A Sampling Method for Measurement of Large Root Systems with Scanner-Based Image Analysis

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ABSTRACT

Measurement of relatively small (<100 m total length, <6 g fresh wt.) root systems has been simplified by image analysis, but measuring larger root systems remains time-consuming and inaccurate (Zoon and Van Tienderen, 1990). Environmental effects on root development have been documented by a number of researchers (Drew and Goss, 1973; Onderdonk and Ketcheson, 1973). For example, Onderdonk and Ketcheson (1973) studied the effect of soil temperature on direction of corn root growth relative to the soil surface. They found that maize roots grew more vertically at soil temperatures above or below the 10 to 30°C range. At a sufficiently low soil water contents, mechanical impedance blocked root elongation most in soils with a high bulk density (Drew and Goss, 1973). When soil offers resistance to root penetration, axial expansion of cells is reduced in favor of radial expansion (Goss, 1977).

Diameter, color, growth potential, and surface texture are the main features of individual roots showing systematic variation (Fitter, 1996). Many of the root characteristics, such as length, average diameter, surface area, and mass, have been used to assess the quantity of roots and the functional size of the root system. Total root mass is usually deemed easier to measure than root length or surface area and has frequently been used to compare root systems (Carley and Watson, 1966; Murphy and Smucker, 1995). Box and Ramseur (1993), however, found more highly significant differences between treatments when wheat (Triticum aestivum L.) roots were compared on a root length rather than root mass basis. Total root mass alone cannot adequately describe many root functions involved in plant–soil relationships. However, total root length, surface area, and branching patterns have been shown to influence nutrient uptake (Raper et al., 1978). Consequently, estimates of root length per unit of soil are sometimes used in quantitative studies of water and nutrient uptake (Cowan, 1965; Brewster and Tinker, 1970).

A wide range of methods (Rowse and Phillips, 1974; Richards et al., 1979; Zoon and Van Tienderen, 1990) have been proposed for estimating root length, most...
based on the line-intersect principle first developed by Newman (1966) and modified by Marsh (1971) and Tennyson (1975). Roots are randomly dispersed over a flat, grid plane surface; root-gridline intersections are counted and the number is then used in a formula to calculate root length. The method is based on the assumption that the longer the root, the more often it will intersect the grid. A drawback of the line-intersection method is that it assumes a random root distribution, so errors in root length estimates can arise when this assumption is not met. In addition, when root overlapping occurs, root length can be underestimated. As the method relies on visual counting of root-gridline intersections, it can be time-consuming and somewhat subjective, especially when measuring samples with a large proportion of fine roots (Smit et al., 1994). Fine roots are also often underestimated when measuring roots with image analysis because they are not successfully detected during analysis due to their small diameter and near transparency (Burke and LeBlanc, 1988). Such roots account for a substantial proportion of total root length in a number of species (80% in barley, *Hordeum vulgare* L. [Hackett, 1968], and about 70% in maize [Pallant et al., 1993]).

Computer-assisted electronic image analyses have made root analysis less time-consuming and allowed more accurate and less subjective measurement of root characteristics than the human eye is capable of making (Collins et al., 1987; Cunningham et al., 1989; Stutte and Stryjewski, 1995; Box, 1996). Electronic methods can be categorized according to the image acquisition system used: (i) video camera (Otman and Timm, 1984; Cunningham et al., 1989) or (ii) optical scanner (Arsenault et al., 1995; Kaspar and Ewing, 1997). Improvements in lighting sources and technical developments in scanner technology have allowed enhanced image contrast over larger areas (Arsenault et al., 1995; Box, 1996). Some image analysis software has incorporated overlap correction methods, and no longer requires exposure threshold adjustment from the user (Arsenault et al., 1995; Kaspar and Ewing, 1997). This has resulted in greatly reduced labor requirements compared with previous techniques.

Notwithstanding significant advances in root study made possible by computer-assisted image analysis, root length measurement is still time-consuming, mainly because of the great length of roots that can be found in a single root system or in a small volume of soil. Dittmer (1937) reported a total root length of 622.8 km for a single winter rye (*Secale cereale* L.) plant grown in a 4.3-L wooden container for four months. Pavlychenko (1937) recorded, 80 d after emergence, the length of the entire root system of a single plant of wild oat at 0.388 km, of ‘Marquis’ wheat at 0.249 km, of a prolific spring rye at 0.37 km, and of ‘Hannchen’ barley at 0.405 km.

