Evaluation of Fine Root Length and Diameter Measurements Obtained Using RHIZO Image Analysis

Jürgen Bauhus and Christian Messier*

ABSTRACT

Image analysis systems facilitate rapid measurement of root length and diameter, but their accuracy is not easily determined. The objective of this study was to develop a set of simple experiments for evaluating the accuracy of fine root measurements obtained using image analysis. Using the system RHIZO (trademark of Régent Instruments, Québec), we tested the accuracy of (i) length measurements made over a range of root lengths per unit area, (ii) average diameter measurements and length per diameter distributions in string, wire, and fine root samples of varying diameter, and (iii) diameter measurements on short segments of diagonally oriented objects. Our results suggest that preliminary testing of image analysis systems is absolutely necessary for producing reliable root measurements. Total length was accurately determined for typically encountered length per unit areas of <1.5 cm cm^{-2}. For samples with higher values, however, the method underestimated total length by >5%. It is therefore recommended that users of image analysis systems determine this maximum length per unit area for accurate determinations of total root length. In samples that contained different string diameters, the total sample length and average string diameter could accurately be measured. However, the length per diameter class was underestimated by >20% when the string diameter was less than one pixel smaller than the upper limit of the diameter class. Adjustment of diameter intervals and increasing the scanner resolution are required to reduce this underestimation. Both the length and the angle of the short segments analyzed were found to influence diameter measurements. Similar sets of experiments are proposed for a rigorous evaluation of the performance of other image analysis systems on root measurements.

Root length is one of the most important and widely used parameters for describing fine root systems and predicting their response to changes in the environment. The ratio of length to mass, that is the specific root length, has also been widely used as an indicator for fine root morphology (Robinson and Rorison, 1983; Fitter, 1985). Once roots have been separated from the soil, the determination of their weight is not difficult. Methods for measuring root length, however, can be tedious and onerous. The use of image analysis systems, instead of techniques such as the line-intersection method (Newman, 1966; Tennant, 1975), has facilitated rapid root length measurements (e.g., Ottman and Timm, 1984; Barnett et al., 1987; Burke and LeBlanc, 1988; Pan and Bolton, 1991; Kirchhof, 1992). Despite the widespread use of image analysis systems for root measurements, tests on the accuracy of these systems are rarely conducted; thus, significant errors in the data may be undetected. Length calculations done using image analysis systems can vary greatly, depending on the calculation technique (Burke and LeBlanc, 1988). Root overlap and abutment can also result in an underestimation of root length, because part of the root system is excluded from calculations (Barnett et al., 1987; Pan and Bolton, 1991; Murphy and Smucker, 1995). This root overlap effect may be avoided by careful placement of the roots in the image to be analyzed, though this is also tedious. Although statistical methods can provide correction factors for root overlap, they are based on rough estimates from empirical data. In addition to the uncertainties about root length measurement, very little information is available on the accuracy of root diameter measurements.

Our objective was to develop a set of experiments that can be used to evaluate the accuracy of fine root measurements obtained using image analysis. For this purpose, we evaluated the performance of the image analysis system RHIZO (Régent Instruments, Québec) with regard to three issues associated with fine root measurements: (i) the accuracy of root length measurements for samples in which roots overlap, (ii) the accuracy in the distribution of length over different diameter classes, and (iii) the accuracy of diameter measurements for root segments when using a small window size for image analysis. This latter objective arose from a problem observed in the estimation of root diameter for single segments that lie at an angle from vertical or horizontal planes of the window.

MATERIALS AND METHODS

Introduction to RHIZO

The image analysis system RHIZO is unlike most other systems in that it identifies areas of root overlap and makes corrections for them (Arsenault et al., 1995). RHIZO also assigns root lengths to predefined diameter classes, thus providing diameter distributions of total root length. The system acquires one direct digital image, a grayscale image obtained from the scanner, and then creates two other types of images for its analysis. A threshold image is created that removes all parts of the original image that fall below a user-defined

J. Bauhus, Dep. of Forestry, Australian Natl. Univ., Canberra, ACT 0200, Australia; C. Messier (through July 1999), Ministry of Forests, Research Branch, Forest Biology Section, P.O. Box 9519 Stn. Prov. Govt., Victoria, BC V8W 9C2, Canada [thereafter: Groupe de Recherche en Ècologie Forestière (GREF) interuniversitaire, Dép. des Sciences Biologiques, Univ. du Québec à Montréal, C.P. 8888, succursale centre ville, Montréal, QC H3C 3P8, Canada]. Received 28 Aug. 1997. *Corresponding author (christian.messier@gems5.gov.bc.ca through July 1999; thereafter: messier.christian@uqam.ca).

