

Running head : Imaging software for root architecture analysis

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Journal research area:

Breakthrough Technologies

A Novel Image Analysis Toolbox Enabling Quantitative Analysis of Root System Architecture.

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Footnotes :

The research findings reported here were supported by the BARN project of the Belgian Science Policy, the ARC05/10-329 grant of the Communauté française de Belgique and fellowships to GL (FRIA, Belgium) and XD (FNRS, Belgium).

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Abstract

We present in this paper a novel, semi-automated image analysis software to streamline the quantitative analysis of root growth and architecture of complex root systems. The software combines a vectorial representation of root objects with a powerful tracing algorithm which accommodates a wide range of image sources and quality. The root system is treated as a collection of roots (possibly connected) that are individually represented as parsimonious sets of connected segments. Pixel coordinates and grey level are therefore turned into intuitive biological attributes such as segment diameter and orientation, distance to any other segment or topological position. As a consequence, user interaction and data analysis directly operate on biological entities (roots) and are not hampered by the spatially discrete, pixel-based nature of the original image. The software supports a sampling-based analysis of root system images, in which detailed information is collected on a limited number of roots selected by the user according to specific research requirements. The use of the software is illustrated with a time-lapse analysis of cluster root formation in lupin (*Lupinus albus*) and with an architectural analysis of maize root system (*Zea mays*). The software, SmartRoot, is an operating system independent freeware based on ImageJ and relies on cross-platform standards for communication with data analysis softwares.

Keywords: root system architecture, image analysis, root growth, quantification, cluster root, maize

1 Introduction

...any non-specialist faced with an image analysis problem rapidly realises that a unique image transformation usually fails to solve it.

P. Soille, 1999

It is now widely accepted that root system architecture (RSA) is a fundamental component of agricultural and natural ecosystems productivity (Lynch, 1995 ;Hammer et al., 2009 ;Hodge et al., 2009). Concurrently, recent progress in our understanding of the molecular bases of root growth and development in model systems (de Smet et al., 2006 ;Péret et al., 2009) and novel insights on the role of RSA in field resource capture (Draye et al., 2010) yield new prospects of manipulating RSA in crop species (de Dorlodot et al., 2007). This situation reinforces the need for robust, evolutive and high-throughput root phenotyping hardware and software solutions.

While new imaging technologies such as magnetic resonance imaging (MRI) (Jahnke et al., 2009) and X-ray computed tomography are being developed to extract RSA information from soil cores, classical imaging using flatbed scanners or cameras remains most widely used. Digital imaging is indeed affordable, features a wide range of image resolution, can be adapted to an array of experimental systems (e.g. hydroponics, aeroponics, rhizotrons, gel plates, gellan gum) and has been extended to time-lapse and 3D applications (French et al., 2009 ;Hund et al., 2009 ;Yazdanbakhsh and Fisahn, 2009 ;Iyer-Pascuzzi et al., 2010 ;Clark et al., 2011).

In the same time, the interest in RSA phenotyping has gradually evolved from static and global traits (e.g. root mass or length density) to dynamic and local traits (e.g. growth rates, tropisms, insertion angles) (Ge et al., 2000 ;de Dorlodot et al., 2007). Accordingly, a panel of software have been implemented targeting specific traits and experimental constraints (reviewed recently in French et al., 2009 and Le Bot et al., 2010). These software can be assigned to manual, semi-automated and fully automated methods according to the amount of user interaction.

In manual methods, users typically draw the skeleton of the root system using freehand graphical tools, as in DART (Le Bot et al., 2010) or WinRHIZO Tron (Regent Instruments, 2011). These methods exclude software generated errors and should provide accurate estimation of most

local and global traits, but are highly time consuming. They are often the only solution for complex root systems and for rhizotron images.

Semi-automated methods usually combine automated thresholding and skeletonisation algorithms with some extent of user intervention, mainly to re-touch and annotate software-generated root structures, as in EZ-Rhizo (Armengaud et al., 2009) and RootReader3D (Clark et al., 2011). The number of errors is usually negligible with seedling images but increases with the amount of root overlap. In principle, many traits can be estimated accurately if the software is used with the type of images and RSA for which it was designed.

Finally, automated methods rely on pre-defined procedures to perform image analysis without any user interaction. Such methods use specialised algorithms and tend to be application-specific, unlike manual or semi-automated methods that generate explicit root structure information. The number of errors is kept to a minimum as long as the type of root system and images fit the requirements of the software. Examples of automated methods include WinRhizo (length and topology; Arsenault et al. 1995), RootTrace (growth, gravitropism and branching; French et al. 2009; Naeem et al. 2011) and a recent system developed at Duke University (various morphological descriptors; Iyer-Pascuzzi et al. 2010).

In line with the distinction between methods, it is often the case that some areas of root images lend themselves to automated analysis better than other areas where the image quality is lower or where the amount of root overlap is higher and for which semi-automated or manual methods would perform better. Sometimes, a spatial segmentation of images even arises from the experimental design as with areas (or periods) where root growth is obviously altered by local (or transient) conditions. As long as the research focus is on local or dynamic traits (e.g. growth rate, growing and branching angles, diameter or inter-branch distance), a sampling-based processing strategy would be applicable to the areas of interest. In terms of efficiency, working on subsets of root systems would indeed allow to devote more attention to the subset, or to handle more (or more complex) systems at constant cost.

In this study, we introduce a novel, multi-purpose and semi-automated image analysis toolbox (SmartRoot) which speeds up the quantification of root growth and architecture of complex root systems and from a wide variety of applications. The software combines a highly intuitive user interface with a new tracing algorithm and supports sampling-based image processing. SmartRoot is a platform independent software (Windows, MacOS, Linux) implemented as a plugin for the

popular ImageJ software (Rasband, 2011) and relies on established cross-platform standards (Java, SQL and XML). Some of its features are illustrated with a time-lapse analysis of cluster root formation in lupin (*Lupinus albus*) and with an architectural analysis of maize root system (*Zea mays*).

2 Results and discussions

2.1 Multi-dimensional representations of roots

SmartRoot shares some features with Geographic Information Systems (GIS) in particular the ability to capture, store, present and process data that are linked to location. As with GIS, information is stored in separate data layers. The first layer comprises the source image viz. the 2D-array of raw pixels values. The second layer contains all root morphological information in a vector format, with individual roots approximated by segmented lines (fig. 1). A third data layer contains the topological relationships between roots, while the fourth data layer contains user- or software-generated annotations or beacons inserted along roots.

A consequence of the vector representation of roots is that any position along a root has two corresponding sets of coordinates: the classical $[x, y]$ absolute coordinates and very intuitive $[r, d]$ relative coordinates specifying the root identifier (r) and the (geodesic) distance to the root base (d). Using the second coordinate system, it is easy to calculate inter-branch distances, to combine physical dimensions with topological information and to match corresponding positions on successive images in a time-lapse analysis (see below).

