Li et al. 2017, Sofi et al. 2018). Therefore, studies centered on high-throughput phenotyping have led to the development of techniques that enable efficient identification of superior progenies in plant breeding programs. Among the tools that can be used for high-throughput phenotyping, the methods Shovelomics and WinRHIZO are worth mentioning. Both are destructive, since the evaluated plants are removed from the cultivation environment (Trachsel et al. 2011, Pornaro et al. 2017, Appiah-Kubi et al. 2022, Etana and Nebiyu 2023). To date, the application of these field phenotyping methods in plant breeding programs is poorly documented.

In addition to the doubts about the best method for root phenotyping, the ideal moment (phenological stage) for evaluations is also a concern of plant breeders. The reason is the high plasticity of root system traits. The term plasticity means the ability of plants to adapt phenotypes in response to the environment (Fried et al. 2019, Lorts et al. 2020). Plants are capable of modifying the root architecture (root angle, length and hair density) to adapt to different growth environments, where drought, salinity and acidity or other stresses may occur. This adaption occurs by means of combined physiological, biochemical and genetic mechanisms that regulate the expression of the root system (Ranjan et al. 2022). Under water stress for example, plants can stimulate the growth of lateral roots and root hairs (Koevoets et al. 2016). Based on this adaptation to the cultivation environment, the root system of common bean has been evaluated in critical moments of development, e.g., at flower bud growth (R_5), full flowering (R_6) and the grain-filling stage (R_8), (Galvão et al. 2019, Burrridge et al. 2020, Wu et al. 2021). The question about the ideal moment of the crop cycle when phenotyping should be used arises because, regardless of the phenological stage, plant breeders need to distinguish the phenotype of the progeny from that of its ancestors, in order to understand whether breeding achieved any genetic gain for target traits or not.

Although plant breeders generally agree that root system measurement is essential and should be taken into consideration in breeding programs, there is no literary consensus as to which root phenotyping method should be used and at which point of the crop cycle assessments should be carried out. Thus, the objective of this study was to evaluate the effect of combining phenotyping methods with different phenological development stages to assess and compare the root system of fixed and segregating common bean genotypes, aiming to improve the selection of plants with enhanced root traits.

MATERIAL AND METHODS

To accurately assess the impact of phenotyping methods and phenological stages on root system evaluation in common bean genotypes, phenotyping studies were carried out since the growing seasons of 2019/20 and 2020/21 (Cerutti et al. 2023). In these tests, a strong influence of the physiological plant age on root expression was observed. For the same purpose, another field experiment was initiated during the 2021/22 growing season in Lages, Santa Catarina (SC). The experimental area is part of the State University of Santa Catarina (UDESC), Centro de Ciências Agroveterinárias (CAV) (lat 27° 47' S, long 50° 18' W, alt 950 m asl). The overall average of maximum and minimum temperatures is 21.8 °C and 11.8 °C, considering a historical series of 85 years (1940 a 2025) and annual precipitation 1500 mm according to information from the Climatic Atlas of the Southern Region of Brazil (Wrege 2012). The soil of the experimental area is classified as Cambissolo Húmico Aluminico Léptico, characterized by a clayey texture (Embrapa 2018). The soil was analyzed to a depth of 0.40 m, with the following results: pH = 5.9; organic matter content = 3.2%; P = 3.1 mg dm⁻³ and K = 124.0 mg dm⁻³. Phosphorus and potassium contents were determined by the Mehlich-1 method (CQFS-RS/SC 2016).

In this growing season, 36 field treatments were set up, resulting from the interaction among the levels of three experimental factors: genotypes, phenotyping methods, and phenological stages of development. The levels of the genotype factor (structured specific qualitative) were given by two parents (BAF07 and BRS Embaixador), and the progeny BAF07 x BRS Embaixador F_6 . Parent BAF07 is an accession of Active Germplasm Bank of UDESC of the Mesoamerican gene pool that belongs to the black bean group. Genotype BRS Embaixador is a common bean cultivar already planted in Brazil, of the Andean gene pool and the market group colored (red). Controlled crosses to establish the progenies BAF07 x BRS Embaixador F_6 were carried out in 2016. In that year, six common bean parents were artificially hybridized in a full diallel design. The combination between the parents resulted in 30 F_1 progenies. These progenies were taken to the field

for evaluation of the root system and generation advancement (bulk method in all years). Among these combinations, progeny BAF07 x BRS Embaixador proved to be interesting for some agronomic traits, e.g., root distribution (% of the soil occupied by roots) and grain yield and quality (Cerutti et al. 2021, Cerutti et al. 2023).