The issue of sampling arises when measuring entire root systems becomes impractical. Here two challenges present themselves: (i) how to obtain one or more representative (i.e., not skewed in the proportion of different root sizes or morphologies compared with the complete root system) subsamples, and (ii) the number and combined size of subsamples necessary to accurately estimate the length of the entire root system.

In most cases, it is difficult to obtain homogeneous subsamples because of heterogeneous mixing of root segments. A mixing device is required to allow homogeneous mixing of samples. Reports on subsampling of entire washed root systems are scarce, with most studies focusing on collecting root subsamples in the field by obtaining soil cores (Mackie-Dawson and Atkinson, 1991). To our knowledge, there have been no previous reports on subsampling of entire root systems for the measurement of total root system length by scanner-based image analysis. McGonigle (1994) described a technique for obtaining a single subsample of a given mass from washed roots. Root pieces were suspended in water and vigorously stirred using a glass rod. Samples were collected with a glass beaker as often as was necessary to obtain the required fresh weight. In any such technique, a too-small sample size might not allow the detection of small but important differences between treatment effects, whereas an excessively large sample size would constitute a waste of time and resources (Bros and Cowell, 1987; Pillar, 1998).

Several procedures are available to determine an appropriate sample size. The standard procedure involves analytical formulae in which the estimated variance of the sample mean is assumed to be equal to the population variance ($s^2$) divided by the sample size, when the sample size is sufficiently large and the sampling is simple and random (Sokal and Rohlf, 1995). Because $s^2$ is generally unknown, it must be estimated from the current sample or a preliminary sample of data (Cochran, 1977).

As an alternative, Efron’s (1979) nonparametric bootstrap procedure is based on intensive computation and data resampling to generate empirical distributions (Mooney and Duval, 1993). It allows the estimation of confidence intervals and confidence limits to assess sample sufficiency (Bros and Cowell, 1987; Efron and Tibshirani, 1993; Mooney and Duval, 1993; Pillar, 1998).

Many other factors are involved in the determination of the sample size, such as time and cost of processing root samples, variability among root samples, and the desired level of precision. Because of the absence of pertinent information in the literature, this study reports on the application of Cochran’s (1977) and Efron’s (1979) procedures to determine appropriate sample sizes for root measurements with scanner-based analysis systems.

Our objectives were to (i) develop and evaluate a system for collection of homogeneous root subsamples, (ii) determine the required sample size for measurement of large root systems, and (iii) evaluate genotype effects on sample size, all based on measurement with a scanning-based image analysis system.

**MATERIALS AND METHODS**

**Plant Material**

Root materials were collected from an experiment established in the research greenhouse of the Macdonald Campus of McGill University, Ste.-Anne-de-Bellevue, Quebec, Canada. Three different maize genotypes and three levels of N fertilization were factorially combined and replicated three times in a randomized complete block design. The maize genotypes studied were leafy reduced stature, leafy normal stature, and
Pioneer 3905 (nonleafy normal stature). The genotypes were chosen to provide contrasting root systems based on preliminary observations by A. Modarres (personal communication, 1996). The plants were grown in 63-L containers filled with a soil–sand mixture (2:1 v/v) with three N levels (0, 127.5, and 255 kg N ha\(^{-1}\)) but, only roots of plants grown at the 127.5 kg N ha\(^{-1}\) level, and only one root system of the three replicate plants, were analyzed for each of three maize genotypes.

Greenhouse growth conditions were 24/16°C (day/night) air temperature and 85% relative humidity. The light–dark cycle was 16/8 h. When daylength needed to be extended artificially, 430-W Phillips sodium lamps were used, resulting in a light intensity of 800 μmol m\(^{-2}\) s\(^{-1}\) at the canopy level. Light intensity was measured with a 1-m-long LI 190SB quantum sensor (Li-Cor, Lincoln, NE). Plants were grown from March to May 1998.