The information stored in data layers is displayed on the graphical user interface (GUI) as a separate set of six Photoshop-like layers containing the source image, the skeleton (segmented line) of individual roots, the nodes of the segmented lines, the border of individual roots, their area, a geodesic ruler along each root and a visual representation of the annotations and beacons (see supplementary Figure 1).

Although invisible to the user, the separation between data layers and GUI layers disconnects the design of the GUI from any constraints relating to data structure (and vice-versa) and has proven to be instrumental in the evolution of SmartRoot since its first implementation.

2.2 Root tracing principles

SmartRoot features an automated individual root tracing algorithm triggered by a mouse click anywhere along the root in the image source GUI layer. It determines the centre (midline) of the root near the picked position and proceeds with the stepwise construction of a segmented line approximating the root midline, progressing forwards and backwards to the tip and basis of the root. The algorithm estimates the root diameter at each node of the segmented line and uses this information to set the orientation of the segmented line (from the root base of the root tip). Afterwards, a name is given to the root (see above, the (r) coordinate). This interactive procedure is at the core of the sampling-based processing mentioned in the introduction.

SmartRoot uses adaptive distances between nodes, increasing the node density for tiny roots and in curved regions of roots in order to maintain the accuracy of the segmented representation of the roots while minimising the number of nodes (fig. 2). Indeed, large roots tend to show smaller curvature than small ones and can be therefore represented with fewer nodes. The implementation of adaptive distances is detailed below (see Step 1). As it is used hereafter, the term *node* denotes the intersection of successive segments of the segmented line. It is thus different from topological nodes referring to the branching points along the roots.

The optimal placement of nodes occurs during the segmented line construction and proceeds as follows (fig. 3).

Step 1: Before adding a new node to the segmented line, the algorithm investigates the pixel values along a search path, consisting of an arc of circle centred on the current node and oriented opposite the previous node (fig. 4A). The initial amplitude of the arc is set to 90° to increase the likelihood of finding the prolongation of the root while avoiding branches or neighbouring roots. The initial radius (L_i) of this arc is set at two times the diameter of the current node.

Step 2: Pixel values along the search path are compared against a threshold value adjusted to local greyscale gradients (for details see the supplemental PDF 1). If more than one candidate position are detected, the best one is selected based on diameter similarity with the current node (fig. 4A).

Step 3: If the algorithm does not find a candidate position for the next node (fig. 4B), it changes the search path amplitude to 120° and decreases the arc radius (fig. 4C). If this fails, the algorithm considers that the end of the root has been reached and the tracing in that direction stops.

Step 4: The algorithm then seeks for multiple points (fig. 5) along root borders near the candidate position to localise root borders with a sub-pixel resolution (comparing interpolated pixel values to the local threshold determined previously, fig. 6). This procedure increases the accuracy of diameter estimation of tiny roots or in highly branched regions (for discussion on diameter estimation accuracy, see supplemental PDF 2). The distance between borders at the candidate position is then tested against three conditions.

- If that distance is smaller than 0.8 times the diameter of the previous node, the tracing stops. This condition aims at finding the very end of thick roots which have a long and conical shaped apex.
- If that distance is larger than 1.5 times the diameter of the previous one, it is considered that a neighbour root has come into contact with the root being traced, thereby creating an apparent diameter increase. A new node with a diameter equal to that of the current node and aligned to the closest root border is then created (for illustration, see supplemental Figure 2).
- If that distance is larger than 4.0 times the diameter of the previous node (fig. 7) the tracing stops. This condition occurs when the basipetal tracing of a lateral root reaches the junction with the parent root. The test may lead to different types of errors that are discussed in supplemental PDF 3, but which can be easily corrected manually.
- Otherwise, a new node with a diameter equal to the inter-border distance is created at equidistance of the two borders.

Newly created nodes are appended to the segmented line and the construction proceeds until the algorithm stops.

The parameters of the algorithm have been determined empirically using a wide range of root system architectures and image qualities. They can be taken as fairly generic although they could still be optimised for specific images.

The user can edit the newly created root by simple drag-and-drop actions on the different nodes (e.g. adding, moving, deleting or adjusting diameters). As the editing takes place in the nodes layer, it does not modify the source image and makes this operation very intuitive. Several roots can be picked and traced at once by stretching a line drawing tool across them.

2.3 Topology

Topological relationships can be set by attaching lateral roots to their parent. This information is used typically to calculate branching or insertion angles, position of insertion and inter-lateral distances. It is also very useful for monitoring the sequence of lateral root formation by individual parent roots in a series of time-lapse images. The topological information is also part of the specification of root types (primary, first order,...) and provides the shortest path connecting a root to the root-shoot junction.

Due to the large number of laterals along a parent root, SmartRoot features an algorithm to detect, trace and attach most laterals of an already traced parent root (for discussion on tracing time of laterals, see supplemental PDF 4).

This algorithm creates a search path parallel to the root border, at a distance arbitrarily set to the root diameter. Similarly to the root tracing algorithm, pixel values along this search path are compared against their local threshold value and a new lateral root is created whenever a satisfying position is found (fig. 8). For every new lateral built, a number of optional exclusion criteria (such as diameter, insertion angle and length of the newly build root) are evaluated. This filtering reduces the number of false positives but may eventually lead to a small number of false negatives (missed laterals). Adjusting the settings of the filtering reduces significantly the number of errors.

2.4 Root annotations

The $[r, d]$ coordinate system mentioned above enables the referencing of virtually any type of information with specific longitudinal positions along roots. This referencing is implemented in SmartRoot through annotation tools designed for various purposes. The study of cluster root formation given below illustrates this capability.

Annotations can be used to point to the most distant lateral along a root, whose distance to the tip can be used as a proxy to the root growth rate (Lecompte et al., 2001 ;Pagès et al., 2010). This annotation is automatically added/updated when a new most distal lateral is added to a root. Annotations can be used in pairs to delineate regions along the root, as needed in experiments involving heterogenous nutrient supply, where a separate morphological analysis of regions submitted to different conditions is desirable. Annotations can also be used as simple beacons to request subsequent exportation of local information (direction, diameter, distance to the tip, path to

the root system origin) (see below). Finally, annotations can also be used as a generic, free text commenting tool.

2.5 Time series analysis

A major feature of SmartRoot is the ability to handle sequences of time-lapse images, thereby supporting root growth and development analyses. The general principle of time-lapse handling is that information stored across different images (e.g. annotation, branches or length) and corresponding to different time points, can be cross-linked, displayed on a single image (generally the last of the time-series) and exported in a single database query (see below).

