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REGULAR ARTICLE

Estimating root elongation rates from morphological measurements of the root tip

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Abstract To measure the elongation rate of individual roots in soil remains a challenge. A novel method for estimating elongation rates of excavated roots is presented. Morphological markers are identified along the tip of excavated roots, and their distance relative to the apex is measured. These markers correspond to developmental stages which follow known temporal patterns. Hence, their distance relative to the apex reflects root elongation during the period corresponding to their development. The method was tested on maize roots grown in a range of conditions and substrates. It was found that distances from markers to apices were proportional, with some variability, to elongation rates. Remarkably, the linear relationships between these distances were neither affected by substrate, nor by growing conditions. Using several markers allows covering time periods ranging from 0.3 day to 3 days as well as cross validation of estimates. Provided further testing, under a wider range of environmental conditions, is conducted, the concepts presented in this paper may serve to define a new measurement technique.

Keywords Root elongation · Measurement · Method · Methodology

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Introduction

To measure root growth in situ is essential to understand how plants cope with their environment, and to predict plant production, especially in suboptimal environmental conditions (mineral and water limitation, heavy soils, no-tillage cultivation, etc). However, it remains a major challenge.

Investigations on root development and functioning are still hampered by the fact that it is extremely difficult to observe and analyze roots in the soil environment (Polomski and Kuhn 2002). Roots are very fine and fragile organs covered with tiny root hairs and sticky material deposited at their surface (root exudates), which generally follow tortuous growth pathways and are intimately embedded in the soil. Moreover, the soil medium is opaque, cohesive, and dense. These root and soil properties make the in situ observation of roots a very hard and tedious

process. Quality data sets are therefore difficult to obtain, particularly when dynamic variables related to developmental processes are required.

Methods for estimating root elongation in the soil at temporal scales ranging from hours to days are available, but they are rather scarce and imperfect (Smit et al. 2000a). The most commonly used are those based on the periodic observation of roots through a window placed against the soil (rhizotron), or through a transparent tube inserted into it (mini rhizotron) (Devienne-Baret et al. 2006; Eizenberg et al. 2005; Majdi 1996; Smit et al. 2000b). These methods lead to non-invasive (and thus continuous) measurements, but they only allow observing roots that are growing against the observation window, i.e. in a very particular interface medium with specific characteristics (contact, porosity, temperature, aeration, humidity, etc.). Results obtained in such conditions may not be representative of the typical bulk growing medium. Magnetic resonance imaging (MRI) and X-ray tomography methods (Asseng et al. 2000; Danjon and Reubens 2008; Heeraman et al. 1997) are attractive alternatives, but up to now they can only be used to study small samples (a few centimetres to a few decimetres) and to specific growing substrates. The observation of excavated roots, although destructive and time-consuming, is also widely used as a simple direct method to assess the structure of whole or part of root systems. In comparison to the previous ones, a major advantage of this method is that it can be used to get data on roots growing in any part of the soil volume. However, to obtain dynamic results from usual excavation method, periodic sampling with a time interval corresponding to the specific objective of the study is needed. This requires either collecting homologous (comparable) root samples, or being able to estimate the age of sampled roots. These requirements are often difficult to meet. Dynamic variables (e.g. elongation rate) investigated on excavated roots are therefore estimated from inter-individual comparisons (e.g. difference in length). The discrimination between inter-individual, and soil-, or kinetics- induced variations therefore requires the set up of rather complex experimental designs.

The objective of this study is to present and discuss a novel method for estimating root elongation rate, and to assess its performance. The method's general principle is to derive the elongation rate of an individual root excavated at a given date from a precise point observation of its tip's morphological characteristics. Although destructive, the proposed method potentially allows the estimation of unbiased (representative) root elongation rates, provided the sampling procedure does not introduce any additional bias.

Empirically it has been known for a long time that white and long root tips correspond to fast growing roots, and conversely, that short and brown tips correspond to slow growing roots. However, this is only a qualitative appreciation of root growth. Previous work also showed that a particular characteristic of the root tip, the length of the apical unbranched zone, is correlated to root elongation rate (Lecompte et al. 2001; Pagès and Serra 1994; Pellerin and Tabourel 1995). Lecompte et al. (2001) used this close correlation to estimate the root elongation rate over a period of several days. Watt et al. (2003) noticed that elongation rate and the distance between the root tip and the zone of root hair development were correlated, providing a morphological indicator of root elongation rate in the field. As an extension, the purpose of this work is to assess the predictive value of indicators readily measurable on excavated root tips for estimating the elongation rate of individual roots at various time scales (from hours to days). This methodological study was carried out using maize roots, which exhibit large variations in elongation rate depending on their position within the root system. The method's robustness was tested using measurements made on roots grown under a range of environmental conditions.

