Sample size for measurement of root traits on common bean by image analysis

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Abstract – Evaluation of root traits may be facilitated if they are assessed on samples of the root system. The objective of this work was to determine the sample size of the root system in order to estimate root traits of common bean (*Phaseolus vulgaris* L.) cultivars by digital image analysis. One plant was grown per pot and harvested at pod setting, with 64 and 16 pots corresponding to two and four cultivars in the first and second experiments, respectively. Root samples were scanned up to the completeness of the root system and the root area and length were estimated. Scanning a root sample demanded 21 minutes, and scanning the entire root system demanded 4 hours and 53 minutes. In the first experiment, root area and length estimated with two samples showed, respectively, a correlation of 0.977 and 0.860, with these traits measured in the entire root. In the second experiment, the correlation was 0.889 and 0.915. The increase in the correlation with more than two samples was negligible. The two samples corresponded to 13.4% and 16.9% of total root mass (excluding taproot and nodules) in the first and second experiments. Taproot stands for a high proportion of root mass and must be deducted on root trait estimations. Samples with nearly 15% of total root mass produce reliable root trait estimates.

Index terms: *Phaseolus vulgaris*, root area, root length, sampling.

Tamanho da amostra para determinação de caracteres radiculares do feijoeiro pela análise de imagens

Resumo – A avaliação de caracteres radiculares pode ser facilitada se realizada em amostras do sistema radicular. O objetivo deste trabalho foi delimitar o tamanho de amostras para determinação de caracteres radiculares no feijoeiro (*Phaseolus vulgaris* L.) pela análise digital de imagens. Cultivou-se uma planta por vaso, que foi colhida na emissão de vagens, em 64 e 16 vasos correspondentes a duas e quatro cultivares no primeiro e segundo experimentos, respectivamente. Amostras das raízes foram digitalizadas até completar o sistema radicular, estimando-se a área e comprimento radiculares. A digitalização de uma amostra demandou 21 minutos, e de todo o sistema radicular, 4 horas e 53 minutos. No primeiro experimento, a área e comprimento radicular estimados com duas amostras apresentaram, respectivamente, correlação de 0,977 e 0,860 com estes caracteres mensurados na raiz inteira; no segundo experimento, a correlação foi 0,889 e 0,915. O aumento da correlação com mais de duas amostras foi desprezível. As duas amostras corresponderam a 13,4% e 16,9% da massa radicular total (excluindo nódulos e pivotante) no primeiro e segundo experimentos. A raiz pivotante constitui elevada proporção da massa radicular e deve ser descontada na estimativa de caracteres radiculares por amostragem. Amostras com cerca de 15% da massa radicular total fornecem estimativas confiáveis de caracteres radiculares.

Termos para indexação: *Phaseolus vulgaris*, área radicular, comprimento radicular, amostragem.

Introduction

The root system plays important role in plant adaptation to edaphic limitations, such as water stress and low nutrient availability. Common bean (*Phaseolus vulgaris* L.) cultivars with larger root system and root to shoot ratio had increased growth in soil with low available P (Yan et al., 1995). Besides the bean genotype with higher P absorption efficiency had a branched root system with numerous basal roots, while the inefficient genotype had a smaller and less branched root system (Lynch & Van Beem, 1993). Therefore, genotypic selection for enhanced root growth would be a strategy for increasing P acquisition and grain yield in tropical soils usually with low available phosphorus.

Genotypic differences were reported in common bean for root biomass, root to shoot ratio, root area and radius (Fawole et al., 1982; Yan et al., 1995; Araújo et al., 1998), root architecture and topology (Lynch & Van Beem, 1993; Bonser et al., 1996), basal root gravitropism (Liao et al., 2001), and root distribution along the soil profile (Guimarães et al., 1996), revealing the possibility of selecting bean cultivars for root traits. Screening for root traits may be facilitated if interest traits are expressed in the seedling stage and are stable over time, if they can be assessed on a sample of the root system, and if they present substantial genotypic variation (Lynch & Van Beem, 1993).

The evaluation of large root systems is a laborious and time-consuming task, making difficult its insertion into breeding programs. Root quantification can be improved through suitable sampling methods which combine rapidity and precision, allowing the examination of a relatively large number of plants. However, the design of an effective scheme to sample the root system is difficult, because of the complex branched root structure, the spatial variability of root distribution, and the opaque growing environment (Bengough et al., 2000). The problem of large variation between replicate root samples is widely known, often requiring too many samples to obtain an accurate estimate of the mean (Bengough et al., 2000). Computer electronic image analysis have made root examination faster and more accurate, and the desktop scanners available nowadays provide high quality optical resolution for recording root images, but root measurement is still time-consuming due to the great root length that can be found in a single plant (Costa et al., 2000; Richner et al., 2000).

