ACTA STEREOL 1998; 17/3: 335-341
ORIGINAL SCIENTIFIC PAPER

ESTIMATION OF PLANT ROOT LENGTHS DURING GROWTH

Dvoralai Wulfsohn[1], Jens R. Nyengaard[2]

[1] Department of Agricultural and Bioresource Engineering, University of Saskatchewan, 57 Campus Drive, Saskatoon, SK S7N 5A9, Canada, and
[2] Stereological Research Laboratory, University Bartholin Building, University of Aarhus, Århus 8000 C, Denmark.

ABSTRACT

The method of 'total vertical projections' was applied to develop a non-destructive, unbiased method for estimating total root length from 2D projections of 3D structure in young developing plants. No assumptions regarding the isotropy or randomness of root structure and branching are required. To illustrate the procedures, crested wheat grass plants (*Agropyron cristatum* L.) were grown in polycarbonate magentas containing a transparent tissue culture medium. Estimates of root length, diameter, and branching were obtained over a four week period at approximately three day intervals. The method of estimating total lengths from vertical projections was found to be very robust with estimated sampling coefficients of error generally less than 5% for 50-100 grid intersection counts. Biological coefficients of variance for total length were between 30-70%, and were largely attributable to variation in the degree of secondary root branching.

Key words: *Agropyron cristatum* L., Branching pattern, Diameter, Growth curves, Total length, Total vertical projections, Surface area, Unbiased stereology, Variability.

INTRODUCTION

Our overall goal is to develop unbiased stereological procedures that can be used by plant scientists to estimate the major geometric parameters of plant root growth under various environmental conditions. Important parameters include total length, volume and surface area of a root system, maximum root depth, diameters, lengths, surface areas and volumes of roots at each level of branching, frequency of branching for each branching level, length of root hairs, and interrhizal area surrounded by roots and their branches (Box, 1996). In this pilot study, the method of 'vertical projections' was applied to monitor during early development the total length, lengths at each level of branching, frequency of branching, and diameters of root systems grown in a transparent growth medium.

THEORY

Gokhale (1990) showed that the mean length of a set of curves in three dimensions per unit reference volume, L_V, can be estimated from 'vertical projections' obtained by rotating the curve about an arbitrary fixed axis (the 'vertical' axis), and projecting the curve through a slice of known thickness onto a plane having its normal perpendicular to the vertical axis (a 'vertical slice'). Cruz-Orive and Howard (1991) extended this concept to develop an unbiased estimator of the total (finite) length, L, of lineal features contained in an unbounded reference space:

$$\hat{L} = 2\cdot\frac{a}{\ell}\cdot\frac{1}{n}\cdot\sum_{i=1}^{n} I_i \qquad (1)$$

where a/ℓ = ratio of test area per cycloid to the total cycloid test length, n = number of vertical projections, and I_i = total number of intersections of cycloid test lines with the lineal features counted for the i^{th} projection. Equation 1 states that the average number of intersections between the curve projections and a cycloid test system, multiplied by some constants, provides an estimate of the total length of curves. To avoid bias, the cycloid test system must be positioned uniform randomly with respect to the projections and must be oriented with the minor principal axis of the cycloids perpendicular to the selected vertical axis. Cruz-Orive and Howard (1991) proved that the method is unbiased mathematically, and from measurements of a bent wire of known length demonstrated that the method is practically unbiased.