The phenotyping method factor (unstructured specific qualitative) consisted of two root evaluation methods, called “Shovelomics” and “WinRHIZO”. The former method was developed by researchers at the Penn State College of Agricultural Sciences for high-throughput phenotyping of plants in the field. In this technique, plant roots are adjusted within a template, and the variables of interest are measured (Trachsel et al. 2011). The WinRHIZO method, on the other hand, relies on scanning software. For this study, the system was operated using an Epson Expression 10,000 XL scanner (Regent Instruments Canada Inc.). This method involves placing the plant roots onto the scanner, where the software quantitatively measures and automatically records the relevant variables from an image generated by the system (Pornaro et al. 2017). The phenological development stages represented the third experimental factor (structured as specific qualitative), comprising the following stages: V_{4-4} (four trifoliate leaves), V_{4-8} (eight trifoliate leaves), R_5 (flower bud growth), R_6 (full flowering), R_7 (early pod formation), and R_8 (grain filling) (Gepts and Fernández 1982). The 36 field treatments were randomized in a simple, partially balanced lattice design (6×6), with two replications, resulting in 72 experimental units. In plant breeding, the use of lattice designs is common in experimental studies due to the large number of treatments that need to be evaluated. Under these conditions, lattice designs prove to be more efficient than randomized block designs, segmenting the experimental area into more uniform blocks contributes to increased homogeneity within each block, thereby reducing variability caused by heterogeneous environmental factors. This approach enables a more precise control of experimental conditions, which is particularly advantageous in evaluations of a large number of treatments. In this experiment, the relative efficiency of the lattice design was compared to that of the randomized block design reached 93.4%. This result not only justifies the use of the former but also underscores its ability to enhance statistical analyses in agronomic studies (Gomez and Gomez 1985). The experimental units, each measuring 2×2 m, were spaced 0.5 m apart and seeded at a density of 13 seeds per meter.

All management practices, including fertilization and control of weeds, insects, and crop diseases, were carried out as needed, following the regional technical recommendations for common bean cultivation (Fancelli and Dourado 2007, CQFS-RS/SC 2016). When the genotypes of each experimental unit reached the specified phenological stages, the root system was evaluated. For both methods, this process involved excavating the soil around the plant stem, from 0.25 to 0.30 m away, to a depth of 0.30 m, in order to extract the soil-root set. Five randomly selected bean plants (observation units) were collected from each experimental unit. The soil was then removed to expose the roots by immersing the plants in water containing 5% neutral detergent (Trachsel et al. 2011). Using the Shovelomics method, the following variables were measured: i) basal root angle (RA, °); ii) vertical root length (VL, cm); iii) horizontal root length/left side (LL, cm); iv) horizontal root length/right side (RL, cm). Using the WinRHIZO method, the following variables were evaluated: v) total root length (TL, cm); vi) projected root area (PA, cm^2); vii) root volume (RV, cm^3); and viii) mean root diameter (RD, mm). After data collection, multivariate analysis of variance was applied for each phenotyping method, considering the four response variables of each method. For this step, the MANOVA (Multivariate Analysis of Variance) option of the general linear procedure (proc glm) was used. To explore the simple effects of the genotype x phenological stage interaction, orthogonal contrasts between mean vectors were established and tested by the F test, at 0.05 probability. Broad-sense heritability (h_b^2) was estimated by the relationship between variances, as follows:

$$h_b^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_{ge}^2 + \sigma_e^2 + 2\text{Cov}_{ge})$$

where σ_g^2 represents the genetic variance, σ_{ge}^2 is the genotype x environment interaction variance, σ_e^2 is the environmental (residual) variance, and Cov_{ge} is the genotype–environment covariance. The environmental effects considered in this study are non-genetic, representing sources of variation not attributable to genetic factors. All these analyses were performed using SAS Studio® software, available on the SAS OnDemand for Academics platform.