For maize (*Zea mays* L.) plants, samples with 20% of total root volume produced satisfactory estimation of total root length by the photoelectric method (Rossiello et al., 1995), whereas 10% of total root volume can be sampled for estimation of the entire root system by image analysis with an accuracy within 10% (Costa et al., 2000). However, root morphology of dicotyledon plants is usually more complex than that of monocotyledons, many dicots possessing a taproot and basal roots from which lateral roots arise, and sometimes adventitious roots arising from nonroot tissues (O'Toole & Bland, 1987). Many studies have focused different techniques for measuring roots, but the design of sampling schemes for roots that are adequate for different situations is rarely considered (Bengough et al., 2000).

The objective of this work was to determine the sample size of the root system for estimating root traits of common bean cultivars by digital image analysis.

Material and Methods

Two experiments were carried out at Embrapa Agrobiologia, as part of a broad study on the inheritance of root traits of common bean under conditions of low P availability. In the first experiment, cultivars Carioca and ICA Pijao were studied, whereas the second one studied cultivars Carioca, ICA Pijao, Ouro Negro and Puebla 152. These cultivars present different architecture, i.e., while ICA Pijao has an erect indeterminate growth habit (type II), Carioca, Ouro Negro and Puebla 152 present a prostrate indeterminate growth habit (type III). Moreover, the cultivar ICA Pijao has a high root area, whereas Carioca and Ouro Negro present a high root efficiency ratio, i.e., total P content per root area (Araújo et al., 1998).

In each experiment one plant was grown per pot with 3 kg of soil, and pots were disposed in randomized blocks in a greenhouse. The first and second experiments comprised 32 and 4 plants per cultivar, respectively, summing 64 and 16 pots. The substrate of both experiments was a 6-mm sieved sandy clay loam soil (Ap horizon of Haplustult soil), with 3 mg kg^{-1} available P (Mehlich-1), 26 $mmol_c$ kg^{-1} Ca+Mg , water pH 5.0, and 8.5 g kg^{-1} C (Walkley & Black). The soil of each pot received 0.5 g kg^{-1} $CaCO_3$ and, nine days later, the following nutrients in a diluted solution (in mg kg^{-1} soil): 30 P (KH_2PO_4), 10 Mg ($MgSO_4.7H_2O$), 2 Cu ($CuSO_4.5H_2O$), 1 Zn ($ZnSO_4.7H_2O$), 0.05 B (H_2BO_3), 0.2 Mo ($Na_2MoO_4.2H_2O$), 1 Fe (Fe-EDTA). The substrate of each pot was homogenized, presenting, at sowing time 7 mg kg^{-1} available P and water pH 5.6.

At sowing, liquid inoculant with the strains BR322 and BR520 of *Rhizobium* was placed into the hole made for the seeds. At 25 days after emergence, 60 mg N per pot were applied as NH_4NO_3. Plants were harvested at the stage of pod setting, 45 days after emergence. The 3-kg pots guaranteed an almost unrestricted root growth since no curling roots were observed at harvest. Leaves, stems and pods were separately oven dried and weighed. Roots were recovered by carefully washing the soil through a 2 mm sieve and rinsed in running water to eliminate soil debris, maintaining the integrity of the root system. Roots were placed into a formaldehyde 2% solution.

Root samples without nodules were mounted between 20x30 cm acetate sheets and scanned in 256 gray-levels and resolution of 150 dpi. On the procedure of sampling, entire basal root axes arising from taproot were placed on the acetate sheet, and lateral roots were carefully spread using a needle. In four plants of each cultivar of the first experiment, and in all plants of the second experiment, such procedure of sampling continued until the entire root system had been scanned. In the 24 remaining plants of the first experiment, only two samples were scanned per plant. Only two trained operators scanned all roots. The scanned root samples were dried and weighed. Nodules were detached, counted, dried and weighed. The taproot was separated from the remaining root system by scissors, and these portions were dried and weighed. Root portions were weighed with a precision of 0.1 mg.