Practical requirements for the method to be applied are: (1) the 'curve' should be rigid (i.e. its shape will not change when observed from different orientations) and bounded; (2) there should be an identifiable vertical axis; (3) it should be possible to view the entire object through its containing medium (i.e. to obtain 'total projections' of the 3D object onto a 2D field of view), and (4) the length density should be sufficiently low such that overlapping effects are not significant. The sampling error variance of the estimator (1) is not known and is complicated by the fact that observations I_i are not independent. A prediction formula for systematic observations on the semicircle has been proposed by Cruz-Orive (1993). For $n = 2$ and the 'total vertical projections' method, the latter estimator of the sampling error variance can be written:

$$\text{est}\, CE_{\text{e}}^2\{\hat{L}_j\} = \frac{(\hat{c}_j - 1)^2}{\hat{c}_j}, \qquad j = 1,2,\ldots,m \qquad (2a)$$

where

$$\hat{c} = \frac{2\cdot(\hat{L}_{j1}^2 + \hat{L}_{j2}^2)}{\left(\hat{L}_{j1} + \hat{L}_{j2}\right)^2} \qquad (2b)$$

where $\hat{L}_j$ = estimated total length of the j-th plant, $\hat{L}_{ij}$ = estimated length of the j-th plant determined from the i-th projection (i = 1, 2), and m = number of observed plants.

MATERIALS AND METHODS

Six crested wheat grass (*Agropyron cristatum*) plants were grown under sterile conditions in closed containers formed by joining two polycarbonate magenta vessels (77 × 77 × 97 mm high GA-7 vessel, Sigma Chemical Company, Saint Louis, MO, USA) containing a transparent tissue culture gel (Murashige & Skoog basal medium with 0.2% Gelrite Gum to substitute for agar) in the lower vessel section. Seeds were germinated in a 1% agar/0.5% sucrose medium until root initials reached 10 mm length ($CV = 44\%$). Pregerminated seeds were then transplanted to the growing medium in the magenta containers. The bottom halves of the containers were covered in black plastic and the surface of the gel was covered with silver foil to prevent exposure of the root to light. Plants were placed in a growth chamber with 22°C temperature and a 16-h photoperiod. The vertical axis was defined as the direction of gravity. The upright sides of the square cross-sectional containers (i.e. 'vertical planes') then defined two projection directions (i.e. $\phi = 0, 90°$). Seeds were inserted into the growing medium at random orientations about the vertical axis, and the containers in turn were oriented at varying degrees about the vertical axis when placed in the growth chamber (to randomize any effects due to variation in lighting intensity), so as to meet the requirement of a random orientation of the first vertical projection plane about the vertical axis. Projection images were viewed using a stereo macroscope (Model SZ 11 stereoscope, Highlight 3001 lighting system, 73AL 0.5X objective lens, Olympus, Denmark) at a final magnification of 16X. The microscope stage was adapted to hold the magenta such that the focal axis of the macroscope was parallel to the bottom face of the container (a 'horizontal plane') and the image of the vertical axis of the container was aligned with the horizontal edge of the computer screen. Live images were acquired using a video camera (Model JAI-2040, iAi Protec) and viewed using the stereological software C.A.S.T.-Grid (v. 1.08, Olympus, DK) which was also used to generate the cycloid test systems. Each field of view was determined by the size of the window generated by C.A.S.T.-Grid on the computer screen and corresponded to 18.5 mm × 12.9 mm at the object scale. Starting from a random x-y position, in most cases, the entire reference space (i.e. that containing a root system) was covered completely by successive shifts of 18.5 mm from right ('top') to left ('bottom') along the vertical direction, and of 12.9 mm in the horizontal direction, using a manual x-y stage. At very early stages of plant growth when roots were very short, in order to increase the total number of grid intersection counts sampling fractions of over 1 were used by stepping distances less than 18.5 mm along the vertical axis. For each field of view the following measurements were made:

(1) The total number of branch segments of order $k = 1$ (primary roots), 2 (secondary or lateral roots) or 3 (tertiary branches) were counted if the node was visible in the image;
(2) The total number of intersections between the cycloid test system and the surfaces of roots were counted separately for each branching order. As root systems developed more complex morphologies, test systems having different a/ℓ were sometimes used for each level of branching to obtain reasonable numbers of counts;
(3) In each frame, root diameter measurements at each level of branching were obtained by measuring widths perpendicular to the root axis with a lineal-ruler. Intersections of the cycloid arcs with the root edges were used to identify uniform random locations for measurements.

