

# Phenotyping methods and phenological stages to quantify the root system of common bean

**Abstract** – The objective of this work was to evaluate root phenotyping methods and the ideal phenological stage to quantify the root system of fixed and segregating common bean populations, in order to select superior genotypes. The experiment was carried out in two municipalities in the state of Santa Catarina, Brazil, and the treatments consisted of six genotypes, the Shovelomics and WinRHIZO root phenotyping methods, and the  $V_{4-4}$ ,  $R_6$ , and  $R_8$  phenological stages. The simple lattice experimental design was used to evaluate the following variables: basal root angle, vertical root length, left and right horizontal root length, total root length, projected area, and root average volume and diameter. For all variables, there was a significant interaction between phenotyping methods and phenological stages, showing their influence on root system evaluation. The Shovelomics and WinRHIZO phenotyping methods are efficient in quantifying the root system of common bean plants and show specificity for phenological stages, regardless of the genotype. The quantification of the root system of fixed and segregating genotypes is analogous in both methods. The Shovelomics method is more efficient in evaluating the root system of common bean at the  $R_8$  stage, and the WinRHIZO method, at the  $R_6$  stage.

**Index terms:** *Phaseolus vulgaris*, Andean gene pool, Mesoamerican gene pool, plant breeding, Shovelomics, WinRHIZO.

## Métodos de fenotipagem e estádios fenológicos para quantificar o sistema radicular de feijoeiro

**Resumo** – O objetivo deste trabalho foi avaliar métodos de fenotipagem de raízes e o estágio fenológico ideal para quantificação do sistema radicular de populações fixas e segregantes de feijoeiro, para selecionar genótipos superiores. O experimento foi realizado em dois municípios do estado de Santa Catarina, Brasil, e os tratamentos consistiram em seis genótipos, nos métodos de fenotipagem Shovelomics e WinRHIZO, e nos estádios fenológicos  $V_{4-4}$ ,  $R_6$  e  $R_8$ . O delineamento experimental látice simples foi utilizado para avaliar as seguintes variáveis: ângulo de raiz basal, comprimento vertical das raízes, comprimento horizontal esquerdo e direito das raízes, comprimento total das raízes, área projetada, e volume e diâmetro médio de raízes. Para todas as variáveis, houve interação significativa entre os métodos de fenotipagem e os estádios fenológicos, o que mostra a influência desses na avaliação do sistema radicular. Os métodos de fenotipagem Shovelomics e WinRHIZO são eficientes para a quantificação do sistema radicular do feijoeiro e apresentam especificidade para o estágio fenológico, independentemente do genótipo. A quantificação do sistema radicular de genótipos fixos e segregantes é análoga em ambos os métodos. O método Shovelomics é mais eficiente para avaliar o sistema radicular do feijoeiro na fase  $R_8$ , e o método WinRHIZO, na fase  $R_6$ .

**Termos para indexação:** *Phaseolus vulgaris*, pool gênico andino, pool gênico mesoamericano, melhoramento de plantas, Shovelomics, WinRHIZO.

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Received  
October 28, 2022

Accepted  
January 24, 2023

**How to cite**  
CERUTTI, P.H.; MELO, R.C. de; CARBONARI, L.T. dos S.; SCHWARZER, P.A.; GUIDOLIN, A.F.; COIMBRA, J.L.M.; PRIMIERI, S. Phenotyping methods and phenological stages to quantify the root system of common bean. *Pesquisa Agropecuária Brasileira*, v.58, e03169, 2023. DOI: <https://doi.org/10.1590/S1678-3921.pab2023.v58.03169>.

## Introduction

Common bean (*Phaseolus vulgaris* L.,  $2n = 2x = 22$ ) is an economically and socially relevant crop (Nkhata et al., 2021). However, most of its production system faces challenges that limit its potential yield, such as sensitivity to abiotic stresses in different production environments (Polania et al., 2017). Plant breeders, therefore, have focused on developing common bean genotypes with adaptive agronomic traits, which include a reduced plant height, an optimized foliar angle and area, and an improved root system (Rocha et al., 2019).