On the scanned root images, the root area and length were measured by the software SIARCS 3.0 (Embrapa Instrumentação Agropecuária). Initially, the image was segmented by the gray-level thresholding technique to produce binary images, and the projected root area was estimated. Further, objects in segmented binary images were reduced to a skeleton or center line, from which root length was measured (Richner et al., 2000). Assuming that roots are cylindrical, the projected root area was multiplied by π in order to obtain surface root area. Using the ratio between the mass of the scanned root sample and the total root system (excluding taproot and nodules), the total root area and length were calculated. Specific root area and length (root area and length per root mass) were calculated for each plant.

Simple correlation between root traits estimated from root samples and measured in the entire root system was estimated for each experiment. In order to compare the values of root traits estimated from root samples with the same values measured in the entire root system, an analysis of variance was performed considering these sampling methods as a source of variation and each pot as a replicate.

Results and Discussion

On average, scanning an entire root system required the mounting of 15 sheets, each sheet demanding 21±6 minutes to assembly a root sample. Thereby, scanning an entire root system of a single 45-day-old bean plant requires 4 hours and 53 minutes (±58 minutes), being a very laborious task that hampers its execution for a large number of plants. The amount of root placed on the sheet affects the accuracy of the estimates, since too many roots increase overlapping and crossing over (Costa et al., 2000), and depending on the size of sample, spreading roots can be very time-consuming (Richner et al., 2000). Bean root samples in the mounted sheets had mean dry mass of 60±11 mg and length of 7.4±1.1 m. Studying a digital line-intercept method, Farrell et al. (1993) observed less variability on wheat (*Triticum aestivum* L.) root samples with total length varying from 2 to 4 m.

A previous assay denoted that weighing root samples by root fresh mass introduced large errors. The sum of fresh mass of root samples corresponded approximately only to 20% of the total root fresh mass at the beginning of root scanning. Continuous manipulation of root system and the photoelectric scanner strongly dehydrated the root system, and almost 80% of its mass was lost as water. Hence, the dry mass must be used for calculating the ratio between the mass of the root sample and the entire root system, although the turgid root fresh weight provides an estimation of root volume (Nye & Tinker, 1977).

In both experiments, every correlation coefficient between the root traits estimated from root samples and measured in the entire root system was higher than 0.76 and statistically significant at 0.001 probability level (Figure 1). In the first experiment, the root area and length estimated with one sample of the root system had correlation of 0.909 and 0.765 with the root area and length measured in the entire root system; using two samples, the correlation was 0.977 and 0.860. In the second experiment, the root area and length estimated

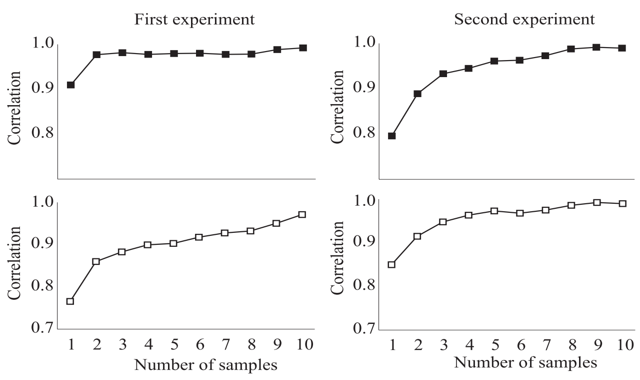

Figure 1. Coefficient of correlation between root traits estimated from root samples and root traits measured in the entire root system of common bean plants, as regard to root area (■) and root length (□). In the first and second experiments, two and four cultivars were evaluated, respectively, with four replicates per cultivar.

with one sample of the root system had correlation of 0.796 and 0.850 with the root area and length measured in the entire root system; using two samples, the correlation was 0.889 and 0.915. As the number of samples increased, the accuracy of root estimates also increased, but more than three samples improved negligibility the correlation; additionally, all correlation coefficients did not differ statistically.

In the first and second experiments, these two root samples corresponded, respectively, to 13.4±3.8% and 16.9±3.5% of the total root mass (excluding taproot and nodules). It must be noticed that a single plant was grown per pot, and each root sample corresponded indeed to one mounted acetate sheet. Studies with maize plants indicated that samples with 10% (Costa et al., 2000) or 20% (Rossiello et al., 1995) of total root volume produced satisfactory estimation of root length. The results of the present work indicate root samples of almost 15% of total root mass for estimating root traits of common bean.