The magenta was than rotated 90° to obtain the second 'projection', and steps 1 to 3 repeated. The estimated length was determined from Eq. (1). Since roots have a non-zero thickness, intersections of cycloids with *borders* of root projections were counted, and the total number of intersections divided by 2 (Cruz-Orive and Howard, 1991). Then, for $n = 2$ and allowing for a sampling fraction other than unity, Eq. (1) becomes:

$$\hat{L} = \frac{a}{\ell} \cdot f \cdot \frac{1}{2} \cdot \sum (I_{1e} + I_{2e}) \tag{3}$$

where $1/f$ = sampling fraction, I_{ie} = number of intersections of cycloids with the root surface for the i^{th} projection.

It was sometimes necessary to focus through the medium in a particular viewing frame, in order to bring root sections into better focus, particularly when obtaining root diameter measurements and when intersections between cycloid arcs and root edges were ambiguous. The effect was a slight lateral 'shifting' of the root image with respect to the grid. This did not affect measurements along the vertical direction; however, to avoid errors due to under- or overlapping across horizontal frames, care was taken to align the image at the same focal plane depth along the edges of contingent horizontal fields. Overlapping was not found to be a problem. There were distinct variations in opaqueness of roots that were in and out of the focal plane and visible diameter differences between primary and secondary roots. For the one plant that had very dense branching originating from a 'knot' that developed, there occasionally was difficulty in distinguishing between secondary and tertiary branches if the nodes were not present in the current sampling frame.

Measurements of root lengths, diameters and branching were made at approximately three day intervals. Experiments were terminated at 32 days, or earlier if a root system reached within a short distance of the bottom of the magenta – once a root system begins to grow along the sides of the containers, the requirement of an 'unbounded' containing medium is violated. (Data for one plant was obtained up to the 11th day when the container was unintentionally dropped.) Root surface areas were estimated from the average length and average diameter (summing the contribution from each branching order) assuming a cylindrical shape for roots.

Equation (2) was used to estimate the sampling error variance for the root length estimate. The biological variance was estimated as the difference between the total variance and the estimated sampling error variance.

RESULTS AND DISCUSSION

Typically ~50 intersection counts were obtained for root measurements done while the roots were still short. Such counts were obtained using sampling fractions up to 1.5, and the finest grid test system obtainable with C.A.S.T. Grid for the 16X magnification (a/ℓ = 1.2 mm) (One could use a higher magnification rather than sampling fractions greater than unity). As root length increased, a sampling fraction of 1 and progressively coarser grids (a/ℓ up to 4.6 mm) were used to obtain total intersection counts of ~100. In the case of one plant that developed a root system with extensive branching (No. 3, see Table 1), sampling fractions of less than 1 were used at later stages to try to keep the total number of counts down. As the root systems developed, different grid line densities were used for various branching levels as appropriate.

Fig. 1 shows a set of growth curves obtained for one plant. This particular plant had two primary initials emerging from the seed at the time of transplant, and after a couple of weeks additional primary roots emerged at an increasing rate. In other cases single roots initiated from the seed and after one to two weeks, once the first primary roots began to grow secondary branches, additional primary roots appeared. With the growth of lateral roots, total lengths and surface areas increased rapidly, and it was not unusual to find them equaling or exceeding the contribution of the primaries over the last week of the experiment. Notice how little deviation (smooth) the length data, in particular the total lengths, have with respect to the growth curves (obtained by regression through the measurement data). This indicates that the total length estimator had very low sampling variability. Fig. 2 presents estimated sampling *CE*'s and biological *CV*'s with time over the experiment. The low estimated values of sampling *CE* according to Eq. (2) arose because very similar total root lengths were obtained from each of the two projections. Given the high biological *CV*'s, such precision is not required and it would be sufficient to estimate root length from a single projection. A similar conclusion was arrived at by Albrechtová *et al.* (1997/98) for *Zea mays* L. However, more plants would be needed (i.e. to reduce the biological *CV*) in order to distinguish any variation in *CV* with time or differences between treatments, if any, in physiological experiments of root growth.

