BRIEF COMMUNICATION

Non-destructive stereological method for estimating the length of rigid root systems

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Abstract

A non-destructive method of total vertical projections for estimating the length of rigid root systems, not introduced yet in plant sciences, is described. It is demonstrated on measuring less and more dense root systems of seedlings of *Zea mays* grown at hypoxic or control conditions. Photographs of six vertical projections (30° apart) of each root system were taken and evaluated. The method being based on proved mathematical formula offers unbiased estimation of the length of a rigid root system, curved in three-dimensional space, by non-destructive means. Thus, it is applicable during ongoing experimentation on plants grown in a solution culture. It was shown that less than 120 intersections between the root projection and test lines in one photograph ensured sufficient precision of the method and that the observer subjectivity could be overcome by presented instructions.

Additional key words: growth analysis, total vertical projections, *Zea mays*.

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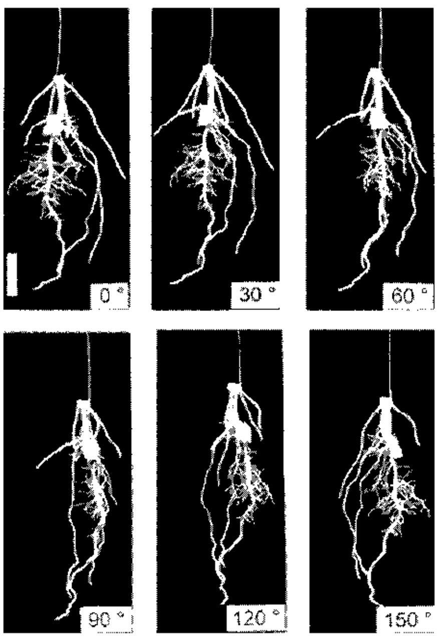
Root length is an important parameter for determination of the absorption of water and nutrients from soil. Methods for measuring root length published up to now are mostly destructive or at least designed only for root systems which can be flattened. Destructive methods are based either on the so-called line-intercept method (Newmann 1966; the principle of this method was shown already by Buffon in 1777 - see, *e.g.*, Weibel 1979), or image analysis processing (Tanaka *et al.* 1995) or on counting intersections of roots and faces of cubes cut off soil containing roots (Lang and Melhuish 1970). The line-intercept method, which is performed manually (*e.g.* Newmann 1966, Tennant 1975, Bland and Mesarch 1990) or automatically, usually with the help of an image analyser or scanner (*e.g.* Harris and Campbell 1989, Zoon and van Tienderen 1990, Kirchhof 1992, Bláha and Janáček 1997), assumes that the roots can be teased apart after washing and positioned flat in a layer of water. It means that this method is not applicable to stiff, suberised roots of woody species, which cannot be perfectly flattened. Therefore, a non-destructive method enabling to measure the length of stiff roots, curved in three dimensional space, is clearly demanded. Moreover, non-destructive approach to root length measurement is missed for the purposes of a gradual observation of root length increments during a physiological experimentation.

For this purpose, the root length was estimated by a recently developed and mathematically proved unbiased method for estimation of the total length of a linear feature in three dimensions from total vertical projections (Cruz-Orive and Howard 1991) based on work of Gokhale (1990) and subsequently demonstrated in practical examples by Roberts *et al.* (1991).

The application of this method to estimation of the root length has not been published yet. The only published example of the method application and testing for plant material up to now is the determination of the branch length of a bonsai tree (Roberts *et al.* 1991). The authors suggested a possible application for the estimation of root length there. However, the method of total vertical projections requires a fixed curve shape and not-too high length density, so that overlapping effects are more or less negligible after projection. Since the root systems usually represent quite a complex structure with many overlapped and overlaid root intersections (Bland and Mesarch 1990), even in some cases exhibiting planar anisotropy, the aim of this study was to test the possibility of overcoming the above shortcomings. To test the method for more structured and congested rigid root systems we needed both the simple and the more structured root systems. Thus, we used root systems of *Zea mays* L. affected by hypoxia in order to produce simpler root systems.