Material and methods

Plant material and experimental setup

Maize (*Zea mays*, cv. DK 475) seeds were germinated in a mixture of peat and vermiculite (1v:1v) kept at a constant temperature of 24°C. At the time radicles were about 4-cm long, seedlings were transferred to root observation boxes (L×W×D: 30 cm×1 cm×50 cm) or cylindrical pots (Ø×D: 10 cm×66 cm) filled with a mixture of vermiculite and peat (1v:1v) or sand and peat (1v:1v). In some of the root boxes, a nylon mesh was inserted between the substrate and the root window in order to prevent root penetration into the substrate and enable their observation through the window (seedlings were placed between the window and the nylon mesh). A gravity stimulus

was applied to some of the pot-grown plants by rotating the cylinder pots so as to transiently reduce the root elongation rate (e.g. Le Roux and Pagès 1996). All experiments were carried out in a growth chamber with 400 µmoles.m^{-2}.s^{-1} PAR, 16 h photoperiod, 25°C and 50–70% RH.

Sampling and measurements

Root elongation rates were measured on plants grown in root observation boxes based on daily recording of the positions of every individual root's apex visible through the window area. Indicator variables (see below) were measured simultaneously on the growing root tips.

Root systems were excavated from pots and root boxes on three successive occasions (9, 10 and 13 days after transplantation) by gently washing the soil off the roots with running water. Root systems of plants that were not gravity-stimulated were excavated on the first and second occasions, while that of gravity-stimulated plants were sampled last. Six roots were sampled from each of the washed root systems (seminal, early-nodal, and first-order lateral). Roots grown in substrate retained a sheath of substrate particles at some distance from the apex (Fig. 1a). This sheath was not washed off as it is an indicator that we measured (see below). Root tips were kept wet in labelled Petri dishes for subsequent measurements and were imaged using a flatbed scanner with a resolution of 9200 dpi. Root diameters were measured on these images using the ImageJ software (http://rsb.info.nih.gov/ij ImageJ, Image Processing and Analysis in Java). Primordia were detected on root tips fixed in Clarke's liquid and cleared in lactic acid, following coloration using the Feulgen's procedure (McLeod 1989).

Variables measured on root tips, referred to as "indicator variables" in the text, were (see also Fig. 1b):

- Length of the apical unbranched zone (L_{lat}), i.e. the distance from the apex to the most distal lateral root longer than 0.5 mm, measured with a ruler;
- Length of the apical zone without initiated primordia (L_{prim}), calculated as the average of the distances from the apex to the three most distal primordia, measured under a binocular microscope with a graticule;
- Distance from the apex to the most distal root hair longer than 0.2 mm (L_{hair}), measured with a binocular microscope;

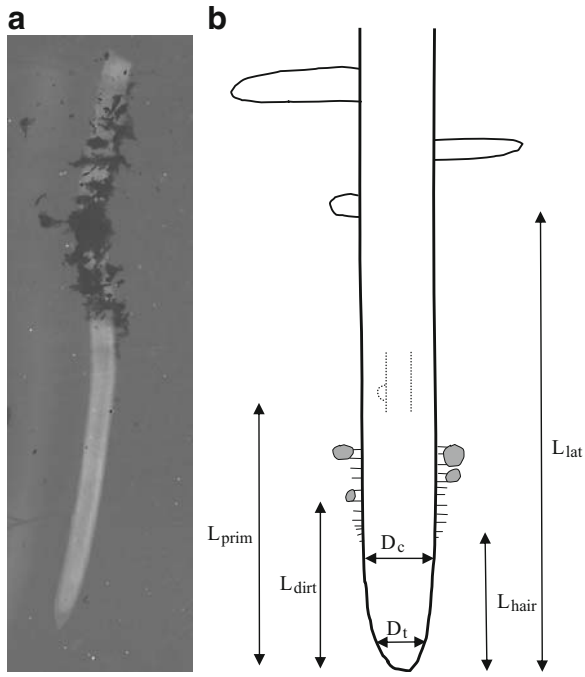

Fig. 1 **a** Picture showing the morphology of a typical root tip, and the adhering substrate at some distance from the apex. **b** Schematic of the root tip presenting morphological characteristics and variables measured on it