Total root lengths, average length of root at each level of branching and calculated surface areas for the root systems 25 days after transplanting are summarized in Table 1. It is clear that the primary contribution to variability in total lengths $L(r)$ and total surface area $S(r)$ is the degree of secondary branching and therefore total length of secondary roots. Similar trends were evident through the experiment.

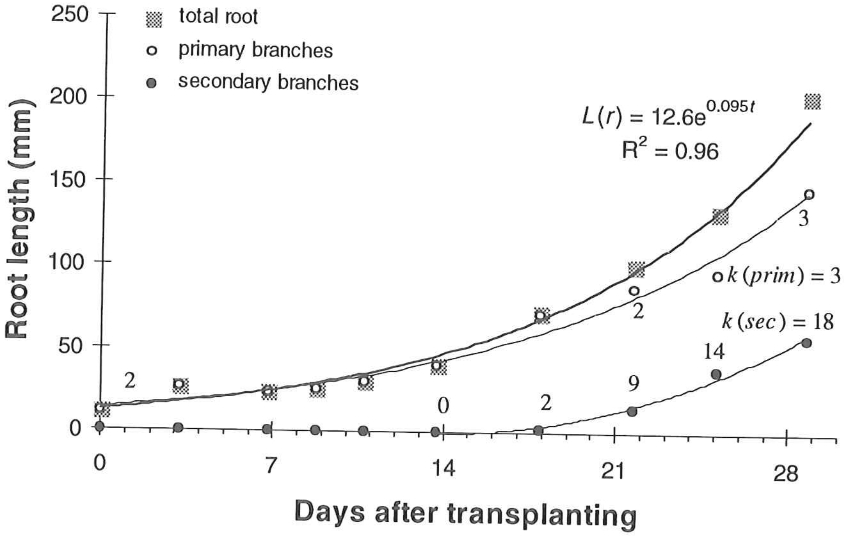

Fig. 1. "Typical" growth curves for primary, secondary and total root lengths, showing number of branches (k) at each branching level.

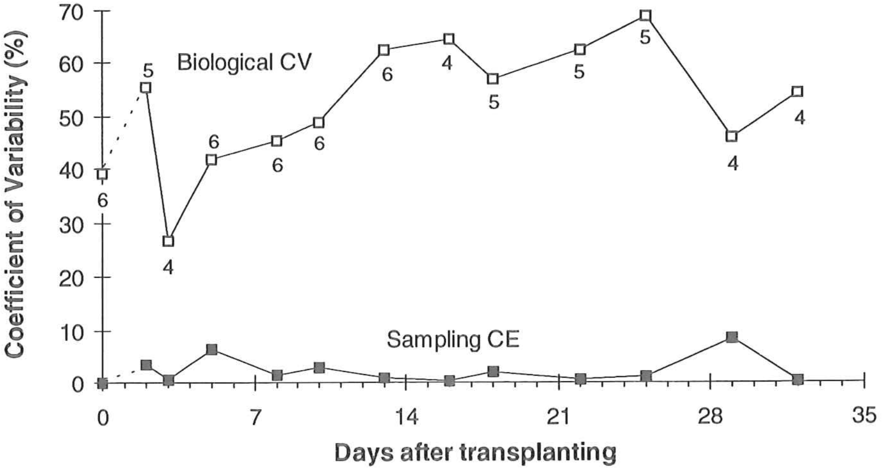

Fig. 2. Biological *CV* and average sampling *CE* versus time for total root length estimates. The numbers indicate the number of plants (replicates) at each time measurement.