In the first experiment, the analysis of variance identified no significant difference between values of root area and length estimated from root samples or in the entire root (Table 1). However, in the second experiment, which comprised four cultivars, the root area estimated from one or two samples was lower than the root area measured in the entire root; the root length using up to three samples was lower than that measured in the entire root (Table 1). Irrespectiving of the number of samples, there was no significant method x cultivar interaction, denoting that possible errors introduced by the sampling method were relatively constant for all cultivars.

Table 1. Values of root area and length estimated from root samples or measured in the entire root system of common bean plants. In the first and second experiments, two and four cultivars were evaluated, respectively, with four replicates per cultivar.

Number of root samples	First experiment		Second experiment	
	Root area (m^2 $plant^{-1}$)	Root length (m $plant^{-1}$)	Root area (m^2 $plant^{-1}$)	Root length (m $plant^{-1}$)
1	0.237	110	0.160*	82*
2	0.228	107	0.166*	86*
3	0.230	111	0.169	89*
4	0.228	111	0.170	91
Entire root	0.240	115	0.175	99

*Significant difference from the root area and length measured in the entire root system by F test at 0.05 level.

Additionally, the angular coefficients of the regression equation of root traits measured in the entire root system on root traits estimated from two root samples were lower than 0.9 but they did not differ statistically from 1.0 (Figure 2). This fact indicates that using two root samples slightly underestimated root area and length as compared to measure the entire root system. Such underestimation of root area and length by the sampling method can be partially ought to the sampling procedure, when entire root axes arising from taproot were excised and scanned. As sampling proceeded, the basal root axes were becoming thinner, and the latter acetate sheets were likely to posses a higher root area and length per unit root mass. However, simple correlation between specific root area and length of the root samples and their temporal position in the sampling procedure was not significant in both experiments, denoting that such presumable sampling error was not systematic.

The matter of extracting homogeneous root samples from a root system was discussed by Costa et al. (2000), who described an automatic method for collection of root samples of maize plants for image analysis. However, their procedure required approximately 43 hours to analyze an entire root system, and even by sampling, more than 3 hours were needed for a single plant, such large amount of time making almost impracticable its use for a great number of plants. Actually, the relative importance of the various root classes for root system function remains uncertain in beans, and more investigation is required to choose a representative root fragment for convenient root analysis (Lynch & Van Beem, 1993).

Total root mass, lateral root mass, root area and root length presented similar coefficient of variation in both experiments (Table 2), denoting that the root sampling

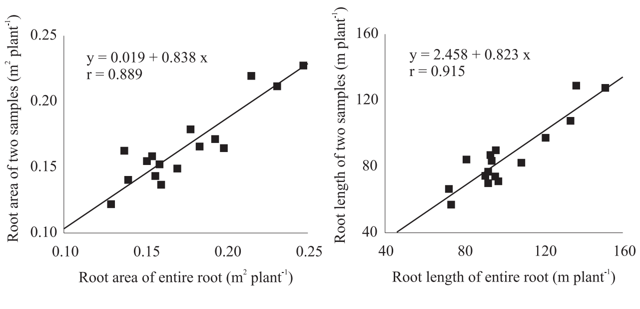

Figure 2. Comparison of root traits estimated from two root samples and measured in the entire root system of common bean plants. Data from the second experiment, with four cultivars and four replicates per cultivar.

and digital image analysis did not introduce additional experimental errors besides those involved in plant growth and harvest. Root length has been used more often to describe root systems, mainly due to the widespread line-intercept method, but root area has been applied in many nutrient and water absorption studies (Nye & Tinker, 1977; Barber, 1984). Richner et al. (2000) argued that surface area measurements by image analysis are expected to compact extraneous objects, which falsely increase the area of a sample even more than the length. The physiological bases for the choice between root area or root length to describe root systems deserve further studies, but the digital image analysis permits the simultaneous measurement of both root traits.

In the first experiment, taproot mass corresponded to 13.8% and 11.4% of total root mass of the cultivars ICA Pijao and Carioca, respectively (Table 2). In the second experiment, the proportion of root mass allocated on taproot ranged from 18.0% in cultivar Ouro Negro to 21.2% in cultivar ICA Pijao. Contrariwise, the contribution of taproot for root area and length was negligible. Stoffella et al. (1979) observed that taproot mass represented 37% of the total root mass of bean cultivars at flowering in a greenhouse experiment. Therefore, the taproot mass must be discounted on estimating root traits from root samples of bean plants. The cultivar ICA Pijao had the highest taproot mass (Table 2). Taproot is likely to be important to uprightness of bean plants (Stoffella et al., 1979), hence the stronger taproot of the erect cultivar ICA Pijao supports an association of root and shoot architectures (Lynch & Van Beem, 1993).