An improved root system is essential for plants to better absorb water and mineral resources (Fenta et al., 2014). However, for genetic breeding programs, evaluating and phenotyping plant root systems in the field is an intense, hard, and expensive work, especially when it covers a large number of segregating populations (Polania et al., 2017). This explains why studies related to that trait have been carried out almost exclusively in controlled environments, such as greenhouses with different types of cultivation in soil or other types of substrates, as hydroponic systems and nutrient solutions (Kabir et al., 2015; Miguel et al., 2015). Additionally, the root phenotyping method and the ideal physiological plant growth and development stage for quantification have not been clearly defined yet (Trachsel et al., 2011).

With the advances in science and technology, analysis methods based on image quantification have been used to phenotype the root system of different crops (Blaser et al., 2020), and plants are usually evaluated in the phenological stages that are considered. Most assessments are carried out at the most critical stage for crop growth and development, such as full flowering or grain filling (Wu et al., 2021).

To obtain more effective results in the genetic breeding of the common bean crop, it is important to consider the interaction between phenotyping methods and evaluation stages, mainly due to the high plasticity of the root system of this species in response to environmental effects (Ehdaie et al., 2012). Considering these interactions, the phenotyping of segregating populations in the field, targeting the root system, may provide more reliable and accurate results, by reflecting the real cultivation condition of common bean.

The objective of this work was to evaluate root phenotyping methods and the ideal phenological stage to quantify the root system of fixed and segregating common bean populations, in order to select superior genotypes.

## Materials and Methods

The experiment was carried out during the 2020/2021 crop season, in the two following sites (municipalities) in the state of Santa Catarina, Brazil: Concórdia, located in the western region of the state ( $27^{\circ}14'S$ ,  $52^{\circ}1'W$ , at 550 m of altitude); and Lages, located in the region of Planalto Serrano ( $27^{\circ}47'S$ ,  $50^{\circ}18'W$ , at 950 m of altitude). These different sites were chosen to verify the environmental effect on the root system of common bean genotypes.

The climate of the experimental region is Cfb temperate, i.e., humid mesothermal and mild summer, according to Köppen-Geiger. In Concórdia, the average air temperature was  $20^{\circ}C$ , the annual rainfall was 1,700 mm, and the soil was classified as a Latossolo Vermelho distroférrico (Santos et al., 2018), corresponding to an Oxisol. Soil analysis of the experimental area showed: pH ( $H_2O$ ) 5.5, 3.3 mg  $kg^{-1}$  organic matter content, 4.0 mg  $dm^{-3}$  phosphorus, and of 130.2 mg  $dm^{-3}$  potassium. In Lages, the average air temperature was  $16^{\circ}C$ , the annual rainfall was 1,441 mm, and the soil was classified as a Cambissolo Húmico aluminico léptico, with a clayey texture (Santos et al., 2018), corresponding to an Inceptisol. The soil analysis showed: pH ( $H_2O$ ) 5.9, 3.2 mg  $kg^{-1}$  organic matter content, 3.1 mg  $dm^{-3}$  phosphorus, and 124.0 mg  $dm^{-3}$  potassium; both elements were determined by the Mehlich-1 method (Silva et al., 2016).

The lineages ('BAF07' and 'BRS Embaixador' parents) and segregating populations ( $F_2-F_5$ ) chosen for the experiment (Table 1) were those that presented the best root system results in the study of Grigolo et al. (2021), which started in 2016, when a complete diallel with six parents was carried out and 30 progenies were taken to the field. The lineages were from different commercial groups and gene pools, and the segregating populations were obtained by self-pollination and conducted by the bulk population method.