**Root Cleaning, Storing, and Staining**

Root systems were sampled only once, at midsilking, for each genotype. The entire root system was carefully removed by sliding it from its container. The stem was cut off and the root system washed, first by immersion in a water-filled container, then by spraying with water until it was almost free of soil and sand particles. Sieves of several mesh sizes (2 mm, 500 μm, and 53 μm) were used to prevent loss of fine roots. Prior to image analysis, roots were further cleaned by immersion in a water-filled basin, and any adhering particles were removed by hand. Root samples to be analyzed within 1 wk were placed in plastic bags and stored at 4°C. For longer-term storage (1 wk to months), roots were kept fully immersed in an ethanol solution (350 mL ethanol per liter of water). Root systems were stained for 15 min with 0.1% (w/v) toluidine blue just prior to analysis.

**Mixing System**

The mixing system was made from an 18-L (385 mm high, 275 mm diam.) plastic water container with the upper part removed (Fig. 1). Four equidistant holes were made in the walls 30.0 mm from the bottom of the container using a 10-mm drill. A 6.3-mm copper tube was placed around the outside of the container. The tube had three tee junctions and an end elbow that corresponded to the four holes in the plastic container. The inlet had an extended 6.3-mm copper tube 250 mm long to connect to an air source. Inside the container, each of the four entries had a 6.3-mm-diam. and 50-mm-long copper-tube injector bent to about 80° at its midpoint. These acted as air injectors and provided enough water movement to force heavier roots to circulate with lighter ones. The air injectors were connected to the outside copper tube with lock fittings (Swagelock, Solon, OH) and were oriented alternately toward the bottom of the container or perpendicular to it. The system was operated at an air pressure of 34.5 kPa. Rubber washers were placed inside and outside the container at the connections to ensure a good seal. A wide range of container sizes could be used for this system, depending on the size of the root material to be sampled. Transparent containers are advantageous because root movement and distribution can be easily monitored.

**Laboratory Procedures for Mixing and Collecting Subsamples**

The entire root system of each of three maize genotypes was cut with scissors into ~10-mm segments. This length was chosen because large diameter roots that were longer than 20 mm were not homogeneously mixed with the other root segments.

The mixing system was connected to an air source; the container was filled with water to 85% of its total volume, and the air was turned on. The stained roots were added in small amounts, and any clumps were teased apart. Approximately 15 min was allowed for roots to mix thoroughly. A small (50-mL) dip net was used to scoop about 3 g of material, which was put into a plastic tea infuser with handles removed. Fresh weight was used because it is relatively easy to determine. Fresh roots are largely composed of water; however, water content varied greatly among subsamples. This variation could affect the homogeneity of subsamples and the computation of the sample size. Thus, prior to root measurement excess water was removed by spinning subsamples in a salad spinner. Up to 20 scoops/infusers were placed into a salad spinner (Plastic Cabano, Quebec, Canada) and spun for 25 s (140 to 160 rpm) to dry them. The performance of the salad spinner was tested using 7 spinning times, from 10 to 40 s spaced at 5 s intervals. Subsamples of 0.5 g FW were then weighed out and any remaining material returned to the mixing system. The scoop/subsampling process was repeated as many times as necessary to collect all subsamples of an entire root system. A total of 957 subsamples (~185 scoops) were obtained: 362 for LRS, 423 for LNS and 172 for P3905.

**Root Measurement via Image Analysis**

Root measurements were performed with the WinRHIZO version 3.9 (Regent Instruments, Quebec City, Quebec, Canada), an interactive scanner-based image analysis system that controls scanning, digitizing, and analysis of root samples. Scans could be analyzed immediately or stored as TIFF files. We used a Windows-based PC, Pentium 100 system, with 32 MB RAM, and a Hewlett-Packard scanner (ScanJet 3c/T), set to 300 dots per inch (dpi; 118.11 dots per cm) scanning...
resolving the scanner had two light sources, one located above, on the scanner cover, and the other below, incorporated in the scanner main body.

Root subsamples were placed in the Plexiglas trays (200 by 300 mm) with a 3- to 4-mm-deep layer of water. Depth of the water layer varied with root size. It was adjusted to help untangle the roots and minimize root overlapping. Before taking measurements, several known masses of roots were placed on the tray, tested, and analyzed one at a time. An adequate mass was that which facilitated root untangling and resulted in a minimum amount of crossing over (i.e., 0.5 g).

The amount of root placed on the tray had an important effect on accuracy; too many roots increased overlapping, crossing over, and the time required for analysis. Use of the appropriate tray size and a smaller amount of root shortened the time required for each scanning. The analysis of a sample, on average, required 45 s to scan and 85 s for the computer process.