After 3-d germination of caryopses of *Zea mays* L. cv. CE 250 at 20 °C in the darkness, seedlings of uniform size were selected and transferred into plastic vessels filled with washed coarse sand. They were placed into a growth chamber with 16-h photoperiod, irradiance of 80 W m^{-2}, and temperature of 20 °C. Hypoxia was evoked by flooding them with distilled water, the level of which was maintained 5 mm above the sand surface during the whole cultivation period. The control plants were watered daily in quantities sufficient to moisten the sand fully but without allowing liquid to accumulate at the bottom of the vessels.

Five root systems were examined in this study: two hypoxic ones (H1, H2), two control ones (C1, C2) from one experiment and one control root system (C3) from the repetition of the experiment. Higher degree of planar anisotropy was found in root systems C1 and H2, remaining ones were more or less isotropic. The whole root systems were cut off 5 d after flooding. Generally, the maize roots are not completely stiff but approximately keeping spatial arrangement when taken out from a solution culture. To achieve a complete rigidity of root systems, the fixation in FAA (the mixture of formaldehyde with 70 % ethanol and acetic acid in the ratio 1:18:1 by volume) for 24 h at least was used. Fixed (*i.e.* rigid and keeping the original architecture) roots were taken out of liquid and placed into a simply arranged photographic recording system comprising a black board (contrast background), a simple hanging system, a protractor (for measuring angles of rotation), and a camera on a stand. The direction of the vertical axis was chosen to coincide with the direction of the root rotation axis (roots were hung on a thread), *i.e.* roughly parallel



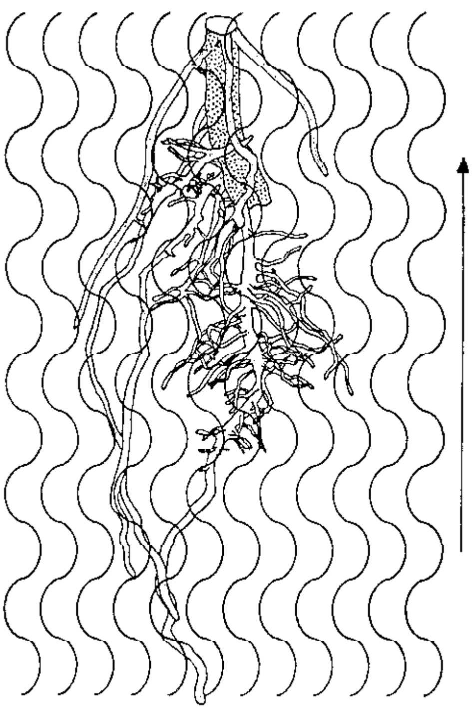


Fig. 1. Six vertical projections of the control root system C1 taken under 30°-intervals of rotation. The arrangement of the root system does not exhibit planar anisotropy. Scale = 2 cm.

Fig. 2. Random superimposition of the cycloid test system on the root projection. Dotted area (caryopsis and mesocotyl) is not included into the measurement. The vertical direction is indicated by the arrow.

to the main root axis. It meant that the root total vertical projections were parallel to the main root axis and could be obtained by photographing the entire root system from different sides. With a starting position given by a uniform random rotation of the root system, six photographs of each root system were taken under 30° -intervals of rotation (Fig. 1). The uniform random rotation can be assured by selecting a random number from the set {0,1,2,..,29}. If this number happens to be *e.g.* 19, then the photographs would be taken under 19°, 49°, 79°, 109°, 139°, and 169°.

The cycloid test system (Fig. 2 and Cruz-Orive and Howard 1991) was superimposed on photographs and intersections between the root system and test lines were counted. The total root length (L) of each root was estimated using the formula of Cruz-Orive and Howard (1991):

$$\text{est L} = 2\,\frac{a}{l}\,\text{M}^{-1}\,\frac{1}{n}\,\sum_{i=1}^{n} I_i$$

where est L is the unbiased estimator of the root length, a/l is the ratio of test area to cycloid test length (equal to 7.069 mm in our case), M is the linear magnification of the vertical projections, n is the number of vertical projections analysed ($n = 6$ in our case), and I_i is the total number of intersections counted for the i-th vertical projection ($i = 1,..., n$).