Cultivar Carioca produced more nodules than ICA Pijao in the first experiment, and in the second experiment Carioca had the highest number of nodules although differences among cultivars were not statistically significant (Table 2). Hence Carioca confirmed its potential for nodulation under low P supply (Araújo & Teixeira, 2000).

Evaluating the adequacy of sampling to characterize bean cultivars as regard to root traits, the root area and length were estimated by using two samples in the 32 replicates of the two cultivars of the first experiment, and in the four replicates of the four cultivars of the second experiment. Cultivar ICA Pijao presented the greatest total root mass in both experiments (Table 2). ICA Pijao also had higher root area and length than Carioca in both experiments, mainly due to its greater lateral root mass, since the cultivars did not differ in specific root area and length in both experiments (Table 2). Comparing wild and cultivated bean genotypes in pot experiments, Araújo et al. (1998) also observed a strong root growth of ICA Pijao, such vigorous rooting confirmed in a field experiment (Araújo et al., 2000). Araújo & Teixeira (2000), using a photoelectric area meter, obtained root area of 0.11 m^2 $plant^{-1}$ for bean cultivars with near 1 g $plant^{-1}$ on root system. Such low values of root area, as compared to Table 2, were probably caused by the less sensible photoelectric device for detecting fine roots.

Table 2. Traits of root system of common bean cultivars evaluated in two experiments; root area and length were evaluated from two samples of the root system. Means of 32 and 4 replicates for each cultivar in the first and second experiments, respectively[(1)].

Cultivar	Number of nodules per plant	Nodule mass (mg $plant^{-1}$)	Taproot mass (g $plant^{-1}$)	Lateral root mass (g $plant^{-1}$)	Total root mass (g $plant^{-1}$)	Root area (m^2 $plant^{-1}$)	Root length (m $plant^{-1}$)	Specific root area (m^2 g^{-1})	Specific root length (m g^{-1})	Taproot:total root ratio (%)
					First experiment					
ICA Pijao	164b	71a	0.187a	1.11a	1.37a	0.245a	116a	0.222a	104a	13.8a
Carioca	230a	84a	0.142b	1.03b	1.25b	0.228b	107a	0.223a	105a	11.4b
CV (%)	62.2	62.6	20.8	17.0	18.7	19.7	22.1	12.3	16.4	15.4
					Second experiment					
ICA Pijao	84a	67a	0.268a	0.95a	1.28a	0.189a	99a	0.200a	104a	21.2a
Ouro Negro	78a	72a	0.174b	0.72b	0.97b	0.157ab	77bc	0.217a	107a	18.0a
Carioca	101a	50a	0.194b	0.72b	0.96b	0.143b	75c	0.199a	103a	20.0a
Puebla 152	67a	38a	0.206b	0.88ab	1.12ab	0.176ab	94ab	0.200a	107a	18.4a
CV (%)	51.2	51.3	13.9	12.6	12.2	14.1	13.3	9.4	7.9	11.2

[(1)]Means followed by the same letter do not differ by Duncan test at 0.05 level.

Bean cultivars did not differ for specific root area and length in both experiments (Table 2). Lynch & Van Beem (1993) also verified no genetic differences in specific root length in bean cultivars at 14 days of growth. Cultivar differences for root area and length in both experiments were mainly due to variations in root mass rather than to differences in root thickness, which could justify the measurement solely of root mass for screening bean genotypes (Table 2). However, Araújo & Teixeira (2000) observed that root radius of bean cultivars varied in different pattern as plant aged, and Lynch & Van Beem (1993) registered that root architectural parameters varied substantially through time. Moreover, root traits such as length, surface area and branching patterns influence nutrient uptake in a more complex manner that can be described by root mass (Barber, 1984). Therefore, in spite of the probable narrow genotypic variation in specific root area and length within bean germplasm, bean root area and length must be considered on more detailed nutritional studies.

Conclusions

1. Sampling procedure saves considerable time for root measurement, enabling the evaluation of a large number of plants.
2. Root area and length estimated from two root samples present correlation higher than 0.86 with these traits measured in the entire root system.
3. Root samples corresponding to almost 15% of total root mass (excluding taproot and nodules) provide reliable estimates of root traits of common bean cultivars.

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