The merging of information across different images is performed in the $[r, d]$ coordinates system and is therefore independent from cartesian coordinates (fig. 9). SmartRoot has therefore the ability to handle time-stamped images of root systems grown in liquid or air, where successive images are generally not superposable. This requires that the roots of interest be traced on every image of the image sequence and be coherently identified. As this can be tedious with complex images, the graphical user interface offers several intuitive viewing tools to navigate simultaneously along a root across different images.

When plants are grown on solid medium (e.g. *Arabidopsis thaliana* in agar plates), the root system structure is generally well conserved and is only augmented from one image to the next. In such cases, the tracings stored in a source image can be imported in a target image, either in a forward way, the imported tracings being augmented in the target image, or in a backward way, the imported tracings being cut or deleted to match the target image. If the source and seed images are not perfectly aligned, the root system structure of the source image can be registered on the target image through a linear transformation (translation and rotation) carried out using three user specified landmarks (Thévenaz et al., 1998).

2.6 Data handling

Information generated using SmartRoot can be exported as built-in tables to any SQL-compliant external database system for further analysis. This capability has been successfully used with Microsoft Access and MySQL database systems. At present, built-in tables include a table of node coordinates and diameter, a table of root global data (length and topological information), a table of annotations and a table of root length densities (as shown in table 1). This basic set of information is generic enough to allow the computation of most variables used in root morphological analysis:

growth, gravitropic behaviour (tip angle as a function of time), wavy patterns (distance between positions having the same direction), radial growth (diameter as a function of longitudinal position and time), morphological response to localised or transient environments, lateral root density,... From a technical point of view, many of those variables can be accessed transparently using SQL views defined once in the external database system.

2.7 Image requirements (type and quality)

Many aspects of image quality (including resolution, contrast and background noise) are known to affect image processing output. Not surprisingly, the best results are obtained with high resolution images as captured using a flat bed (transparency) scanner. This technology potentially provides pictures of high quality (Smit et al., 2000) but is time-consuming and may not be suitable for high-throughput image acquisition. Images of lower quality (e.g. camera pictures) can be analysed with SmartRoot, as long as roots are at least two- to four-pixel wide (see supplemental PDF 2 for a discussion of accuracy). Due to the adaptive thresholding used by the tracing algorithm, background noise and contrast are usually not an issue. The software has been positively evaluated using pictures from different experimental setups, such as aeroponics, petriplates, or rhizotrons.

SmartRoot reads the most popular image file types (jpg, gif, tif and bmp). Image processing is carried out in the greyscale space, with roots appearing darker than background. Colour images are automatically transformed to greyscale and grey level inversion can be performed if required.

2.8 Example 1: Architectural analysis of *Zea mays*

We first illustrate the capabilities of SmartRoot with an architectural analysis as part of a modelling study of carbohydrate competition between the various root types of maize appearing during the first two weeks after germination.

SmartRoot was used to estimate growth and branching parameters (growth rates, lateral root density, inter-lateral distances and branching angles) of the various maize root types (primary, seminal, crown and first order lateral roots, named after (Hochholdinger et al., 2004)) in aeroponics.

Twelve maize plants were grown in aeroponics and their root system photographed at daily intervals during fifteen days. The resolution and quality of the images were low, with an uneven and noisy background (fig.10A). The primary, seminal and crown roots were traced on each picture

of the time-lapse sequences using the line selection tools and the laterals were traced only on the last picture of the sequence using automated lateral root tracing (fig. 10B). Two datasets were established: one containing time-series of root length (primary and seminal) and a second containing a near-complete architectural description of the root system at the end of the experiment.

Using the first dataset, the growth rate of the primary, seminal and crown roots were calculated. Insertion angles (fig. 11A), inter-branch distances (fig. 11B), root diameters (fig. 11C) and the length of the apical unbranched zone (LAUZ) were retrieved from the second dataset. The growth rates of lateral roots were estimated with the SAS software (SAS Institute Inc., Cary, NC, USA) based on their length, their position on the parent axis and the growth rate of the parent axis (Hackett and Rose, 1972 ; Lecompte et al., 2001), assuming lateral root initiation in maize is almost acropetal (Lloret and Casero, 2002). A summary of the architectural parameters is given in table 2.

These parameters (mean value and standard deviation) were used to execute a root architecture model (RootTyp, Pagès et al., 2004) to simulate virtual and dynamic root systems useful for the carbohydrate competition analysis (not shown).

This example made extensive use of SmartRoot tools supporting time-series and topological analysis. It illustrates the possibility of extracting an information-rich dataset from very simple experiments, even with low quality pictures.

2.9 Example 2: Time-lapse analysis of cluster root formation in *Lupinus albus*

This second example illustrates a sampling-based processing and the possibilities offered by the annotation tool. In several species, compact clusters of short roots, first described by Purnell in 1960, are produced in discrete regions along first order laterals, called proteoid roots. Cluster roots increase phosphorus acquisition and enhance survival of these species on their native low-P-soils (for review, see Shane and Lambers, 2005). Little is known about the acquisition of proteoid root identity nor about the mechanisms which trigger the initiation of clusters along proteoid roots. It is currently thought that a systemic signal is involved, leading to discrete events of synchronous cluster root formation (Watt and Evans, 1999 ; Skene, 2000). In order to explore the temporal dynamics of cluster root formation and to identify growth and morphological features that are singular to proteoid roots, the root systems of twelve lupin (*Lupinus albus*) plants grown in

aeroponics have been scanned at daily intervals during sixteen days. The resulting time-lapse sequences have been analysed with SmartRoot.

Despite the high quality of the images, carrying out this type of analysis using standard skeleton-based algorithms would be hampered by the high degree of root overlapping, unless a prohibitively long manual separation of roots is made prior to scanning. This, fortunately, is a typical situation where sampling parts of the root system would be a practical way to escape root separation while providing the data required (fig. 12).

Thirty first-order lateral roots randomly selected on each plant were retraced in all images from their emergence till the end of the experiment. When a lateral turned out to be a proteoid root, the position of its clusters were recorded over time using interval annotation tools (fig. 13). The final dataset comprised a set of informations for individual lateral roots: length over time, diameter and position of the clusters (if any). This dataset was exported to a Microsoft Access database and analysed with the SAS software (SAS Institute Inc., Cary, NC, USA).

Significant differences were found between proteoid and non-proteoid roots for growth rates (respectively 0.76 cm.d^{-1} and 0.27 cm.d^{-1} , $p\text{-value}<0.001$, t-test) and diameters (respectively 0.059 cm and 0.039 cm , $p\text{-value}<0.001$, t-test) (fig. 14). Besides, proteoid roots tended to form only during the first four days after germination while non-proteoid root formation continued over time (fig. 15). These morphological differences suggest that proteoid root identity is already established when the lateral root emerges from the primary root (or that roots with a low growth potential loose the ability to form clusters).