To test the effect of observer subjectivity, six persons repeated all measurements under the same superimposition of the test grid chosen uniformly at random in the beginning. The instructions given to them were based only on the available papers of Cruz-Orive and Howard (1991) and Roberts *et al.* (1991). The effect of grid superimposition was tested by measuring each photograph by one observer under six systematically shifted random positions of the test grid. The uniform random position of the first superimposition was assured by placing the grid reference point so that its co-ordinates were given by two independent random numbers (see also Kubínová 1993).

The root length of hypoxic (H) root systems was lower than the length of control (C) ones (Tables 1, 2). This is in agreement with biological interpretation since hypoxia reduces the development of the root system (Wiedenroth and Erdmann 1985). Results obtained by six observers having a different experience demonstrate that subjectivity of an observer may cause a difference in obtained absolute values (proved by analysis of variance, *ANOVA*, on $P = 0.01$), usually following a pattern: the more experienced observer the higher number of counted intersections. Extreme values reported by less-experienced and well-experienced observer showed even the difference of $P < 0.01$ level by the Scheffé method. However, the differences between variants remained unchanged. The higher estimates of root length obtained by more experienced observers were found also for the manual line-intercept method (Bland and Mesarch 1990, Farrell *et al.* 1993). For measurements obtained by one observer under six different superimpositions of the test grid on photographs (Table 2) *ANOVA* did not prove any effect of superimposition on the results.

To minimise observer subjectivity and to assure proper counting of intersections, we recommend: *1*) Each root projection abstracted by the observer as a one-dimensional curve by simply taking only one arbitrarily chosen border of the root as that curve and simultaneously the cycloid test system abstracted as a one-dimensional curve. For instance, to count only the intersections between the leftmost border of the root projection and the rightmost border of the test lines; *2*) to trace the path of intersected roots to decide how many roots were in fact intersected by the test grid before starting to count; *3*) to ensure a blind identification of measured projections before counting in order to exclude subconscious error of the observer; *4*) evaluation of the whole experiment by one observer.

The method offers an unbiased estimation of the root length using the mathematically proved estimator provided an observer follows the recommended rules listed above. It does not require any sophisticated device for measurement and it is suitable for rigid root systems not measurable by line intercept method. As the same plants could be monitored during the experimentation period, even when roots are not entirely stiff, but can be arranged into a constant shape, this method might be applied for growth analysis. The intersection counting is quite fast, even if a high precision is required. The time for evaluating one photograph depends on the complexity of the root system measured and on the number of counted intersections. In our case, when the number of intersections had a value from 61 to 116 (which assured coefficient of error less than 5 %) the time spent ranged between 60 and 150 s. If required, the method enables to measure the length of roots of different categories (*e.g.* in case of maize: adventitious nodal roots, adventitious mesocotyl roots, adventitious seminal roots) or root orders (*e.g.* by marking the parts of the root system in the photograph by different colours). If a sufficiently contrast background is used, the root projections might be quite easily segmented by an image analyser. After generating the test system by computer graphics and projecting it on the screen of the display of an image analyser, the intersections could be counted (using *e.g.* software provided by *Kinetic Imaging*, Liverpool, Great Britain, or *Laboratory Imaging*, Prague, Czech Republic). However, a manual control of root overlaps might be desirable.

The objection against the application of the method in more extensive experiment could be the requirement of six rotations of one root. Up to now, the recommended and tested number of vertical projections taken has been preferably six, possibly three if the objects do not exhibit a strong planar anisotropy (Roberts *et al.* 1991). In the present study it was found that the number of projections could be lowered to three in all roots examined as the differences in values obtained were below 6 % from the pooled estimate in agreement with the calculated error. Lowering the number of vertical projections to only one may be recommended only for isotropic root systems.

Apparently, the method of total vertical projections offers a lowering of methodical limitations of a current research in experimental botany.

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