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Abbreviations: dpi, dots per inch.

threshold of grayscale value. This procedure separates the background component from the root segments. The threshold image is used for diameter measurements and has only two colors: black for the roots, and white for the background. A second created image is called the *skeleton* image; this consists of a line, one pixel (picture element) in diameter, that is superimposed over each root image (Fig. 1). RHIZO measures root length by scanning the length of the root skeleton. If a straight root is oriented vertically or horizontally, then the root length equals the sum of all the skeleton pixels. If a root is oriented diagonally at 45°, the root length is equal to the sum of the pixel diagonals. If the angle deviates from the above two orientations, then RHIZO uses a simple mathematical correction algorithm. Root diameter is measured from the threshold image at each skeletal pixel by multiplying the smallest number of pixels on a line perpendicular to the root axis by the pixel size. The direction of pixel displacement is again taken into account. Root diameter is not measured in areas of branching or root crossing. In these critical areas, the diameter is extrapolated from measurements taken outside of the crossing or branching areas.

RHIZO accounts for root length and area in overlap situations by using a nonstatistical method (Régent Instruments, 1995). When root overlap occurs, a pixel in the root skeleton ideally has four neighboring skeleton pixels (Fig. 1). While overlap is easy to identify for angles around 90°, at obtuse or acute angles the roots crossing each other may share more than one skeleton pixel for a short distance. Thus, two cases occur in which a skeleton pixel has three neighbors instead of four (Fig. 1). The latter situation must be differentiated from branching, where correction for overlap is unnecessary.

RHIZO (Version 3.0.3) (Régent Instruments, 1995) was installed on a Macintosh microcomputer (Quadra 800, System C1-7.1). Images were acquired using a desktop scanner (ScanJet 3c/T, Hewlett Packard, TX), equipped with two light sources, one from below and the other from above. When roots are scanned with two light sources, they do not cast a shadow (shadows may distort diameter measurements). If not otherwise stated, all measurements were carried out at a resolution of 300 dpi (dots per inch), which is equivalent to a pixel side length of 0.085 mm.

Measurements

Since samples of known length were required for three out of the four experiments, string and wire samples of known length and constant diameter were used. These were placed on the scanner within a defined area of 9 by 8 cm. Each measurement was replicated three times; for each replicate, objects were removed from the scanner and then repositioned. In all experiments assessing the effect of overlap, the string or wire was randomly arranged on the scanner, which resulted in many overlap situations. A micrometer was used to initially determine the diameter of the string and wire. When fine roots were measured, they were placed directly on the scanner, and kept moist between measurements. All statistical analyses were carried out in Systat, version 5.02 (SPSS, Chicago).

Exp. 1: Effect of Length per Unit Area on Length Measurement

The chance of root overlap increases with root density on the scanner. The chance of abutment also increases, since adjacent parallel roots are often closer together than the width of one pixel. We assessed the recovery of length for values of string length per unit area of 1.4, 2.8, and 4.2 cm cm^{-2}, which are equivalent to 1, 2, and 3 m of string, respectively.

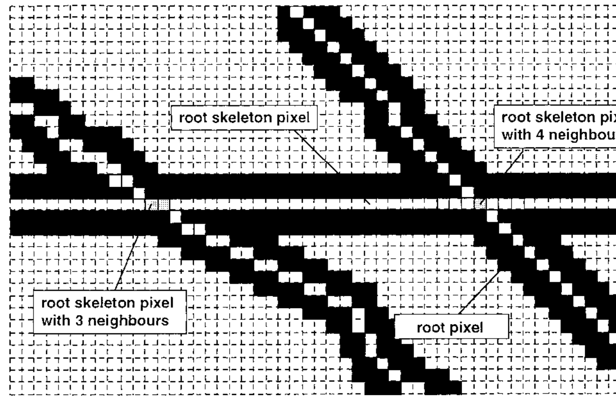

Fig. 1. Areas where root diameter is underestimated when selecting a segment for image analysis that is nonperpendicular to the root.