From the same dataset, the number of clusters for individual plants was counted over time, with a temporal resolution of one day (fig. 16). This analysis revealed a continuous pattern of cluster root formation at the plant level, which does not fully support the systemic signalling hypothesis generating flushes of cluster root formation. However, the temporal resolution of our analysis does not allow to exclude the possibility of synchronous formation of clusters with a daily (or sub-daily) frequency.

Several features of SmartRoot have been instrumental in this experiment: the annotation tools to export cluster boundaries; the sampling-based processing to enable analysis of images overcrowded in roots; the viewing tools for the reliable identification the selected laterals throughout the image sequence.

3 Conclusion

We have presented a novel software supporting in depth characterisation of root morphology, geometry and topology from images or time-lapse image sequences. The software uses several algorithms designed for root tracing and has been validated on a wide range of image spatial resolution, noise and contrast. The strengths and weaknesses of SmartRoot are listed in Table 3 and discussed hereafter.

Currently available imaging tools developed for the analysis of root system architecture share the same type of workflow: automated analysis followed (sometimes) by manual editing. This strategy leads itself to medium- to high-throughput handling of root images, but tends to be restrictive on the complexity of the root systems that can be analysed. With branched root systems presenting a large degree of overlap, the user has to manipulate individual roots in the scanner tray prior to scanning or perform an intensive editing of the root skeleton. This reduces the throughput of the analysis and ultimately leads to restricting root studies to the young seedling stage.

The design of SmartRoot was largely influenced by Yoav Waisel's perspective that root systems consist of populations of roots of different types, ages, topological and spatial locations (Waisel and Eshel, 2002). The focus was therefore placed on individual root behaviour rather than on cumulated variables that are more difficult to interpret. Here, we introduce an innovative type of workflow, based on root system sampling at the time of image analysis. With this approach, manipulating roots prior to imaging is no longer required since parts of the root system where image quality or root overlapping would preclude accurate analysis or imply intensive editing can simply be discarded. The analysis can also be focused on specific root types, which may not be easily recognised by an algorithm, or can be performed stepwise, starting with an evaluation of cheap variables and proceeding with deeper analysis only where needed. The throughput of image processing using SmartRoot will therefore depend on the type of root system, quality of image and desired information (See supplementary material for an evaluation of processing time). A direct consequence of this sampling strategy is an inability to estimate variables such as total root length, at which most root analysis software are usually very good.

A further innovation of SmartRoot, compared to many root imaging software, is the vectorial representation of roots. This representation enables the most useful capabilities/features of the software which support the sampling strategy: intuitive editing and annotation, navigation in a time-

lapse sequence of non-superposable images and an object-oriented data structure allowing an explicit topological description of root systems.

A classical issue in the analysis of root systems image is the discrimination between root branching and root overlapping. Initially, this issue attracted attention because overlaps create biases in the estimation of the total root length (Arsenault et al., 1995). The problem becomes more complicated if the scope of the analysis extends to the recognition of individual roots, in which case the crossing root segments must also be correctly connected. All software that we know, including SmartRoot, attempt to recognise a finite list of situations which they handle in an approximate way. There remains here an important area for the development of more generic algorithms, integrating, for instance, advances in neurone imaging.

There are increasing reports of using colour images to ease the separation of roots from extraneous objects on the image (substrate particles) or to analyse root health. SmartRoot currently lacks colour management, however the multilayer approach will make it easy to implement the stacking of additional raster layers providing data in other range of the electromagnetic spectrum (mainly visible and fluorescence).

To some extent, further developments may come from biomedical imaging, where neuron tracing and cell tracking algorithms have become popular (Meijering et al, 2004). Indeed, neurons and root systems share a similar tree-like structure and the displacement of cells shows parallel behaviors with growing root meristems. Preliminary testing indicates that those methods are not directly applicable on root images that are very different from those obtained in biomedical sciences. However, tracing and tracking tools used for neurons and cells rely on different image analysis concepts and those are worth being investigated in the root domain.

SmartRoot is a platform-independent freeware available at www.uclouvain.be/en-smartroot.

4 Material and Methods

4.1 Experience 1

Plants (*Zea mays*, genotype B73) were grown in aeroponic (de Dorlodot et al., 2005) with a modified Hoagland's solution (doubled Fe content). The pictures, seven megapixels in size, were taken with a regular CCD camera (Canon EOS 450D) every two days in a small black chamber build inside the greenhouse.

4.2 Experience 2

Plants (*Lupinus albus*) were grown in aeroponic system (de Dorlodot et al., 2005) with the same nutrient solution as Johnson *et al.* (1994). Daily pictures were taken during sixteen days with a flat bed scanner (Microtek 9600XL), at a resolution of 300 DPI.

5 Supplemental data

Supplementary figure 1: Display option in SmartRoot

Supplementary figure 2: How does SmartRoot tracing algorithm deal with touching roots.

Supplementary PDF 1: Adaptive thresholding algorithm.

Supplementary PDF 2: SmartRoot accuracy estimation.

Supplementary PDF 3: Handling root crossing.

Supplementary PDF 4: SmartRoot tracing time estimation.

6 Acknowledgments

We thanks H  l  ne Cordonnier, Tristan Lavigne, Aur  lie Bab   and Geoffrey Berguet for their extensive use of SmartRoot during its development. We also thank Lionel Dupuy, Tony Pridmore and anonymous referees for their constructive comments on previous versions of the manuscript.

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Table 1: Built-in tables available for export in SmartRoot. The root length density table is designed for rhizotron images acquired as described in Cheng et al. (1991) and Busch et al. (2006).

Export options	Export data
Global root data (one record / root)	image filename / root ID / length / surface / volume / branching order / topological position / number of children / branching density / position of first and last child on the root axis / insertion angle / insertion point
All marks (one record / mark)	image filename / root ID / annotation type / position along the root / annotation value
Nodes data (one record / node)	image filename / root ID / node coordinates / node diameter / position along the root
Root length density	image filename / x-y coordinates of the considered area / root length density in this area

Table 2: Summary of the architectural data of 15-days old maize plants grown in aeroponics (mean $\pm$ s.e.m.). Crown roots were about 2-cm long at the end of the experiment and were not analysed. LAUZ: Length of Apical Unbranched Zone; LR: Lateral Root. Roots are named according to Hochholdinger et al.(2004). The growth rate of LR is estimated in their linear growth phase. The validity of the insertion angle is questionable since images are 2D projections of 3D root systems.