The experiment was randomly carried out in a  $6 \times 6$  partially balanced simple lattice design, with 36 treatments in a  $6 \times 2 \times 3$  arrangement of genotypes,

phenotyping methods, and phenological stages, with two replicates. Each site consisted of 72 experimental units, with 1.5 m width and 1.5 m length, spaced at 0.5 m from each other. Sowing density was 13 seeds per linear meter, with a final population of 260,000 plants per hectare. In the municipality of Lages, the seeds were sown on 11/26/2020, and, in Concórdia, on 12/23/2020. All other management practices during the cycle followed the technical recommendations for the common bean crop (Fancelli & Dourado Neto, 2007; Silva et al., 2016).

The levels of the genotype factor, or structured specific qualitative factor, consisted of the two used lineages. The 'BAF07' lineage, belonging to the Mesoamerican group, has small seeds weighing 200–250 g per 1,000 seeds, S-type phaseolin, and plants with types II and III growth habits. The 'BRS Embaixador' lineage, belonging to the Andean gene group, presents large seeds weighing 350–500 g per 1,000 seeds, T-type phaseolin, and plants with types I and IV growth habits. These lineages also contrasted for the root distribution trait, which was observed in complete diallel arrangements due to the distinct general combining abilities between the lineages.

The phenotyping method factor, or specific unstructured qualitative factor, consisted of two methods, called Shovelomics, with modifications, and WinRHIZO. The Shovelomics method was developed by researchers at the Pennsylvania State University, aiming at the rapid phenotyping of the root system in the field. This is a simple, robust, and inexpensive tool in which roots are placed on a template to directly obtain information on the root system variables

(Burridge et al., 2016). WinRHIZO method consists of a scanning software system operated by the Epson Expression 10,000 XL scanner (Epson America, Inc., Los Alamitos, CA, USA), on which the plant root system is placed for the software to automatically measure its variables.

The evaluation of the stage factor, a specific unstructured qualitative factor, comprised the three following phenological stages of the common bean crop: V<sub>4.4</sub>, four trifoliate leaves; R<sub>6</sub>, flowering; and R<sub>8</sub>, grain filling (Gepts & Fernández, 1982). The environmental factor was considered the combination of the non-genetic factors: method, stage, and site.

For both used methods, when the plants of the experimental units reached the phenological stages for phenotyping, the root system and the surrounding soil were removed from the field by carefully excavating the soil at approximately 0.25 to 0.30 m around the plant stem and at a depth of 30 cm. After this, the root system was exposed, and excess soil was removed. The roots were immersed in a recipient with water and neutral detergent, at a concentration of 5%, to clean the root system.

Subsequently, for the adapted Shovelomics method, the following root system variables were calculated: root basal angle; vertical length, in centimeters; left horizontal length, in centimeters; and right horizontal length, in centimeters. The root basal angle is considered the most important variable of the original method (Trachsel et al., 2011).

For the WinRHIZO method, the following variables were evaluated: total length, in centimeters; projected area, in square centimeters; root volume, in cubic centimeters; and root average diameter, in millimeters. The application of the multivariate analysis of variance was essential, since the information collected consisted of samples with different variables evaluated in each experimental unit.

The response variables of the methods were standardized with the log y+1 transformation, due to their different magnitudes. To verify the possible combination of the information collected in the experiments, the waste matrices of the sites were compared using the Mantel's t test. The acceptance of the normality hypothesis between matrices (t=1.85; p=0.960) indicated the suitability of the performance of the joint analysis. In addition, the hypothesis of multivariate normality was tested, assuming

**Table 1.** Six common bean (*Phaseolus vulgaris*) genotypes (G1–G6) used to evaluate root phenotyping methods and the ideal phenological stages to quantify the root system.

Identifi- cation	Denomination	Commercial group	Origin
G1	'BAF07' <sup>(1)</sup>	Black	Lineage
G2	'BRS Embaixador' <sup>(2)</sup>	Red	Cultivar
G3	F <sub>2</sub> Progeny	Black	Self-pollinated F <sub>1</sub>
G4	F <sub>3</sub> Progeny	Black, brown	Self-pollinated F <sub>2</sub>
G5	F <sub>4</sub> Progeny	Black, brown	Self-pollinated F <sub>3</sub>
G6	F <sub>5</sub> Progeny	Black	Self-pollinated F <sub>4</sub>

<sup>(1)</sup>Accession from the Active Germplasm Bank of Universidade do Estado de Santa Catarina. <sup>(2)</sup>Commercial cultivar developed by Empresa Brasileira de Pesquisa Agropecuária.

normality at a significance level of 0.05 by Mardia's test ( $p=0.739$ ).