These values provide a range from low to high densities that can normally found in routine measurements. The string (diameter 0.72 mm) was cut into pieces 4 and 6 cm long that were randomly arranged in a 72-cm^2 area. We also determined the capability of RHIZO to assign all of these root segments to the 0.5- to 1.0-mm diameter class, when using the diameter class width of 0.5 mm. Differences between measured lengths and correct length were submitted to *t*-tests.

Exp. 2: Effect of Diameter on Length and Diameter Measurements

The area of the root skeleton image that is potentially subjected to root overlap increases with the diameter of the overlying root. We examined the recovery of a known string length of 50.5 cm for three different diameters: 0.26, 0.76, and 1.57 mm. These three diameters represent a typical wide range of fine roots found in plants. Length was measured separately for each string diameter; the average diameter, total length, and length per diameter class for three different diameter-width classes (0.3, 0.4, and 0.5 mm) were then determined. String segments of all three diameters were subsequently used together, and similar measurements were made on this composite sample. Results are reported for diameter classes increasing in 0.3-mm steps. In addition, we compared the average diameter measured by RHIZO with the calculated weighted mean of the known diameters in the composite sample. Since the accuracy of diameter measurements is more sensitive to pixel size than is the length measurement, the effect of pixel size on assigning length to diameter classes was assessed at both 300 and 600 dpi resolutions and using identical sample arrangements.

Differences in the percent of correct length measured for different diameter classes were assessed using ANOVA and a post hoc Tukey's test. The significance of differences in lengths in diameter classes between measurements using 300 and 600 dpi resolution was tested with paired *t*-test.

Exp. 3: The Effect of Angle and Frame Size on Diameter Measurements

The part of the image being analyzed by RHIZO can be defined by the user with a horizontally oriented square frame. If one segment of a root is to be analyzed (e.g., the measurement of length and diameter between two branching points) and the frame is not perpendicular to the root cylinder, the root diameter may be underestimated (see Fig. 2). The proportional error that results from underestimating the diameter at each end of the segment increases with decreasing segment length. We tested the effect of both angle and segment length

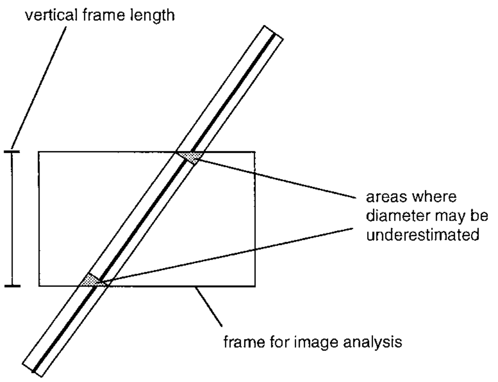

Fig. 2. Threshold image with superimposed root skeleton image for two overlapping roots on a pixel grid. In the situation on the right, the two roots share only one pixel, which has four neighboring pixels. In the situation on the left the two roots share two pixels, which have three neighboring pixels each. The latter situation must be distinguished from branching points.

on diameter determinations using a straight pencil graphite cylinder 0.72 mm in diameter. It was placed on the scanner at angles of 0°, 12°, 30°, and 45° from the vertical. Diameters were determined three times using frames of 0.5, 1, and 2 cm vertical lengths at different locations along the cylinder.

Whether the differences in measured diameters were significant or not was tested with ANOVA, using segment length and angle as factors. Individual differences were examined with a post hoc Tukey's test.

Exp. 4: Reproducibility of Fine Root Measurements and Comparison with the Tennant Method

Root length was determined individually and with minimal overlap for six fine root fragments taken from white spruce (*Picea glauca*) and a deciduous shrub. Beginning with two fragments, the length per unit area was gradually increased through addition of further root fragments, up to 1.93 cm cm^{-2}, a length per unit area value that would normally not be exceeded in our routine measurements. Measurements of individual and combined fragments were each replicated three times. Length measurements of combined root fragments with overlap were compared with those calculated from the addition of individual root fragments of known length (measured with minimal overlap) and with lengths determined by the Tennant method (Tennant, 1975). The Tennant method is a line-intersect method that is independent of the area of measurement and the length of the intercept line. Root length is calculated as the product of the number of intercepts and a length conversion factor which is based on the size of the grid used. RHIZO's length measurements were compared with those obtained using the Tennant method and with the length calculated from the addition of individual fragments using ANOVA and post hoc Tukey's multiple comparisons. Measurements were considered accurate if the probability level for the difference between RHIZO values and the other calculated values was <5%.