Primary	
Growth rate [mm/d]	37.0 ± 0.2
Diameter [mm]	1.4 ± 0.3
Branching density [LR/cm]	5.9 ± 2.3
LAUZ [mm]	146.8 ± 54.8
Seminals	
Growth rate [mm/d]	31.0 ± 0.6
Diameter [mm]	0.9 ± 0.4
Laterals	
Growth rate [mm/d]	0.6 ± 0.17
Diameter [mm]	0.48 ± 0.16
Insertion angle [$^{\circ}$]	65.6 ± 14.3

Table 3: Summary of the strengths and weaknesses of SmartRoot.

Weaknesses	Strengths
Lack of global parameters	Sampling-based analysis
Time of user interaction → depending on depth of analysis	Low requirements → on image quality, plant type or age
Batch analysis not supported	Time-series handling
Color management not supported	Annotation
	Vector-based representation of roots
	Database connection
	Platform independent
	Highly interactive

Figure legends

Figure 1: SmartRoot stores information in four separate data layers. A, Source image (raster). B, Morphology (vectorial). C, Topology (| | = parent root ; | = branch root ; • = connection). D, Annotations (illustrated here with a beacon, see text). The double arrows indicate matching position in different coordinate systems.

Figure 2: Effect of the distance between nodes (circles) on the accuracy of the segmented line (dashed) approaching a curvilinear root object (gray). A. Large and fixed distance between nodes. Arrows point to poorly represented regions of the root. B. Small and fixed distance between nodes. C. Adaptive internode distances lead to parsimonious segmented lines..

Figure 3: Diagram of SmartRoot tracing algorithm

Figure 4: Illustration of the stepwise construction of the segmented line. A. Pixel values are evaluated along a 90° arc centered in front of the last node (NODE i). The resulting profile is compared against a local threshold (see text). The candidate positions are filtered based on their diameter similarity with NODE i diameter (here, the candidate 2 is excluded). B. This algorithm fails when the root curvature is too strong. C. Increasing the amplitude and decreasing the radius of the arc allows the search algorithm to find the successor node.

Figure 5: Illustration of the multipoint border search and centering algorithm. The open circle represents the initial position of the node. The dashed arrows represent the different trajectories used to find the root border. The plain arrow represents the shortest segment containing the node and joining root borders. This segment is used to estimate the local root diameter and to re-center the node on the root axis.

Figure 6: Consequences of sub-pixel resolution on the variability of diameter estimation. A. Close-up of the original image, near the border of a root. The dashed line marks the position of the threshold limit between root and background pixels. B. Segmented image after discrete thresholding. C. Interpolation of grey levels of A generates a smooth transition between root and background pixels . The position of the threshold limit can be approximated locally with a line. D Segmented image after thresholding of the interpolated image.

Figure 7: Detection of root junctions. A sudden increase of the orthogonal distance between the end of the segment and the closest borders marks the termination of the segmented line construction (see text).

Figure 8: Lateral root detection algorithm. A. The algorithm creates search paths parallel to each root border, at a distance arbitrarily set to the local root diameter ($d = \varnothing$). B. As for the root tracing algorithm, pixel values along this search path are compared against their local threshold value and a new lateral root is created whenever a satisfying position is found.

Figure 9: Analysis of time-lapse image sequences of plants grown in liquid or air medium (when root systems at different time points cannot be superposed). Corresponding positions are established based on the $[r, d]$ coordinates (see text).

Figure 10: Analysis of 15-day old maize root systems grown in aeroponics. A, Low resolution source image from a CCD camera. B, Representation of root midlines representation after tracing.

Figure 11: Histograms generated with the graph builder tool box of SmartRoot (maize example). A, Insertion angle. B, Inter-branches distances. C, Diameter (dark grey: first order laterals; light grey: primary roots).

Figure 12: Analysis of a 16-day old lupin root system grown in aeroponics. A, High resolution (600 DPI) source image from a flat-bed scanner. B, Representation of the midline of selected roots after tracing.

Figure 13: Evolution of a single proteoid root containing a single, long-lived root cluster (lupin example).: length of the proteoid root (plain line), emergence of the root cluster (arrow) and evolution of the cluster boundaries (dashed lines).

Figure 14: Relationship between growth rates and diameter of lateral roots (lupin example). A clear discrimination is seen between non proteoid (open circles) and proteoid roots (closed circles).

Figure 15: Histogram of the timing of lateral root formation (lupin example). Proteoid roots are in dark grey and non-proteoid roots in light grey.

Figure 16: Sequence of cluster root formation (cumulative) (lupin example). The graph shows the emergence of new clusters on proteoid roots of six different plants.