Table 1. Total and average length of roots at each level of branching, average diameters, total lengths and surface areas of roots 25 days after transplanting.

Plant No.	$L(prim)$ mm	$\ell_k(prim)$ mm	$\bar{d}\,(prim)$ µm	$L(sec)$ mm	$\ell_k(sec)$ mm	$\bar{d}\,(sec)$ µm	$L(r)$ mm	$S(r)$ mm^2
1	112	37.3	239	41	1.9	186	152	108
2	75	25.0	187	19	1.9	165	94	54
3	175	43.8	198	283*	4.2*	152	458	283
4	157	31.4	229	57	2.5	159	215	141
5	96	32.0	228	37	2.6	179	134	90
Mean	123	33.9		83	2.6		211	135
CV (%)	34.1	20.8		121.	36.0		69.0	65.3

* Combined secondary and tertiary roots.

CONCLUSIONS

The method of 'total vertical projections' provides a robust, efficient way to determine lengths, branching frequencies and diameters of developing root systems grown in transparent growth media.

For the *Agropyron cristatum* L. root systems analyzed in this study, the biological *CV*'s of total length ranged 40-70%. Minimum and maximum *CV*'s over this range were likely associated with variation in the 'initial' lengths (at the time of transplanting) and to variation in the degree of

secondary root branching, respectively. The method provided very low sampling *CE*'s, estimated consistently under 10% (and usually well below 5%) for 50-100 grid intersection counts. It would therefore have been sufficient to estimate root length from a single projection. More plants would be needed to detect any change in the coefficient of variation of root growth with time.
The described procedure provides a research tool for some fundamental studies of factors affecting root growth. Its main advantages are that it is non-destructive, it provides rapid, unbiased estimates of root parameters, and it can be implemented at low cost since computer-generated grids are a convenience, not a necessity. A major drawback is that the plants were not grown in soil. Full potential of the method of 'total vertical projections' as a research tool in root studies may be achieved by analyzing NMR or CT images of root systems grown in soil. The current limited resolution of images obtained this way is a major constraint (see Box, 1996). Advances are being made in the use of such imaging technologies for root studies, and it is conceivable that the integration of these methods will be viable over the next few years.

ACKNOWLEDGEMENT

The authors are grateful to the following: A. J. Cutler, Plant Biotechnology Institute (PBI), National Research Council of Canada, Saskatoon, Saskatchewan, for suggesting this project and for guidance with respect to the plant growth program; Tim Squires, PBI, who developed the basic plant growth protocol; Albert O. Meier, Department of Anatomy, and Annette Larsen, Stereological Research Laboratory, University of Aarhus, for expert photographic assistance; Annette Thomsen, Human Genetics Group, University of Århus, for access to laboratory facilities. We also benefited from discussions with H. J. G. Gundersen on the stereological procedures. This work was carried out while the first author was on a sabbatical leave from the University of Saskatchewan, held at the Stereological Research Laboratory.

REFERENCES

Albrechtová J, Kubínová L, Votrubová O, Eliášová, K. Non-destructive stereological method for estimating the length of rigid root systems. Brief Communication, Biologia Plantarum 1997/98;40(2):311-316.

Box JE, Jr. Modern methods for root investigations. In: Waisel Y, Eshel A, Kafkafi U, eds. Plant roots. The hidden half. 2nd ed. Ch. 10. New York: Marcel Dekker 1996: 193-237.

Cruz-Orive L. Systematic sampling in stereology. Bull Intern Statis Inst, Proc 49th Session, Florence, 1993;55(2):451-468.

Cruz-Orive LM, Howard CV. Estimating the length of a bounded curve in three dimensions using total vertical projections. J Microsc 1991;163(1):101-113.

Gokhale AM. Unbiased estimation of curve length in 3-D using vertical slices. J Microsc 1990;159(2):133-141.

Presented at the 7th European Congress for Stereology, Amsterdam, April 20th to 23rd, 1998.