Calculation of Required Sample Size

Subsamples of each of the three root systems were used to determine the optimum number to analyze for each maize genotype. For the purpose of this study, sample size is the number of sampling units (e.g., Plexiglas trays with 0.5 g of spun fresh roots) that were collected from the mixer and taken from the entire root system for measurement with image analysis. In total, 957 root subsamples were analyzed. Root length, the root characteristic that varied the most, was selected as that on which the calculation of sample size would be based. The number of scanned subsamples sufficient to obtain the desired level of precision or margin of error was determined based on stability of root length estimation. This stability was established by the relationship between confidence interval, number of scanned subsamples, and precision level.

Two procedures were used to evaluate the required number of scanned subsamples to obtain a given level of precision or margin of error. First, the following formula (Cochran, 1977) was used. It is readily derived from the lower and upper bounds of a 100(1 − α)% confidence interval for the mean of a normal population when the variance is known:

\[ n = \frac{z_{1-\alpha/2}^2 \sigma^2}{d^2} \]

where \( n \) is the required sample size, \( z_{1-\alpha/2} \) is the \( (1 - \alpha/2) \)-quantile of a standard normal distribution, \( \sigma^2 \) is the population variance, and \( d \) is the margin of error.

Second, the bootstrap resampling procedure (Efron and Tibshirani, 1993; Mooney and Duval, 1993; Pillar, 1998) was conducted with Sampler, a software for sample size optimization (available from vpillar@ecologia.ufrgs.br). Outputs generated by this software include confidence intervals for a given \( \alpha \) level or 100(1 − \( \alpha \))% confidence level. The procedure is described in Pillar (1998). Iteratively larger sample sizes were used and tested for their ability to represent the whole root system. At each sample size, a large number of bootstrap samples can be drawn. Confidence intervals were defined for each sample size by ordering the means of the bootstrap samples from the smallest to the largest value. We worked with 5000 bootstrap samples. For a 95% confidence interval (\( \alpha = 0.05 \)), the lower and upper bounds were, respectively, the 125th and 4876th values of the series of ordered means. This resulted from 2.5% of the 5000 sample mean values being less than or equal to the lower bound, and, similarly, 2.5% of the 5000 sample mean values being greater than or equal to the upper bound. The required sample size is then the minimum sample size at which the specified margin of error, \( d \), is larger than the half-length of the confidence interval for a given confidence level, 100(1 − \( \alpha \))%.

Data Analysis

The normality test was performed with SAS Release 6.12 for Windows (SAS Inst., 1989) using the SAS PROC UNIVARIATE procedure. Regression analysis was also used to determine the relationships between variables.
RESULTS AND DISCUSSION

Effectiveness of the Mixing Device

The device developed for randomly mixing roots was found to allow the collection of homogeneous subsamples (Fig. 1). The container was large enough to contain an entire root system (i.e., up to 600 g fresh wt.) at one time. The air pressure was sufficient to allow homogeneous root stirring of all root diameters. The device is an enhancement of the sampling method described by McGonigle (1994).

Tubing placement around the mixer allowed excellent root mixing (per visual observations). Placing the tubing not more than 30 mm from the bottom of the container allowed downward-directed pressurized air to reach the bottom of the container.

Homogeneity of Root Subsamples

There was a negative exponential relationship between spinning time and fresh weight of subsamples (Fig. 2). Fresh weight was made up of the root material and adhering water. Spinning samples for 15 s (140–160 rpm) reduced the fresh weight by 10%. Longer spinning did not further reduce fresh weight; however, to ensure complete water removal from the samples, we used a 25-s spin time. There was a close relationship between fresh weight and root length (Fig. 3). Because root mass is easier to measure than root length or surface area (Carley and Watson, 1966; Murphy and Smucker, 1995), root mass could be used to calculate root length.

Sampling Reliability

The within-subsamples coefficient of variation found for root lengths of the 957 subsamples collected from the mixer for each of the three maize genotypes were relatively low: 25.5% for LRS, 21.2% for LNS, and 19.5% for P3905. The root length of the collected subsamples for each hybrid did not differ from a normal distribution ($P < 0.05$). The method was, therefore, sufficiently reliable to be used in subsequent calculations of required number of subsamples (sample size) to reach a given level of precision.