- Distance from the apex to the most distal substrate particle adhering on the root surface (L_{dirt}), measured with a ruler;
- Root diameter in the zone where it becomes cylindrical, i.e. at about 3 mm from the apex (D_c), measured on scanned images of root tip samples;
- Root diameter at a distance of 0.75*D_c from the apex (D_t), in the centre of the tapering zone, measured on scanned images of root tip samples;
- Apical tapering (A_T), defined as the relative difference (D_c-D_t)/D_c;

Among the set of variables measured on root tips, two groups can be distinguished. The first group (L_{lat}, L_{prim}, L_{hair}, L_{dirt}) encompasses variables which are dependent upon developmental stages (lateral root emergence, primordium initiation, root hair formation, formation of sticky epidermis), whereas the second group (D_c, D_t, A_T) includes variables which characterize the shape of the most distal part of the root tip, in the growing zone (meristem and elongation zones).

Not all root tip samples could be successfully retrieved: some were lost or damaged during excava-

tion, while in other cases images were of insufficient quality and had to be discarded. The final number of plants and excavated roots for each condition are given in Table 1. The number of available data depended on the indicator, since some could be measured only on branched roots (L_{lat}) or on excavated roots (L_{prim}).

Data analysis

All data analyses were performed with the R statistical software (R Development Core Team 2008; http://www.R-project.org/).

Smoothing tools (such as the local weighted regression smoother "lowess") were used to explore trends between variables, particularly between candidate indicators and our variable of interest, i.e. elongation rate. Statistical inference and model selection were performed by means of covariance analysis (using the "lm" procedure in R). The various indicators were included in the models as continuous quantitative variables, and the growing conditions were included as discrete factors.

Results

The suitability of morphological indicators to predict root elongation rate was first tested in various conditions in root observation boxes. Then, the robustness of the method was further validated by comparing the relationships between indicators in extended conditions, comparing root boxes and pots, and applying gravi-stimulation.

What are the best predictive indicators?

Linear relationships were observed between the variables of the first group and the elongation rate, as illustrated for L_{hair} and L_{lat} (Fig. 2). The linearity of these relationships implies that, at a given time t, the selected morphological variables and elongation rate (estimated over the [t-24 h, t] interval) are linked by a constant factor over the range of elongation rate values. The best correlation observed was the one between elongation rate and L_{hair} (Table 2).

Variables related to the shape of the tip (D_c, D_t, A_T) also exhibited a positive dependency upon elongation rate, yet the quality of these correlations was much lower (D_c, D_t) or even not significant (A_T). Elongation rate variability for a given apical diameter was rather large, mainly due to some of the thickest roots having a slow to intermediate growth rate (Fig. 3). This plot suggests that the diameter tended to set an upper limit to elongation rate.

Covariance analyses and linear regressions were performed in order to select, out of the first group of variables, i.e. L_{lat}, L_{prim}, L_{hair}, L_{dirt}, the set of indicators that best predict root elongation rates. With all tested models, the intercept was not significant, confirming the strict proportionality of the indicator variables to elongation rate. The full model (using all variables without intercept) explained 96.8% of the total variance, with a single significant slope coefficient (L_{hair}) revealing a high redundancy between the indicator variables. The amount of variance explained by all reduced models varied only slightly. Additional linear models based on indicators of the tip shape (second group of indicators: D_c, D_t, and A_T) were also

Table 1 Experimental design, number of plants and excavated roots in each growth condition

Container	Substrate	Gravity stimulation	Number of plants	Number of roots
Cylinder Pot	Vermiculite/peat	no	2	10
		Yes	1	6
	Sand/peat	no	2	10
		Yes	1	6
Root box	Nylon mesh	no	2	12
		yes	1	6
	Vermiculite/peat	no	2	10
		yes	1	4
	Sand/peat	no	2	10
		yes	1	6