RESULTS

Exp. 1: Effect of Length per Unit Area on Length Measurement

Underestimation of the string length increased with the length per unit area (Fig. 3). At a string length per unit area of 1.4 cm cm^{-2} (for a string 1 m long), the image analysis system registered the correct string length with 100% accuracy. At string lengths per unit area of 2.8 and 4.2 cm cm^{-2} (for 2 and 3 m string), total string lengths were underestimated ($P < 0.05$) by 5 and 10% respectively. The percentage of total measured length allocated to the correct diameter class (i.e., between 0.5 and 1.0 mm) was strongly affected by the length per unit area. It decreased from 76% correct at 1.4 cm cm^{-2} to 49% at 4.2 cm cm^{-2}.

Exp. 2: Effect of Diameter on Length and Diameter Measurements

The measured average diameter never deviated by >3% from the correct diameter, and the diameter of the string did not affect measurements of total length

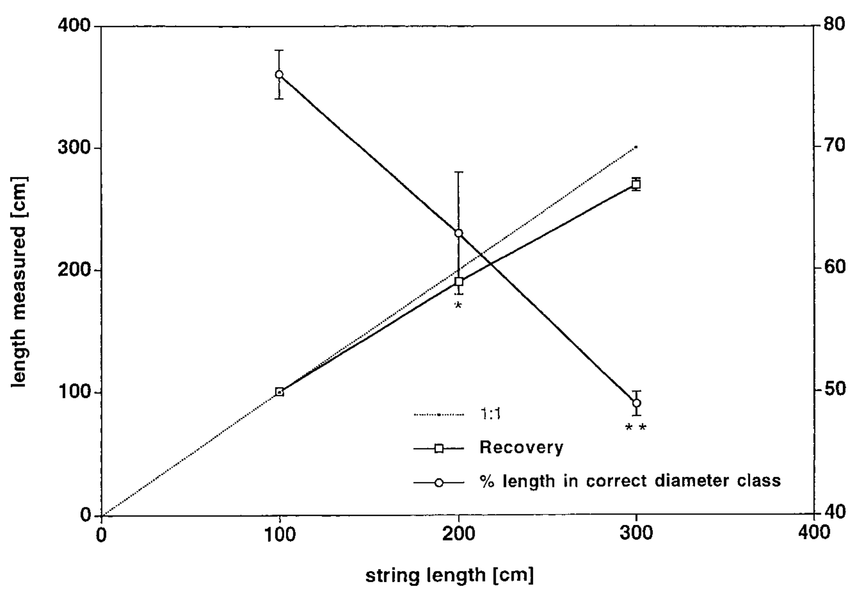

Fig. 3. Recovery of correct length with increasing length per unit area. String length of 100, 200, and 300 cm (0.72 mm diam.) equalled 1.4, 2.8, and 4.2 cm cm^{-2} length per unit area. The 1:1 line represents 100% accuracy. Percentage of string length correctly assigned to the 0.5- to 1.0-mm-diameter interval is depicted on the right-hand *y*-axis. Diameter classes increased by 0.5 mm. Error bars represent standard deviation, $n = 3$. *, Measured length is significantly different from the correct length at the 0.05 and 0.01 probability levels, respectively (*t*-test).**

in the sample (t-test, $P > 0.01$). The measured lengths were 99.5, 99.3, and 100.3% of the correct length for the thin (0.26 mm), medium (0.76 mm), and thick (1.57 mm) diameters. However, the choice of diameter class (interval) did affect the lengths that were assigned to the correct diameter classes (Fig. 4). The closer the correct string diameter to the upper limit of the diameter class, the greater the error in the length over diameter classification. The greatest deviations from the correct length in the correct diameter class were obtained when the upper class limit was 0.3 mm for the 0.26-mm-diameter string (a difference of 0.04 mm), the upper class limit was 0.8 mm for the 0.76-mm-diameter string (a difference of 0.04 mm), and the upper class limit was 1.6 mm for the 1.57-mm-diameter string (a difference of 0.03 mm). In all of these cases, the difference between the correct diameter and the upper diameter class limit was less than the width of a pixel (0.085 mm). In the thin and medium string diameter samples, the variability of the length over diameter measurements also increased with decreasing difference between the correct diameter and the upper diameter class limit (Fig. 4).