RESULTS AND DISCUSSION

A significant effect was observed by Wilks’ Lambda (λ) test ($p > 0.05$) for the interaction genotype x phenological stage (Table 1), both for Shovelomics ($p=0.023$) and WinRHIZO ($p=0.001$). This fact indicates the influence of the phenotyping tool (method) and the moment when root evaluations are performed (physiological age). In terms of understanding a

Table 1. Multivariate analysis of variance, using Wilks' Lambda test (λ), was performed to assess the effects of the experimental factors: replication, blocks within replications, genotype, and phenological stages (V_{4-4} ; V_{4-8} ; R_5 ; R_6 ; R_7 ; and R_8) for each phenotyping method, considering the response variables associated with each method

Sources of variation	Shovelomics		WinRHIZO	
	λ	P-value	λ	P-value
Replication (Rep)	0.98	0.686	0.98	0.589
Rep (Block)	0.72	0.133	0.78	0.577
Genotype (G)	0.87	0.013	0.95	0.603
Stage (S)	0.63	0.001	0.22	0.001
G x S	0.67	0.023	0.62	0.001

Root traits measured using the Shovelomics method included basal root angle (RA, °), vertical root length (VL, cm), horizontal root lengths on the left (LL, cm) and right sides (RL, cm). The WinRHIZO method evaluated total root length (TL, cm), projected root area (PA, cm²), root volume (RV, cm³), and mean root diameter (RD, mm).

breeding strategy for a root system ideotype in the case of common bean, the analysis of the phenotypic variation in the root system within the genetic background is particularly important, due to the differences between the root system phenotypes of the Andean and Mesoamerican genotypes (Jochua et al. 2020).

The multivariate analysis of variance indicated that the first two canonical components captured around 75% of the total variation in the genotype $\times$ phenological stage interaction, as measured by the Shovelomics method. The canonical coefficients of this interaction were -0.10 (left horizontal length); 0.16 (basal root angle); 0.20 (right horizontal length) and 1.06 (vertical length). This indicates that basal root angle and vertical length are response variables that contribute to the interaction effect. Considering the same interaction by the WinRHIZO method, the first two canonical components captured 63 and 20% of the total variation, respectively, and the highest canonical coefficients were observed for the variables mean root diameter (0.60) and projected root area (7.70). Studies focusing on understanding root expression in common bean have particularly emphasized traits such as water stress tolerance, have highlighted the importance of developing or identifying improved genotypes for root length, angle and mean diameter (Kachiguma et al. 2021, Wu et al. 2021).

In addition to the interaction, the main effect of the phenological stage had a strong influence on the phenotyping of the root system. The Wilks' Lambda (λ) value estimated for the Shovelomics method was approximately three times higher than that for WinRHIZO. This estimate difference was expected since the methods are based on different variables and scales of these response variables. However, this effect reinforces the need to consider the most appropriate moment to evaluate the root system, according to the phenotyping method.

The distinction between the most suitable phenological stages for phenotyping in relation to the evaluation method and the studied genotype may be related to the genotype factor itself. The reason is that the parents diverge for some traits, for example: gene pool, tegument color and growth habit. Genotypes with an indeterminate growth habit are positively associated with root development (Velho et al. 2018). Both genotype BAF07 and the progeny have an indeterminate growth habit. Plants with this type of development have a gradual transition between stages and often maintain vegetative growth even after the emission of reproductive structures, i.e., a continuous form of development. This peculiarity is considered an adaptative strategy to adverse conditions (Wu et al. 2021). The lack of significance of the genotype factor when using the WinRHIZO method may be influenced by the environmental factor, as plants of different genotype have distinct characteristics of rhizosphere exploration and, consequently, root development. However, in view of the similarity between the progeny and its parent, these differences may be minimal, leading to insignificance of this factor (Bulayaba et al. 2020).

In addition to the multivariate analysis, orthogonal contrasts between the mean vectors of the Shovelomics (Table 2) and WinRHIZO (Table 3) methods were used to estimate the simple effects of the genotype $\times$ phenological stage interaction. In the composition of these simple effects, the phenological stage factor was fixed, and comparisons of interest between the genotypes were performed. According to Table 2, three contrasts were significant, two between parents (stages R_6 and R_8) and one between parents and the progeny (stage V_{4-4}). For the WinRHIZO method, only two contrasts were significant - one between the parents (R_7) and the other between parents and the progeny (R_8). In the contrasts that differentiated the phenotyping of parents and the progeny, the variables vertical root length (VL) and

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projected area (PA) had the highest canonical coefficients, indicating the relevance of these variables for genotype discrimination (Table 3).