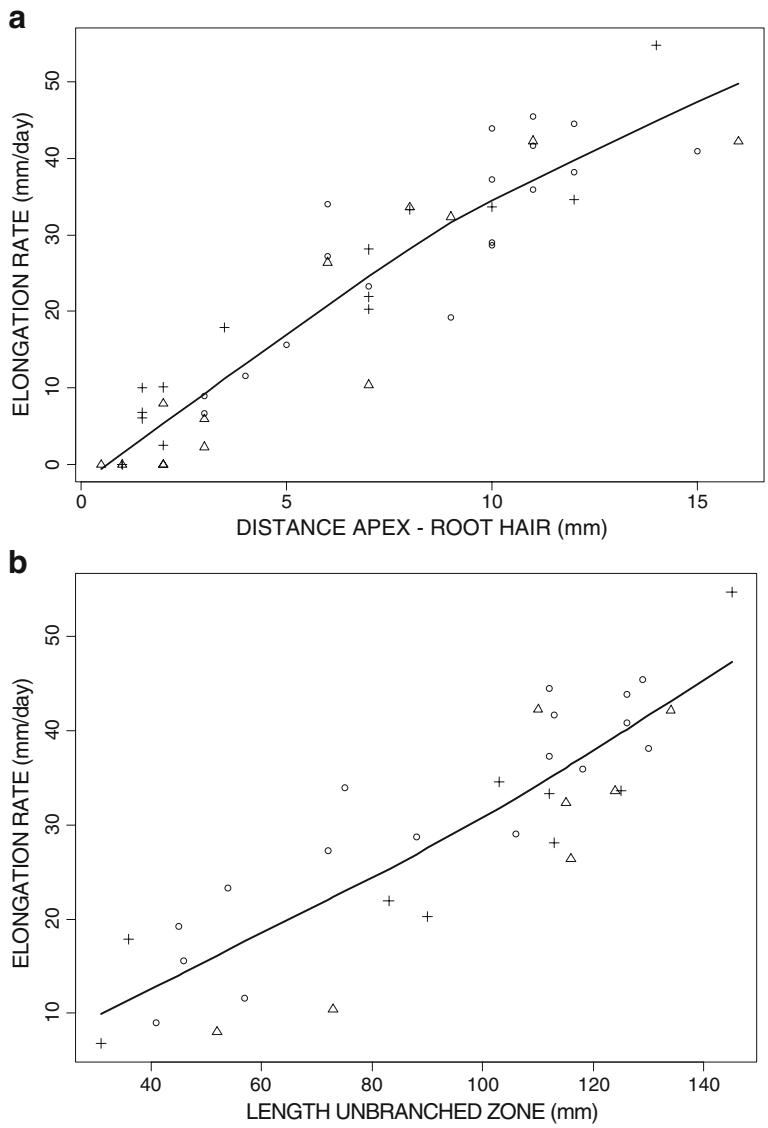

Fig. 2 Relationships between indicators and elongation rate, presented in two examples. **a** Elongation rate versus distance from the apex to the nearest root hair at least 0.2 mm long (L_{hair}); **b** Elongation rate versus length of the unbranched zone (L_{lat}). Each point represents a root measured in a root box. Symbols indicate substrates (circles: nylon mesh; triangles: peat/vermiculite; plus: peat/sand). The curves represent the trends, as evaluated by a local regression smoother (lowess)

tested. However, none of them improved the quality of elongation rate prediction. Table 3 presents the coefficients and tests for the best two- and single-variable based models.

Since the L_{hair} variable was available on a larger number of roots (L_{dirt} could not be measured in root boxes with nylon mesh), we subsequently used the model including only L_{hair} as a reference.

Is the prediction dependent upon the nature of the substrate?

To address the issue of the sensitivity of our predictions to environmental factors known to affect

Table 2 Correlation coefficients between elongation rate and indicator variables of the first group

Indicator	Correlation coefficient	Degrees of freedom	Probability value
L_{hair}	0.926	44	2.2e-16
L_{dirt}	0.857	24	2.2e-08
L_{lat}	0.871	31	4.5e-11
L_{prim}	0.743	41	1.2e-08