When combining strings of all three diameters in one sample (a length per unit area of 2.1 cm cm^{-2}), RHIZO measured 99.7% of the correct length. The length associated with the correct diameter class was more accurate

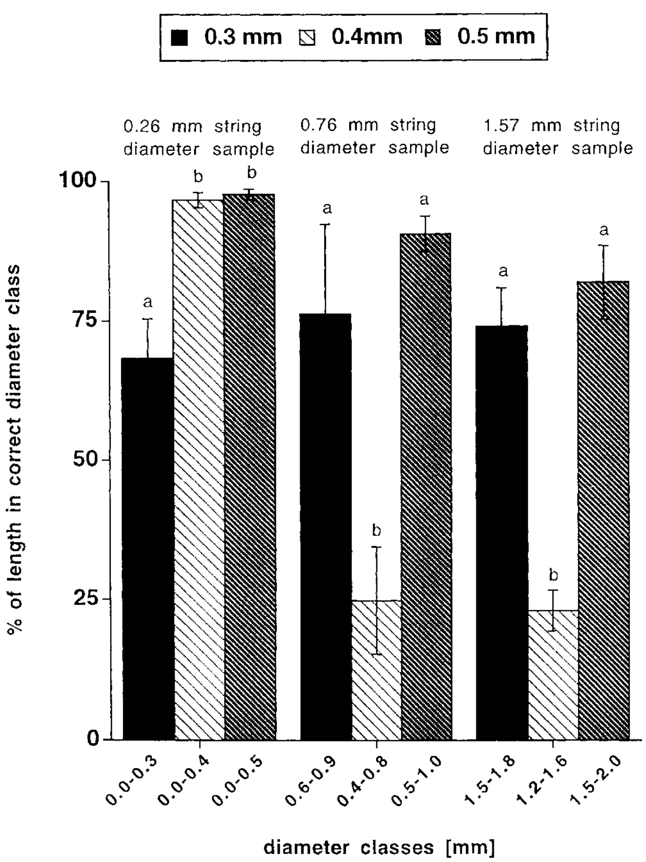

Fig. 4. Percentage recovery of length in correct diameter classes for class widths of 0.3, 0.4, and 0.5 mm for strings of different diameters (0.26, 0.76, and 1.57 mm diam.). Each sample consisted of only one string diameter. Error bars represent standard deviation, n = 3. Means sharing the same letter for the same string diameter are not significantly different at the 0.05 probability level (ANOVA and post hoc Tukey's test).

for the thick (81.9%) than for the medium (77.8%) and thin (72.2%) string; note that all these values are significantly different from the correct values of length over diameter (t-test, $P < 0.001$). Figure 5 shows that the peaks in the length over diameter distribution are clearly recognizable. Increasing the resolution from 300 to 600 dpi significantly improved the length over diameter measurements for the thin and medium diameter strings. Most of the length missing in a correct diameter classification was assigned to the next higher diameter class, but 10% of the string length for the thin and medium diameter was assigned to classes above 1.5 mm (not shown in Fig. 5).

Despite the significant underestimation of length for the correct diameter classes, the average diameter of the sample was accurately determined. The average diameter was 98% of the weighted mean diameter at a resolution of 300 and 96% at 600 dpi.

Exp. 3: The Effect of Angle and Frame Size on Diameter Measurements

Both the vertical frame length and the angle of intersection of the graphite cylinder significantly influenced the diameter measurements (F-ratios 6.1 and 7.9, and P-values 0.007 and 0.001 respectively; ANOVA) (Fig. 6). However, only at an angle of 45° and with a vertical frame length of 0.5 cm was the diameter significantly different from all other combinations of angle and frame size. As indicated by the standard deviation between measurements, the variation also increased at large angles for short segments.