In the context of breeding, it is fundamental to distinguish the performance of progenies in relation to their parents, since a cultivar can be developed from a controlled cross with complementary traits of agronomic interest (De Paula

Table 2. Multivariate contrasts performed using the Wilks' Lambda test (λ) for the simple effects of genotype x stage interaction for *Shovelomics* phenotyping method in common bean

Contrasts	λ	P-value	RA	VL	LL	RL
V₄₋₄						
Parents (BAF07 x BRS Embaixador)	0.96	0.334	0.98	-0.02	-0.23	-0.24
Parents vs. Progeny	0.93	0.033*	0.42	0.89	0.44	-0.26
V₄₋₈						
Parents (BAF07 x BRS Embaixador)	0.97	0.507	-0.53	0.69	-0.59	0.51
Parents vs. Progeny	0.99	0.896	-0.24	0.53	0.81	-0.71
R₅						
Parents (BAF07 x BRS Embaixador)	0.99	0.871	-0.31	0.72	0.16	0.71
Parents vs. Progeny	0.95	0.164	0.10	0.95	0.51	0.09
R₆						
Parents (BAF07 x BRS Embaixador)	0.85	0.002*	0.47	0.78	0.61	-0.61
Parents vs. Progeny	0.97	0.512	0.30	0.66	0.02	0.78
R₇						
Parents (BAF07 x BRS Embaixador)	0.94	0.059	0.15	0.40	0.85	0.50
Parents vs. Progeny	0.98	0.739	-0.29	-0.67	0.64	0.50
R₈						
Parents (BAF07 x BRS Embaixador)	0.93	0.038*	0.48	-0.17	1.03	-0.51
Parents vs. Progeny	0.96	0.322	0.42	0.66	0.53	0.44

*: Significant at 5 % by the *F* test. Phenological stages (V4-4 - R8). The response variables included basal root angle (RA, °), vertical root length (VL, cm), horizontal root length on the left side (LL, cm), and horizontal root length on the right side (RL, cm)

Table 3. Multivariate contrasts performed using the Wilks' Lambda test (λ) for the simple effects of genotype x stage interaction for *WinRHIZO* phenotyping method in common bean

Contrasts	λ	P-value	TL	PA	RV	RD
V₄₋₄						
Parents (BAF07 x BRS Embaixador)	0.97	0.419	1.93	2.33	-4.94	2.61
Parents vs. Progeny	0.95	0.126	0.70	2.88	-3.01	2.56
V₄₋₈						
Parents (BAF07 x BRS Embaixador)	0.99	0.945	4.14	- 3.10	-1.51	2.48
Parents vs. Progeny	0.98	0.793	-1.07	6.75	- 6.50	1.62
R₅						
Parents (BAF07 x BRS Embaixador)	0.96	0.262	-0.13	- 2.26	3.93	-0.90
Parents vs. Progeny	0.98	0.595	-7.00	11.6	-4.99	0.58
R₆						
Parents (BAF07 x BRS Embaixador)	0.97	0.516	4.24	-7.4	4.91	-1.07
Parents vs. Progeny	0.98	0.795	-3.06	7.29	-3.47	1.16
R₇						
Parents (BAF07 x BRS Embaixador)	0.93	0.038*	0.40	2.18	-1.49	0.86
Parents vs. Progeny	0.97	0.490	-4.80	12.62	-7.98	2.49
R₈						
Parents (BAF07 x BRS Embaixador)	0.95	0.112	3.35	-7.59	6.04	-0.41
Parents vs. Progeny	0.86	0.003*	-5.85	11.25	-5.23	-0.06

*: Significant at 5 % by the *F* test. Phenological stages (V4-4 - R8). The response variables included total root length (TL, cm), projected root area (PA, cm²), root volume (RV, cm³), and mean root diameter (RD, mm).

Table 4. Broad-sense heritability coefficients (h_b^2) for the response variables obtained by the phenotyping methods (Shovelomics and WinRHIZO) in the respective root systems

	h_b^2			
Shovelomics	RA	VL	LL	RL
	0.39	0.43	0.41	0.20
WinRHIZO	TL	PA	RV	RD
	0.32	0.40	0.22	0.26

The Shovelomics method included the evaluation of basal root angle (RA, °), vertical root length (VL, cm), and horizontal root lengths on the left (LL, cm) and right (RL, cm) sides. In turn, the WinRHIZO method measured total root length (TL, cm), projected root area (PA, cm²), root volume (RV, cm³), and mean root diameter (RD, mm).