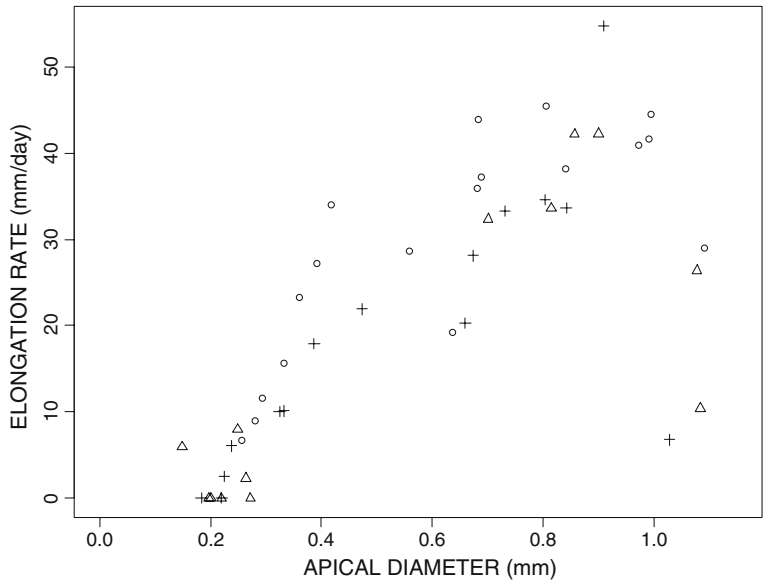

Fig. 3 Root elongation rate versus apical diameter (D_c). Each point represents a root measured in a root box

root elongation, the substrate factor (with 3 levels: Nylon mesh, Vermiculite/Peat without nylon mesh, and Sand/Peat without nylon mesh) was added to the previously selected model.

We found that this substrate factor did not have any significant interaction effect on the slope nor any influence on the intercept. Further, we found that the overall quality of the prediction was not significantly improved by this additional factor, and the estimated slope parameters were not significantly different from one substrate to another (data not shown). The prediction could therefore be considered as independent of the substrate, at least with the range of substrates that we tested.

Table 3 Predictive models of the elongation rate using either L_{hair} (a) or both L_{hair} and L_{dirt} (b) as independent variables

Coefficients:			
Estimate	Std. Error	t value	Pr(>|t|)
(a)			
L_{hair} 3.3591	0.1131	29.70	<2e-16
—			
Residual standard error: 6.043 on 45 degrees of freedom			
Multiple R-Squared: 0.9515, Adjusted R-squared: 0.9504			
F-statistic: 882.2 on 1 and 45 DF, p-value: <2.2e-16			
(b)			
L_{hair} 2.2664	0.2953	7.676	6.51e-08
L_{dirt} 1.0308	0.2599	3.966	0.000575
—			
Residual standard error: 4.607 on 24 degrees of freedom			
Multiple R-Squared: 0.9668, Adjusted R-squared: 0.964			
F-statistic: 349.2 on 2 and 24 DF, p-value: <2.2e-16			

Are the relationships between indicator variables dependent upon the substrate?

The relationships between indicator variables were considered using the whole data set (including plants cultivated in pots on which elongation rate could not be recorded). The four indicator variables from the first group (L_{hair}, L_{dirt}, L_{lat} and L_{prim}) were found to be significantly correlated (Table 4).

As for the predictive models, the stability of these relationships across growing conditions was assessed. A linear regression model was fitted to each pair of indicators, and the existence of an effect of the substrate factor on the intercept and the slope was

Table 4 Correlation coefficients between indicators L_{hair}, L_{dirt}, L_{prim} and L_{lat}. Numbers in parentheses are degrees of freedom. All correlations are highly significant

	L_{hair}	L_{dirt}	L_{prim}
L_{dirt}	0.808 (57)		
L_{prim}	0.771 (74)	0.749 (54)	
L_{lat}	0.864 (55)	0.736 (37)	0.746 (55)

Table 5 Estimated values of time scales represented by different indicators

Variable	Estimated duration (in days)	Estimated duration (in degree.days, base 8°C)
L_{hair}	0.28	4.8
L_{dirt}	0.31	5.3
L_{prim}	0.86	15
L_{lat}	3.1	53

tested. None of these linear relationships was affected by the substrate factor. Overall, the stable relationships which exist between these indicators clearly argue in favour of their robustness for prediction purposes. Moreover, no substrate effect could be detected on apical tapering (A_T).

What are the time scales corresponding to the occurrence of our indicator variables?