The used statistical model was represented by:  $Y = XB + E$ , where Y is the matrix of the set of response variables, X is the design matrix, B is the parameter estimation matrix, and E is the matrix of residual variances and covariances. Initially, the hypothesis of the multivariate normality of the data was tested with Mardia's test, based on measures of asymmetry and kurtosis. After the multivariate analysis of variance, contrasts between mean vectors were performed to explore the simple effects of the interaction between the studied factors. All analyses were carried out using the SAS software (SAS Institute Inc., Cary, NC, USA), and the general linear model procedure, with the multivariate analysis of variance option.

## Results and Discussion

Based on the analysis of variance for the two experimental sites, a significance of 0.05 was detected for method  $\times$  stage  $\times$  site triple interaction and for the stage  $\times$  site, method  $\times$  site, and method  $\times$  stage interactions (Table 2). The significance of the triple interaction was obtained between the methods, combined with the stages of evaluation, which can be altered according to the levels of the site factors.

This significant variation can be supported by phenotypic differences in plants in different environments, that is, the environmental factor may prevail in the observed interaction (Walter et al., 2015).

The significance of the method  $\times$  stage  $\times$  site factors indicates that a particular phenotyping method should be used in a specific phenological stage for the evaluation of populations in plant genetic breeding programs. The use of a suitable root evaluation method, combined with a specific phenological stage, can be more effective in the selection of promising genotypes for several agronomic traits of interest to breeders. Regarding the effect of the method  $\times$  stage  $\times$  site interaction, the first two canonical discriminant functions captured 94 and 6% of accumulated variance, respectively (Table 2). The variables basal root angle, total root length, vertical length, and root projected area presented standardized canonical coefficients ranging from -0.03 to -0.46 and reduced the discriminative potential between the studied factors.

The variables right horizontal length, left horizontal length, root volume, and root average diameter showed values from 1.58 to 5.60, which makes them important for the discrimination of determinant factors to the interaction.

The combination of variables from different phenotyping methods is essential for the selection of common bean plants with a root system adapted to adverse cultivation conditions. Polania et al. (2017) reported a positive association between root mean diameter and total volume, a simple linear correlation of  $r=0.39$ , and a negative association between root mean diameter and total length ( $r=-0.61$ ), when using WinRHIZO. These associations indicate that plants with a greater average root diameter occupy a larger volume of soil. The more soil volume a plant occupies, the more nutrients from this medium it absorbs; consequently, these plants are more likely to adapt to environments with water or nutritional restrictions. Therefore, since these traits carry important information for genetic breeding programs, they should be identified in the stages of plant selection, segregating population conduction, and genotype selection.

**Table 2.** Joint multivariate analysis of variance using Wilks' lambda test ( $\lambda$ ) of the factors replicate, replicate within blocks, genotype, phenotyping method, phenological stage, and cultivation sites of common bean (*Phaseolus vulgaris*).

Source of variation	$\lambda$	Pr > F
Replicate	0.98*	0.044
Block/Replicate	0.93	0.673
Genotype (G)	0.95	0.074
Method (M)	0.01*	0.001
Stage (E)	0.65*	0.001
Site (S)	0.92	0.001
G $\times$ M	0.95	0.080
G $\times$ E	0.94	0.626
M $\times$ E	0.86*	0.001
G $\times$ S	0.97	0.505
M $\times$ S	0.93*	0.001
E $\times$ S	0.94*	0.001
G $\times$ M $\times$ E	0.95	0.930
G $\times$ M $\times$ S	0.96	0.329
G $\times$ E $\times$ S	0.93	0.377
M $\times$ E $\times$ S	0.93*	0.001
G $\times$ M $\times$ E $\times$ S	0.96	0.965

The null hypothesis is  $H_0: \mu_1 = \mu_2 = \dots = \mu_k$ . \*Significant by the F-test, at 5% probability. Pr, probability.