et al. 2019). By the Shovelomics method, the distinction between progeny and parents occurred at an early V_{4-4} stage, where vertical root length was the main differentiating variable. This fact was possibly associated with the trait root system formation of parents and progeny, in addition to the peculiarity of the Shovelomics method. The genotypes of the Andean and Mesoamerican gene pools differ in the expression of root phenotypes. Andean have a longer taproot and more basal roots than the Mesoamerican lines in the vegetative development stages. These in turn have hypocotyl-derived adventitious roots that can increase the biomass of shallow roots in later stages of the crop cycle (flowering), and thus produce more adventitious roots than the Andean lines (Strock et al. 2019, Haus et al. 2020). This fact was confirmed by Pearson's simple correlation estimate, indicating a significant association of -0.39 between the number of adventitious roots and taproot length in Mesoamerican lines, while this association was not observed in Andean genotypes (Strock et al. 2019). The differentiation by the Shovelomics method in the early development stages can also be explained by the particularity of the method, since the evaluations are done manually. In this way, as the plant root system is less developed at this than in the reproductive stage, the results of root phenotyping become more accurate.

The divergence in plant development observed in relation to the gene pool and the development phase (vegetative and reproductive) of the genotypes is in line with the distinction between parents and progeny in grain filling phase (R_g) detected by the WinRHIZO method. Mainly due to the higher amount of adventitious roots of genotype BAF07 and progeny BAF07 x BRS Embaixador than of genotype BRS Embaixador, the use of WinRHIZO for root phenotyping has the advantage of a better measurement of this dense root mass, which is not easily done by hand (Wang and Zhang 2009, Pornaro et al. 2017).

Root phenotyping depends on the genetic nature (gene pool), together with the trait root formation of each pool and the growth habit of the genotypes. On this basis, one can use the phenotyping methods and their traits to propose an ideotype for the root system. The composition of a root system ideotype can indicate the development of a phenotype with a trait assembly (York et al. 2013). The root phenotypes for maintaining or increasing grain yield under limiting environmental conditions are particularly efficient in resource acquisition (Lynch 2018). Since phosphorus, nitrogen and water are the most limiting resources for growth and development, root phenotypes can be improved specifically for each purpose. For not very mobile nutrients, genotypes must be selected that contain traits of a root system that is more densely developed in the soil surface layers. The opposite applies for the selection of improved genotypes for uptake of mobile resources such as nitrogen and water (Lynch 2018, Strock et al. 2019).

In addition to the significance of developing genotypes with enhanced root traits for a given environmental condition, the fraction of genetic variation transmitted over generations must be taken into account. The visual selection of any trait depends on heritability estimates, i.e., on the level of environmental influence on the phenotypic expression. In the case of common bean (mainly autogamous reproduction mode), high heritability (h_b^2) values indicate a predominant association of genetic variance with additive genetic variance, which may facilitate the selection of superior plants. In this study, the mean heritabilities for the different response variables of the phenotyping methods Shovelomics and WinRHIZO were 0.35 and 0.30, respectively (Table 4). These values are considered moderate to low (<0.50). Heritability estimates of segregating populations grown in a greenhouse, for evaluation of the variables root length, volume and area, averaged 0.40 (Cerutti et al. 2021). Under field conditions, for the variable root distribution (%), heritability was also 0.40 (De Melo et al. 2016). In hydroponic medium, the heritability values were somewhat higher (0.89; 0.75; 0.67 and 0.87, respectively, for root length, area, volume and mean root diameter) (Ambachew and Blair 2023).

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The moderate to low heritability estimates found by field phenotyping proved the strong effect of the environment on the root system, reinforcing the need to define effective methods for evaluation. In this way, selection with a view to establishing a root ideotype must be applied in more advanced inbreeding generations, under low selection pressure in the initial cycles. This selection can be further refined by considering the most suitable phenotyping method, based on the genetic nature of the genotypic constitutions within the breeding program.

CONCLUSION

Root quantification in common bean was influenced by the phenotyping method associated with the phenological stage. By Shovelomics phenotyping, fixed genotypes could be differentiated from their segregating progeny in the vegetative phase (V_{4-4}), and at the end of the crop cycle (R_8) by the WinRHIZO method. The definition of the most adequate method and moment of root phenotyping of common bean is a strategy in the development of an ideotype with improved root traits.

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DATA AVAILABILITY

The datasets generated and analyzed during the current research are available from the corresponding author upon reasonable request.

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