The slope of the regression of L_{hair} (or other indicator variables of the first group) versus elongation rate (inverse to the relation shown in Fig. 2) can be interpreted as the time required for a cell formed in the centre of the meristem to be displaced to the position of the morphological marker, i.e. to reach the corresponding developmental stage (e.g. root hair formation in the case of L_{hair}). Based on such an analysis of the slopes of the various linear regressions we could establish between elongation rate and the variable indicators of the first group, we found that the duration of these developmental stages varied by one order of magnitude, from about 0.3 day (L_{hair} and L_{dirt}) to 0.9 days (L_{prim}) and 3 days (L_{lat}) (Table 5).

From this analysis, it follows that beyond likely differences in their predictive value, the indicator variables we considered in this study do not integrate the same time scales. This point was clarified based on the analysis of plants exposed to a gravity stimulus one day prior to harvest. Based on previous experiments (Le Roux and Pagès 1996), slightly reduced elongation rates were expected during a period of one day following the stimulus.

Figure 4 shows the measured elongation rates before and after the gravity stimulus, for roots of plants grown in root boxes. As expected, the elongation rate tended to lower after exposure to the gravity stimulus (in more than 85% of cases). The mean relative difference was of about 10%.

The relationship between L_{lat} and other variables (L_{hair}, L_{dirt}, L_{prim}) was also affected by the gravity stimulus, as revealed by linear regressions (L_{lat} versus other variables) using the complete data set and considering the gravity stimulus as a Boolean factor (yes or not). The shorter term indicators (L_{hair}, L_{dirt}, L_{prim}) were significantly reduced on stimulated plants ($p<5\%$, data not shown), indicating that these were more affected than L_{lat} by the growth reduction.

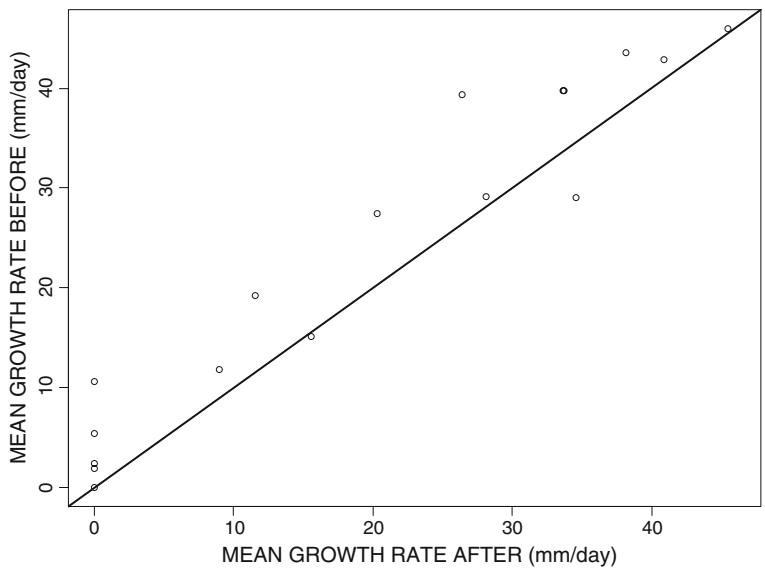

Fig. 4 Relationship between elongation rates of the same individual roots before and after gravi-stimulation

Discussion

This work has confirmed that it is possible to obtain a good prediction of root elongation rate by means of morphological indicators that can be readily measured on excavated root tips. Beyond the fairly high accuracy of the method (selected indicators accounted for more than 96% of the elongation rate variation), its major interest is that it allows unbiased estimation of elongation rates in real soil conditions. Roots can be excavated at any time from their growth medium and indicators directly measured and recorded. This contrasts with most other root observation methods in which roots are constrained to grow into a specific medium to allow their observation (Majdi 1996; Smit et al. 2000b).

The indicators which gave the best prediction were those variables which quantify the distance from the apex to a series of morphological markers corresponding to specific developmental stages. Interestingly, these indicators behaved as simple linear functions of elongation rate without intercept. Because young cells originate at the distal end of the root and move virtually from the apex during their differentiation (as a result of the continuous production and elongation of new cells at the distal end), the linearity of this relation and its specificity to each indicator implies that the corresponding differentiation period requires a specific and constant time lag. The predicted elongation rate is then obtained by dividing the observed position of the indicator by its specific differentiation period. This principle can be applied on several developmental processes that can be detected easily and unambiguously by a morphological indicator either on the root surface (e.g. root hair formation, emergence of lateral roots) or within the root (e.g. primordium initiation).