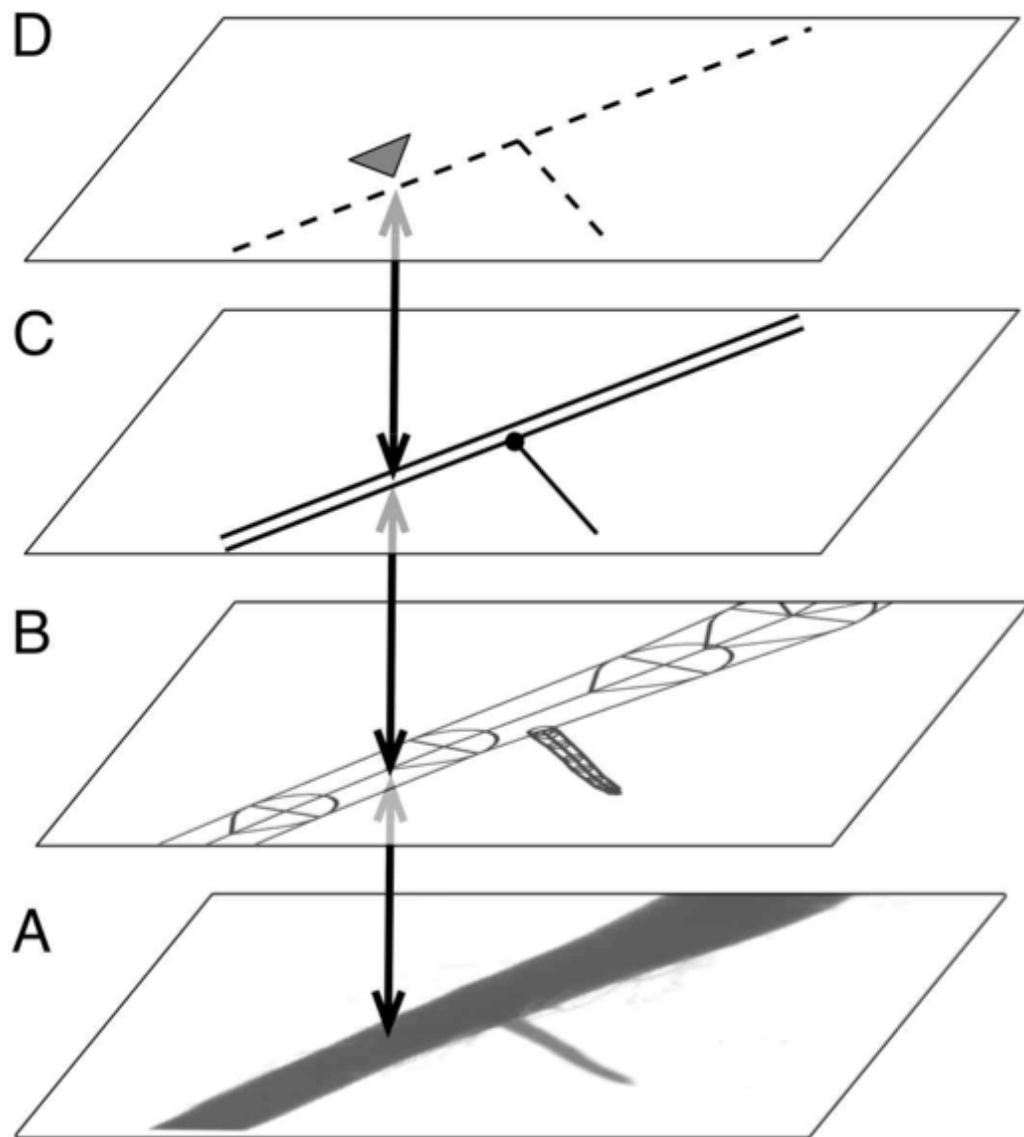


Figure 1: SmartRoot stores information in four separate data layers. A, Source image (raster). B, Morphology (vectorial). C, Topology (| | = parent root ; | = branch root ; • = connection). D, Annotations (illustrated here with a beacon, see text). The double arrows indicate matching position in different coordinate systems.

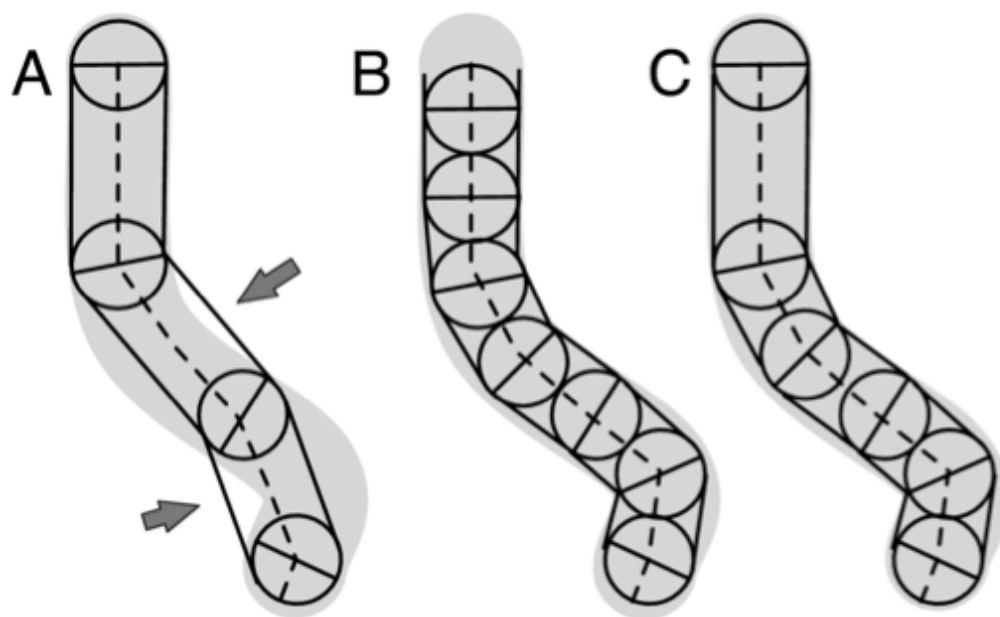


Figure 2: Effect of the distance between nodes on the accuracy of the segmented line approaching a curvilinear root object. A, A too large distance leads to a poor representation of the root shape in curved regions (arrows). B, A higher accuracy is achieved with smaller distances. C, SmartRoot adjusts the distance between nodes to the local curvature.