Size of Measured Subsamples

The amount of root placed on the tray for scanner image acquisition had an important effect on measurement accuracy. For instance, too many roots increased overlapping, crossovers and the time required for analysis (Fig. 4). The use of the largest available tray size (300 by 200 mm) and small amounts of root (0.5 g of fresh spun root) shortened the time for each scan. With this amount of root, the average measured root lengths varied from 289 to 560 cm for each subsample (Fig. 5). The mass of subsamples varies according to the system used for analysis. Farrel et al. (1993) reported 20- to 30-mg oven-dried subsample masses in a study using a digital line-intercept method. They also found less variability using fresh-cut root subsamples with total lengths between 200 and 400 cm. The subsamples we used for P3905 were within this range.

Determining Required Sample Size

The two procedures gave nearly the same estimates of the required sample size. The estimates derived with the standard procedure were 1.3- and 1.1-fold higher for LRS and LNS, respectively, than those of the bootstrap method, while the two methods resulted in similar values for P3905 (Fig. 6).

There was an asymptotic relationship between confidence interval and the sample size required to reach a
Table 1. Total root length, surface area, and average diameter of measured root systems of maize genotypes.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Length</th>
<th>Surface area</th>
<th>Average diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>km</td>
<td>m²</td>
<td>mm</td>
</tr>
<tr>
<td>LRS</td>
<td>1.75</td>
<td>1.89</td>
<td>0.042</td>
</tr>
<tr>
<td>LNS</td>
<td>2.37</td>
<td>2.60</td>
<td>0.035</td>
</tr>
<tr>
<td>P3905</td>
<td>0.49</td>
<td>0.68</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Fig. 7. Relationship between sample size and half-length 95% confidence intervals for root length of three genotypes and for the mean of all genotypes.

The mixing system developed was adequate for collection of random homogeneous root subsamples. Both standard and bootstrap procedures successfully determined the optimal sample size for repeatable root subsamples. The device and its principle can be applied to root sampling of many species. There was no clear reason to choose one procedure over the other, unless the data were not normally distributed, in which case the bootstrap procedure should be used.

CONCLUSIONS

The mixing system developed was adequate for collection of random homogeneous root subsamples. Both standard and bootstrap procedures successfully determined the optimal sample size for repeatable root subsamples. The device and its principle can be applied to root sampling of many species. There was no clear reason to choose one procedure over the other, unless the data were not normally distributed, in which case the bootstrap procedure should be used.

This is the first report on an air stirring-based device for obtaining root subsamples, and the first to give a clear indication of the optimal sample sizes for measuring root with now available scanner-based image analysis systems. These indications of sample size were developed to analyze the entire root system of LRS, 49 h for LNS, and 20 h for P3905. Using our method of sampling, the analysis of required sample sizes for the three maize genotypes took approximately 6, 4, and 3 h for LRS, LNS, and P3905, respectively. This included time for collecting subsamples from the mixer, untangling roots in the tray, and the actual root measurements. An additional 1.5 h was required for rewashing prior to root measurement and cutting. Therefore, this sampling procedure saved considerable time in root measurement and allowed accurate quantification of root characteristics when a reliable mixing system was employed. These innovations have the potential to improve precision and reduce costs of root measurement.

Measured Root Characteristics

The total root length, as measured by image analysis, was linearly correlated \( r^2 = 0.98 \) with the total root dry mass of the three genotypes (Fig. 8). This result agreed with findings reported by other researchers (Murphy and Smucker, 1995). The proportion of very fine (i.e., <0.5 mm diameter) roots (Böhm, 1979) to the entire root system varied among maize genotypes. The highest proportions of very fine roots were for LNS and LRS, i.e., genotypes containing the ‘Leafy’ trait (Table 1). Total root lengths for single root systems varied greatly among the three maize genotypes. The greatest root length was found for LNS and was 1.35-fold longer than that of LRS and 4.84-fold longer than that of the commercial conventional hybrid, P3905. A. Modarres (personal communication, 1996) previously hypothesized the longer root system for the leafy type based on casual field observations. The measured root surface area reflected the size of the root systems and showed the same order of size among the three genotypes as indicated for root length.
rived over an extremely wide range of maize genotypes with contrasting root systems (total length and proportion of fine or coarse diameter roots).

Root sampling reduces time and cost of characterizing larger root systems. Calculations indicated that approximately 10% of the total root volume should be analyzed for an accurate estimation of the root length of the entire system.

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