Exp. 4: Reproducibility of Fine Root Measurements and Comparison with the Tennant Method

Measurements of tree and shrub fine root lengths and diameters were highly reproducible (Table 1). The

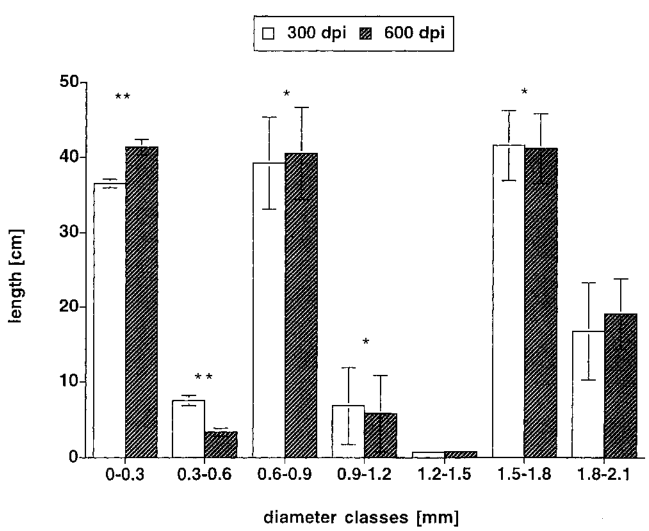

Fig. 5. Distribution of length per diameter class measured by RHIZO for samples consisting of a mixture of different string diameters (0.26, 0.76, and 1.57 mm diam.) at 300 and 600 dpi resolution; length per unit area 2.1 cm cm^{-2}, length of each string 50.5 cm. Diameter classes increased by 0.3 mm. Error bars represent standard deviation, n = 3. *, Length per diameter class differs significantly between 300 and 600 dots per inch (dpi) resolution at the 0.05 and 0.01 probability levels, respectively (pairwise t-test).**

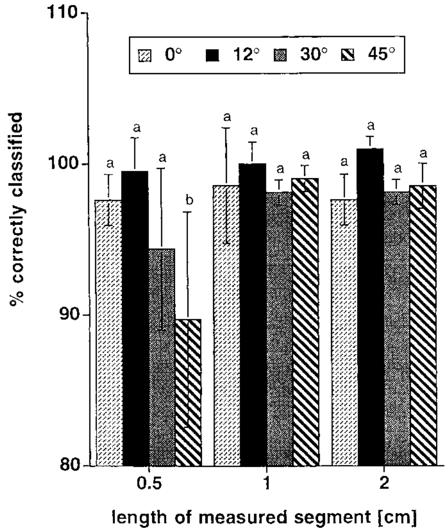

Fig. 6. Percentage recovery of correct diameter of a pencil graphite cylinder (0.72 diameter) for different angles (0, 12, 30, and 45°) and vertical length (0.5, 1.0 and 2.0 cm) of the frame determining the area of the image to be analyzed. Error bars represent standard deviation, $n = 3$. Means sharing the same letter for the same vertical length are not significantly different at the 0.05 probability level (ANOVA and post hoc Tukey's test).

average CV for the mean of three replicated measurements was 1.6%. Root lengths measured for combined and overlapping root fragments were similar to results obtained with the Tennant method and to those calculated through the addition of lengths measured for individual fragments with minimal overlap (Table 1). Root lengths determined by the Tennant method (Tennant, 1975) were significantly higher than values measured by RHIZO and those calculated from measurement of single fragments at three different levels of length per unit area. However, only at the highest length per unit area of 1.93 cm cm^{-2} was the difference between root length measured by RHIZO with overlap and the calculated values larger than 5% of the calculated value. The average difference in length per diameter class between samples consisting of combined fragments, and the calculated length for single fragments, was 9.4% of the correct length. Length per diameter class in overlap situations was both over- and underestimated for the different diameter classes.

Table 1. Tree and shrub fine root length at increasing root densities (0.50 to 1.93 cm cm^{-2}) as measured by RHIZO (Regents Instruments image analysis system) in overlap situations, as calculated from the addition of individual root segments measured with minimal overlap, and as determined by the Tennant method (Tennant, 1975).

	Fine root length					
Method	**0.50**	**0.76**	**0.97**	**1.25**	**1.46**	**1.93**
	cm					
Measured	**74.5a†**	**113.4a**	**145.4a**	**187.0a**	**218.3a**	**289.1a**
Calculated	**74.5a**	**112.1a**	**144.4a**	**184.4a**	**224.5b**	**308.4b**
Tennant	**76.5a**	**117.0b**	**152.1a**	**194.5a**	**227.8c**	**310.3c**

† Within columns, means followed by the same letter are not significantly different at the 0.05 probability level (ANOVA and post hoc Tukey's test).