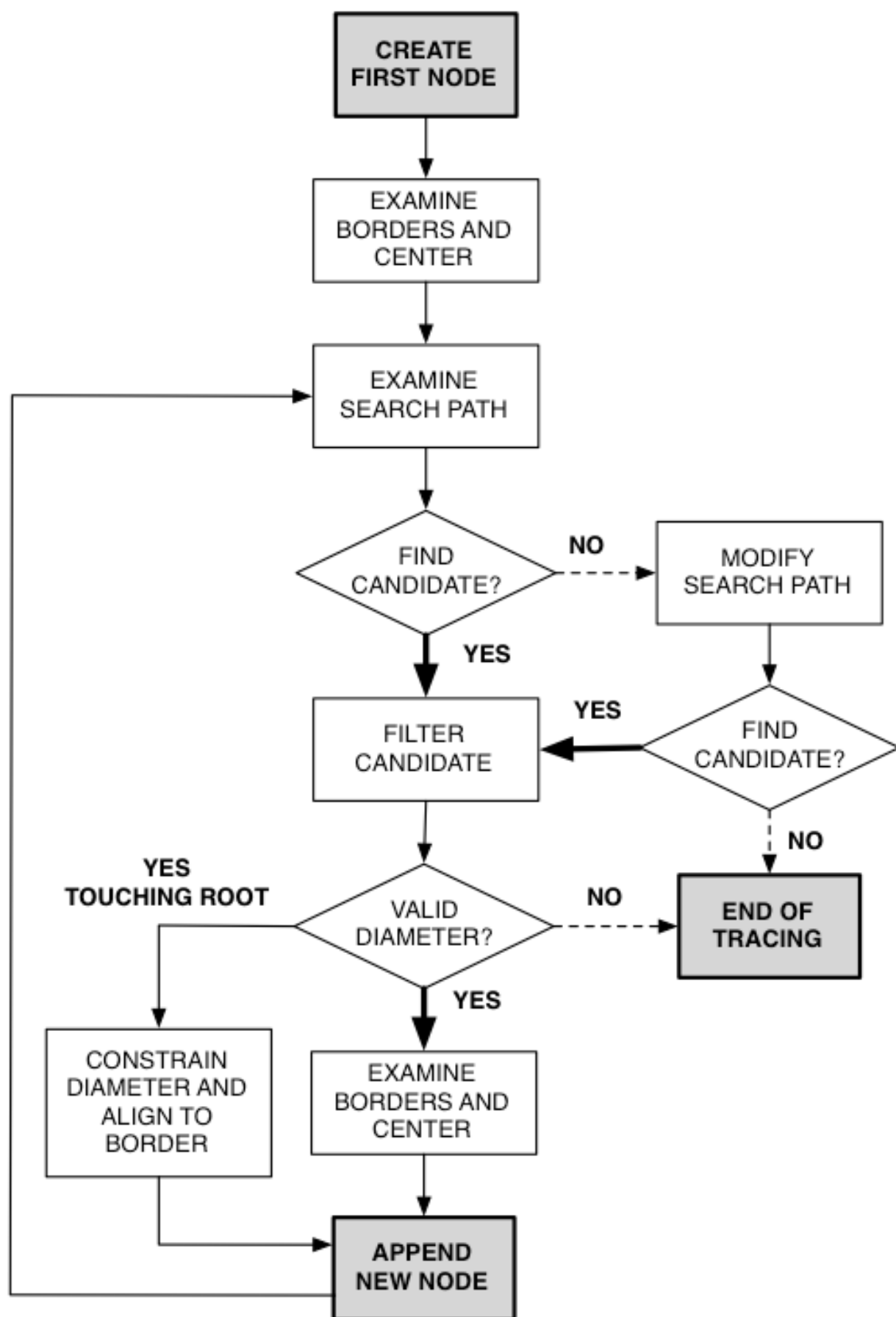


Figure 3: Illustration of SmartRoot tracing algorithm

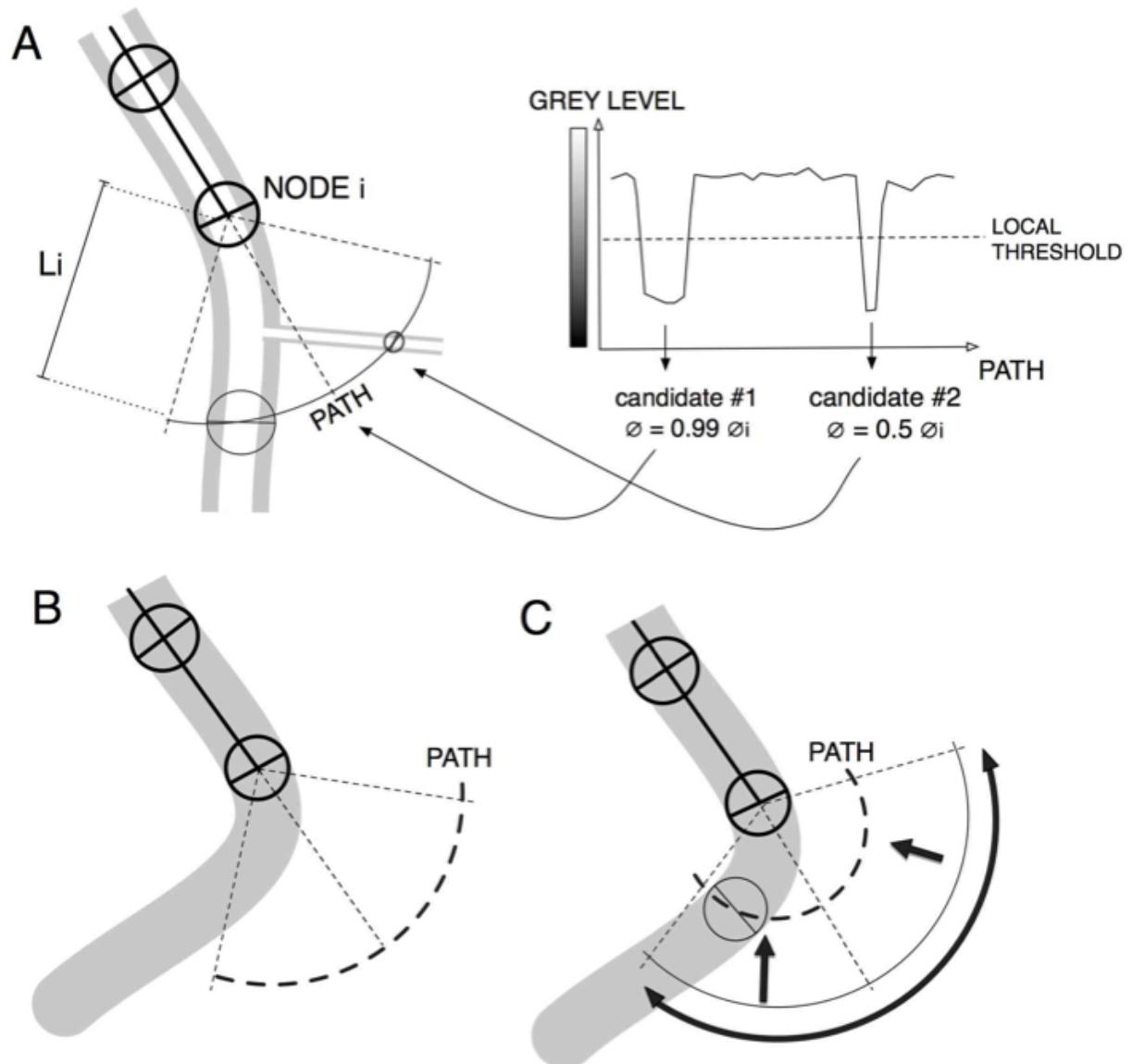


Figure 4: Illustration of the stepwise construction of the segmented line. A, Pixel values are evaluated along a 90° arc centred in front of the last node (NODE i). The resulting profile is compared against a local threshold (see text). The candidate positions are filtered based on their diameter similarity with NODE i diameter (here, the candidate 2 is excluded). B, This algorithm fails if the root curvature is too strong. C. In this case the algorithm increases the amplitude and decreases the radius of the arc.