The main genotype effect and its interactions were not significant at the level of 0.05 by Wilks' lambda test (Table 2). Moreover, the performance of the fixed genotypes, when compared with that of the segregating ones, for the set of root system traits, was not affected by the used phenotyping method, the phenological stage of evaluation, or the cultivation site of the genotypic constitutions. However, a large effect of the crop stage development factor was observed, indicating that non-genetic factors influence the phenotyping of the root system in the field. This result may be relevant for plant breeding programs to select genotypic constitutions in the field, targeting traits of the root system.

The lack of significance of the genotype  $\times$  method  $\times$  stage  $\times$  site interaction indicates that, regardless of the genetic constitution (fixed/segregant), the phenotyping method used by the breeders at a given stage of crop development will not be affected by the environmental effect of the evaluation site. Therefore, the most accessible method of root phenotyping can be selected according to the working conditions and financial resources available to the genetic breeding program.

Rascher et al. (2011) and Fenta et al. (2014) highlighted that evaluating phenotype root system variables on a large scale, over the years, in large sites, is often unfeasible due to the required time and financial resources. In the case of the present work, 144 experimental plots in the field were used in both evaluation sites, meaning that, altogether, the traits of the root system of 720 plants were evaluated, which required 120 hours of work.

The relation among the non-significant effect, the genotype factor and the evaluation sites is intriguing, since the sites of Concórdia and Lages, both located in the state of Santa Catarina, are contrasting regarding soil traits, mean air temperature, precipitation, and altitude. The cultivation cycle in Concórdia lasted 104 days, the highest average air temperature was observed in December, and the accumulated rainfall was 430 mm during cultivation. In Lages, the plants reached the harvest stage after 110 days, the highest temperatures were reported in January, and the accumulated rainfall was 490 mm. These different sites were chosen to verify the environmental effect of the genotype performance, since most works on root systems are restricted to one environment, i.e., one growing site.

The equal performance of the genotypes in relation to the localities may be related to the high plasticity of the root system traits in response to the environment, as previously observed (Nord et al., 2011). Phenotypic plasticity is an important phenomenon to increase the absorption of resources by plants, which expenditure more energy for the formation of aerial organs than of the root system (Fischer et al., 2018; Schneider & Lynch, 2020).

The absence of a significant effect for the main genotype factor and its interactions for all variables measured simultaneously also indicates that segregating populations do not differ from the fixed ones (Table 2). This finding may be due to the number of performed hybridizations, divergence between parents, and likely occurrence of linked genes, which can impair the expression of segregation.

As segregating populations do not differ from fixed populations, root trait can be selected at more advanced levels of homozygosity. This is related to simultaneous improvement of other traits, such as root system development that correlates negatively with grain yield. Therefore, the drastic selection of the root system in early stages of inbreeding can destabilize the balance between these traits.

The obtained results that the parents selected for the hybridization and formation of the segregating populations did not differ significantly regarding the genotypic constitutions of the studied. The proximity to segregating populations despite the contrasting root system of the parents may be related to the additive gene action that governs this trait and to the similar phenotypic plasticity between the genotypes. When it comes to gene group, genotypes from each group have different root phenotypes.

The similar performance of common bean genotypes for traits related to the root system has also been reported in other studies. Melo et al. (2018) found these traits showed a reduced genetic variance throughout the years of genetic breeding, i.e., that they are markedly ruled by additive genetic effects (Mukankusi et al., 2011). These effects make it possible to obtain grains through selection when considering the genetic breeding strategies of autogamous plants, including bean, soybean [*Glycine max* (L.) Merr.], wheat (*Triticum aestivum* L.), and oat (*Avena sativa* L.). Therefore, progenies with a superior genetic performance are a result of screening plants

with superior traits, which tend to be maintained over segregating generations (Gurmu et al., 2018).