DISCUSSION

Experiments 1 and 4 clearly demonstrated that the determination of a maximum value for root length per unit area is critical for accurate routine measurements. Despite the overlap correction in RHIZO, the measured root lengths were significantly different from the Tennant method above a length per unit area of 1.5 cm cm^{-2}. For length per unit area values around or below this threshold, measurements of total length in this study using RHIZO were accurate to within a 5% error margin. Coefficients of variation for the total length never exceeded 3.2%, and are smaller than those of other automated systems for root length measurements (Ottman and Timm, 1984; Barnett et al., 1987). Root length measurements were found to be reproducible under different overlap situations, and at different densities (Table 1). Since the overlap correction is effective at low densities, the underestimation of length that arises at high length per unit area is assumed to be caused by an increase in overlap in the longer string segments at obtuse angles. In addition, the probability that adjacent parallel strings (or roots) will lie closer than the size of one pixel increases with density (Pan and Bolton, 1991). In this situation the parallel strings or roots are not recognized as individual roots, but as a single, large-diameter root. Users of image analysis systems thus have to determine the optimal length per unit area for their particular situations, or alternatively they can determine correction factors to compensate for the underestimation of length at high densities.

Length per diameter class was significantly affected by the choice of diameter class intervals. The examples given in Fig. 4, however, show that length per diameter class is underestimated when the difference between the correct diameter and the upper limit of the diameter class is less than the size of one pixel. In our case, diameter class intervals of 0.5 mm, an interval frequently used by root researchers (Vogt and Persson, 1991), gave the best results for all string diameters. Our results show that RHIZO can assign length to the correct diameter class within a 20% error margin when the operator adjusts the diameter class width. For smaller diameters, as in the tree and shrub fine root samples, error margins tend to be much smaller.

In overlap situations, string length was assigned to diameter classes larger than the correct one, although total length measured in the sample was accurately determined. Thus, misrepresentation of length per diameter classes does not result in false length measurements. This observation is attributed to the decoupling of length and diameter measurements by RHIZO. We assume that in situations where roots cross at obtuse angles or are parallel and lie less than one pixel apart, the individual root diameters are not differentiated. Thus, the diameter assigned to each root is the combined diameter of both roots, which then leads to overestimation. In samples consisting of variable diameters, Kirchhof (1992) also described an underestimation of length in each diameter class, and a total cumulative length of sample that was consistently slightly less than the correct length. It is therefore recommended that users of image analysis systems investigate the relationship between length per diameter class and total root length of their samples.

RHIZO measured the average sample diameter accurately even when the distribution of length per diameter class was inaccurate. This demonstrated that the calculation of average diameter is independent of the length per diameter class assignment.

Diameter measurements were affected when the object was intersected by a frame of short vertical length, at angles of 45°. Although RHIZO may not have been designed to analyze root segments within small frames, our results nevertheless show the need for a diameter correction at locations of intersection between the frame and the root. As this situation can arise for root architectural measurements, users of image analysis systems are encouraged to verify the accuracy of their systems for these kinds of measurements.

CONCLUSION

Our results show that a rigorous preliminary testing is necessary to produce reliable root measurements with an image analysis system. A set of four experiments is proposed for rigorously assessing the accuracy of root length and diameter measurements using image analysis systems: (i) determine the maximum length per unit area for length measurements; (ii) determine the accuracy of average diameter of samples; (iii) determine optimal diameter classes for length over diameter distributions; and (iv) determine the effect of object orientation on diameter.

RHIZO, the system used in this study, measured root length and average diameter accurately for typical values of length per unit area. However, length was underestimated at higher values. It is thus recommended that users of image analysis systems determine the maximum length per unit area for accurate determinations of total root length for the type of roots that they regularly analyze. This could be done by gradually increasing the density of string or wire of known length within a given area on the scanner until the measured length deviates more than is acceptable (e.g., 5%) from the correct length. The calculation of length per diameter class was less reliable in overlap situations where the string diameter was less than one pixel smaller than the upper limit of the correct diameter class.

Our results also suggest that the user should perform some preliminary measurements to determine average diameters of the different orders of fine roots for the species under investigation. This could be done by determining the diameter ranges that contain the highest percentage of the total root lengths. It will be necessary to use different diameter intervals for this, and to adjust the upper limit of diameter classes so that the difference between peaks in the distribution of length over diameter and the upper limit of diameter intervals is more than one pixel. This will allow optimization of diameter class width, which will keep error margins small.

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