From our data it was possible to associate a mean duration for each event (Table 5) and thus to specify various time scales at which the method can be applied. These time scales range from about 0.3 to 3 days. These values are comparable to those reported for the same developmental processes on *Musa* (Lecompte et al. 2001), or maize (Pellerin and Tabourel 1995), or oak tree (Pagès and Serra 1994). These time scales are well-suited to in situ root studies: shorter time scales would not be significant for field studies, and lead to much uncertainty, while longer time scales would not make sense for categories of roots whose elongation lasts only several days. Three days (associated to L_{lat}) appears as a maximal duration for measuring the elongation rate of such roots.

Since several indicators can be used, the method allows some cross validation between several estimates. Discrepancies originating from measurement errors or uncertainties can be detected and further investigated.

Moreover, since associated developmental durations differ greatly depending on indicators (about one order of magnitude), the method offers the possibility to measure not only the elongation rate, but also its temporal variations (growth acceleration or deceleration). This possibility has not been tested in the present study, but it could be the subject of further developments.

The finding of a linear relationship between elongation rate and L_{lat} indicates that the time it takes for a cell to move from the root extremity to the longitudinal position of lateral root emergence is constant. However, different zones (possibly overlapping) make up a cell trajectory from the root tip to the site of lateral root emergence. Two regions that seem especially relevant for the L_{lat} indicator are the growth zone (division – transition - expansion) and the zone between the sites of lateral root initiation and emergence. The linear relationship between elongation rate and L_{lat} should therefore be viewed as an indication that the residence time of cells in all these zones is constant, as it seems indeed unlikely that any variation of the cell residence time in the growth zone would be compensated by an opposite variation of the cell residence time in the other zone. It follows that the reliability of the elongation rate predictions by L_{lat} would ultimately depend on the degree to which experimental conditions affect the residence time of cells in the developmental zones that are relevant to L_{lat}, a reasoning which can be equally formulated for L_{hair}, L_{dirt} and L_{prim}.

This link between the proposed indicators and the regulation of specific developmental processes could be exploited for the validation of the method in a wider range of conditions. In this respect, kinematic studies provide already a wealth of information on the variation of cell velocity arising from environmental, physiological, or genotypic effects (Sharp et al. 1988; Beemster and Baskin 1998; Beemster et al. 2002; Sharp et al. 2004). Cell residence time in the growth zone could in principle be derived from those studies and help in estimating the quality of elongation rate prediction under similar circumstances.

The robustness of this method versus environmental conditions has been partially tested using various substrates and modifying the growth rate with a gravity stimulus. Our first attempts were encouraging, since we could not detect any substrate effect and we could detect growth rate variations induced by a gravity stimulus. Therefore, the same model parameters could be used for the prediction in several substrates. Nevertheless, one cannot exclude the possibility that application of the method in a large range of conditions may require local calibration.

To go further with this validation process, it would be necessary to identify environmental and endogenous factors likely to modify the duration of the developmental events that are considered. Among them, temperature is obviously an important factor, and a cumulated thermal time (generally expressed in degree days) may be required when temperature varies significantly. It might also be necessary to consider trophic aspects as well. L_{lat}, for example, reflects the time required for initiating and developing a lateral root. This time has been shown to be relatively constant in some species (Lecompte et al. 2001), but it may be affected by environmental conditions experienced by the plant, such as a reduction in photo assimilate availability (Willaume and Pagès 2006).

Other variables which have been tested and describe the shape of the very end of the root tip, did not improve the prediction. For example, the apical diameter, which is known to be correlated with the elongation rate, cannot give the same accuracy in the prediction as that obtained from variables of the first group. As shown in previous articles (Pagès 1995; Thaler and Pagès 1996), and confirmed in this study, the apical diameter reflects more a potential growth rate than an actual growth rate. Both variables (apical diameter and elongation rate) coincide only when root elongation is unconstrained. Since root growth is more often than not constrained, either by external or endogenous factors, this variable is of little help to make predictions of elongation rate under a wide range of environmental conditions.

The method can probably be improved and extended using other histological measurements, relying on the differentiation of specific tissues, like endodermis. However, while the method would certainly gain in accuracy if such additional indicators were taken into account, it would also become more complex, hence not as easy and flexible to put in practice.

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