The parents, however, showed significant differences for several agronomic traits due to their distinct gene groups. According to Jochua et al. (2020), genotypes from the Mesoamerican group present root phenotypes that are markedly adapted to water restriction, with a steeper basal root angle and a greater amount of resources applied for the development of the primary root, which, consequently, increases in size and allows of a greater hydraulic conductance in the root system. However, genotypes from the Andean group present phenotypes for adaptation to suboptimal conditions of mineral elements, especially phosphorus, due to their greater number of basal roots, shallower growth angle, and greater lateral proliferation.

The simple effects fixing the evaluation stage factor for each method, in each location (contrasts 1, 2, 4, 5, 7, and 8) and between locations (contrasts 3, 6, and 9), showed that the methods did not differ significantly between sites for the three evaluation stages (Table 3). Therefore, the significance of the triple interaction verified for these factors derives exclusively from the variation between the phenotyping methods and the stages in which the evaluation was carried out.

The used methods varied at all stages of evaluation in each site, showing the greater effect of the stage factor than the site factor for the evaluation of the genotypic constitution of root system traits. Bulyaba et al. (2020) also reported the reduced effect of evaluation sites on the expression of important agronomic traits in bean crops, concluding that the breeder can reduce the levels of this low significance factor and increase that of factors with a contrasting performance.

Therefore, the obtained results indicate it is convenient to increase the levels of the stage factor, to obtain more precise information on the exact combination between the phenotyping method and most appropriate phenological cycle for carrying out the evaluations of the traits of interest. In all studied contrasts, the variables left horizontal length and root volume presented the highest values of standardized canonical coefficients, which gives them a greater power to discriminate both methods in the three evaluation stages (Table 3).

Traits with a greater discriminative power contribute to identify plants with a more developed root system

and better water and mineral absorption. Galvão et al. (2019) highlighted the traits root volume, diameter, and biomass as the main factors responsible for the discrimination of bean genotypes with agronomic potential for water deficit tolerance. Also, in the bean crop, Jochua et al. (2020) observed a positive and significant association between root volume and number of pods ( $r=0.80$ ) and between root volume and pod mass per plant ( $r=0.35$ ). Therefore, associations between easily visible traits, usually intrinsic to the aerial part of the plants, and variables of the root system are fundamental to facilitate the indirect selection of traits of agronomic relevance.

Considering the standardized canonical scores for the two canonical discriminant functions of the three stages used for phenotyping, stage  $R_8$  was the most appropriate for evaluation in the Shovelomics method since its positive canonical coefficients repel the treatments (Figure 1). The  $R_6$  stage showed the highest discrimination of treatments in the WinRHIZO phenotyping method. Therefore, the obtained data are able to indicate the best root evaluation method and phenological stage to carry out the phenotyping of plants for root system traits. In the literature, plants are usually evaluated in the flowering or flower bud stage, the development stages in which they are more

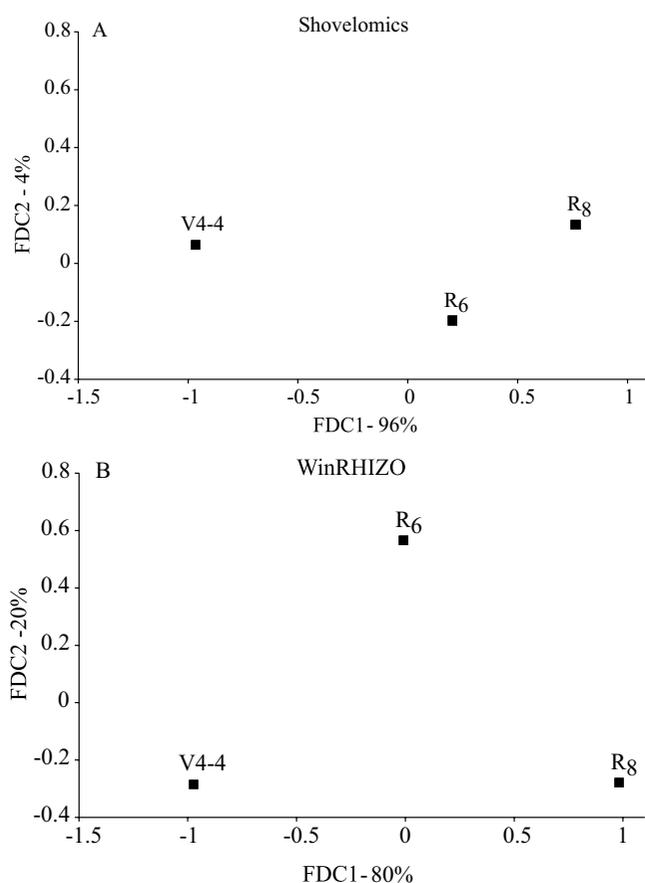
**Table 3.** Multivariate contrasts based on Wilks' lambda ( $\lambda$ ) test for the factors phenotyping method and phenological stage of common bean (*Phaseolus vulgaris*) in the cultivation sites of Concórdia ( $L_1$ ) and Lages ( $L_2$ ), in the state of Santa Catarina, Brazil<sup>(1)</sup>.

Contrast	$\lambda$	Pr>F	CAN1	CAN2	CAN3	CAN4
Stage $V_{4.4}$						
1. $M_1 \times M_2L_1$	0.03*	0.001	0.12	-0.27	7.64	-1.58
2. $M_1 \times M_2L_2$	0.05*	0.001	0.10	-0.25	6.40	-1.62
3. $M_1 \times M_2$	0.99	0.201	0.64	-0.67	1.75	2.34
Stage $R_6$						
4. $M_1 \times M_2L_1$	0.04*	0.001	0.18	-0.23	7.40	-1.75
5. $M_1 \times M_2L_2$	0.07*	0.001	0.09	-0.19	6.23	-1.82
6. $M_1 \times M_2$	0.99	0.550	1.26	0.20	-4.35	2.35
Stage $R_8$						
7. $M_1 \times M_2L_1$	0.04*	0.001	0.07	-0.25	7.55	-1.71
8. $M_1 \times M_2L_2$	0.06*	0.001	0.10	-0.23	5.95	-2.08
9. $M_1 \times M_2$	0.98	0.920	-0.88	-0.58	6.10	0.35

<sup>(1)</sup>Pr, probability; CAN1–CAN4, standardized canonical variables 1 to 4;  $M_1$ , Shovelomics; and  $M_2$ , WinRHIZO. \*Significant by the F-test, at 5% probability.

sensitive to water stress (Galvão et al., 2019). As to the phenotyping method, that of Shovelomics was also recommended by Velho et al. (2017).

As discussed, the task related to the evaluation and quantification of roots in the field is still arduous and challenging for plant breeders (Hochholdinguer et al., 2018). Therefore, advances in research to facilitate a high-throughput phenotyping of a large number of genotypes is essential for advances in genetic improvement programs. The information obtained in the present work, seeking to determine an ideal method and phenological stage for root system phenotyping, can provide significant gains in the selection of bean genotypes with more developed roots.



**Figure 1.** Dispersion of standardized canonical scores for the first two canonical discriminant functions (FDC) estimated for the phenotyping methods Shovelomics and WinRHIZO.

## Conclusions

1. The Shovelomics and WinRHIZO phenotyping methods are efficient in quantifying the root system of common bean (*Phaseolus vulgaris*) and show specificity for phenological stages, regardless of the evaluated genotype.

2. The root system quantification of fixed and segregating genotypes is analogous in both the Shovelomics and WinRHIZO phenotyping methods.

3. The Shovelomics method is more efficient for evaluating the root system of common beans at the R<sub>8</sub> stage, and the WinRHIZO method, at the R<sub>6</sub> stage.

## Acknowledgments

To Fundação de Amparo à Pesquisa do Estado de Santa Catarina (FAPESC), for financial support; to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for financing, in part, this study (Finace Code 001).

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