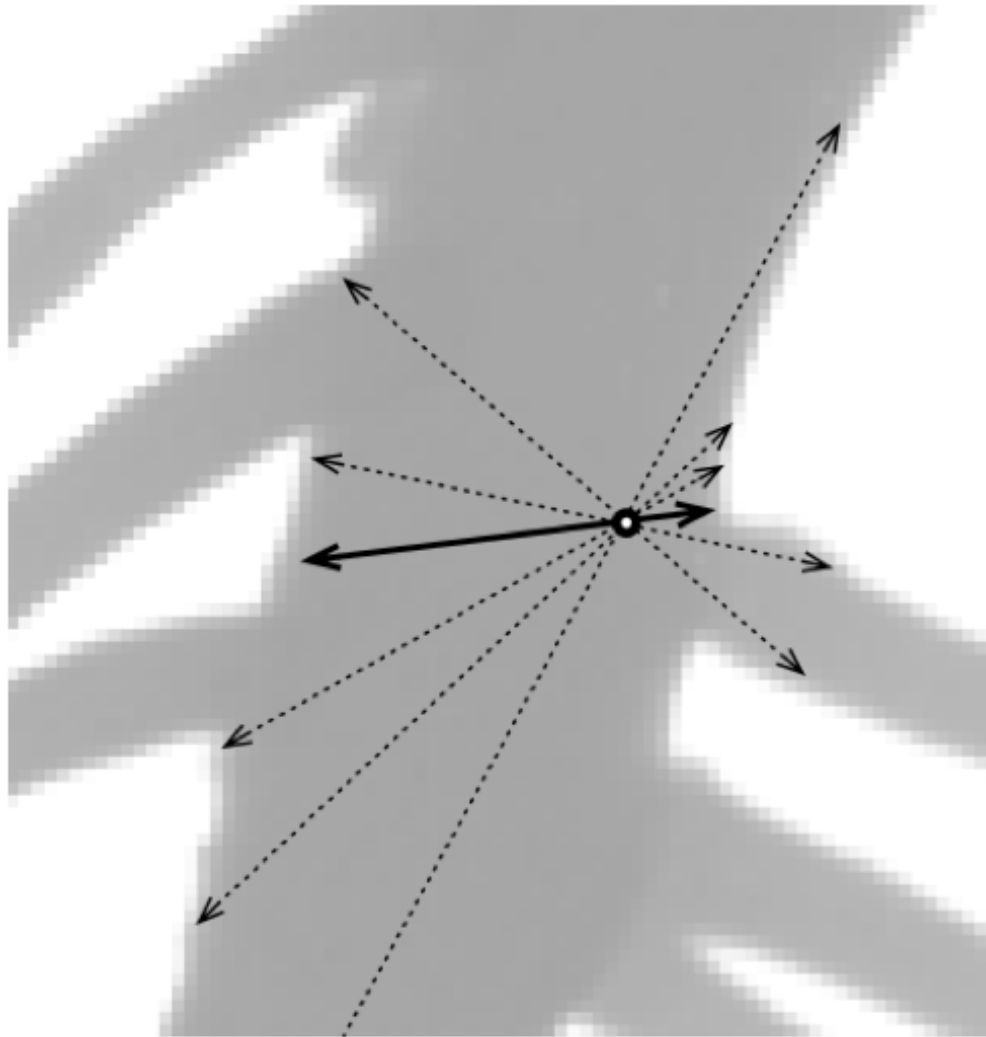


Figure 5: The tracing algorithm seek for the root border at multiple point. The open circle represent the node initiation point. The dashed arrows represent the different trajectories used to find the root border. The plain black arrows represent the shortest distances to the root border.

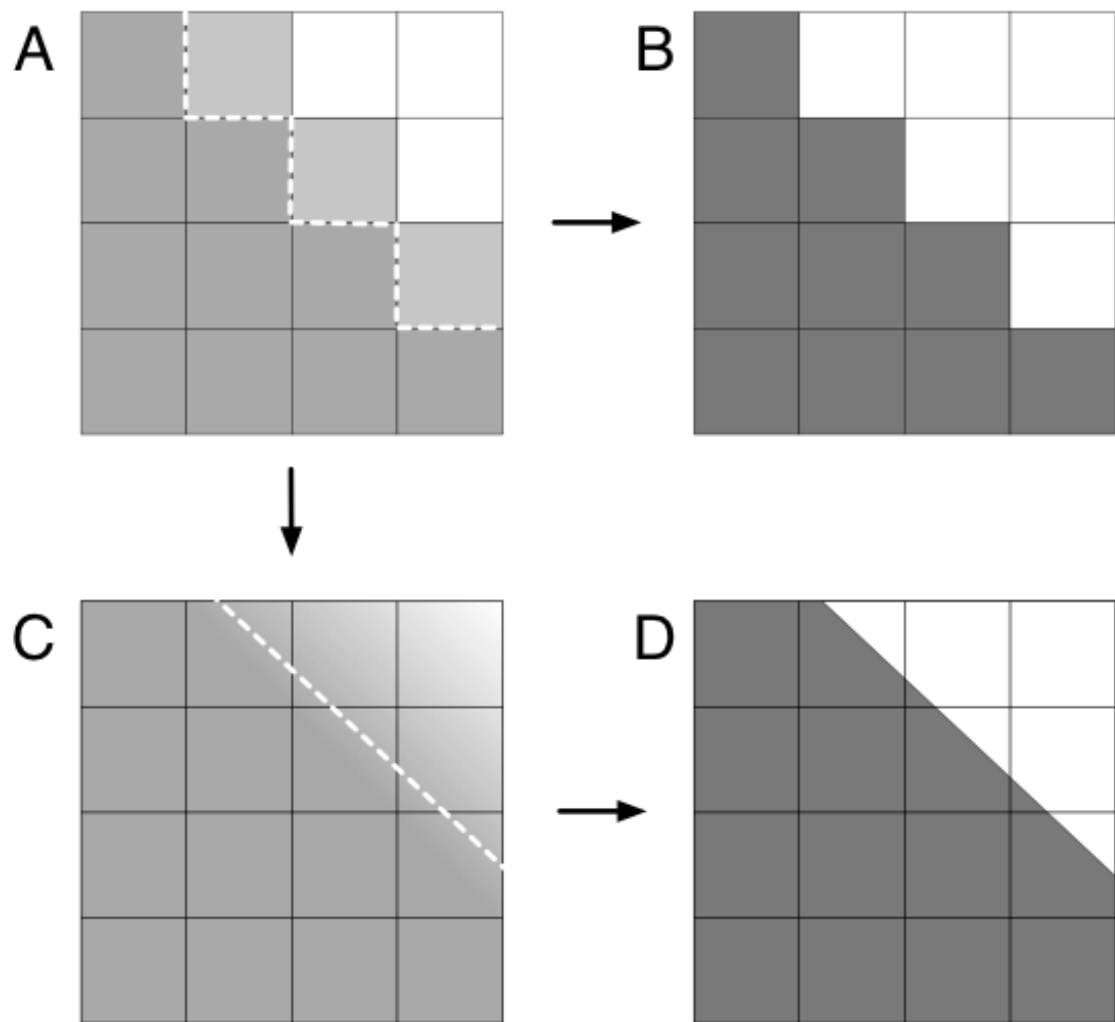


Figure 6: Consequence of multipoint border search and sub-pixel resolution on the variability of diameter estimation. A, Original image, showing the border of a root. The white dashed line represent threshold limit. B, Discrete (pixel-based) thresholded image. C, Modified image with continuous grey level value obtained with a linear extrapolation of the discrete pixel values of A. The white dashed line represent threshold limit. D Continuous thresholded image.

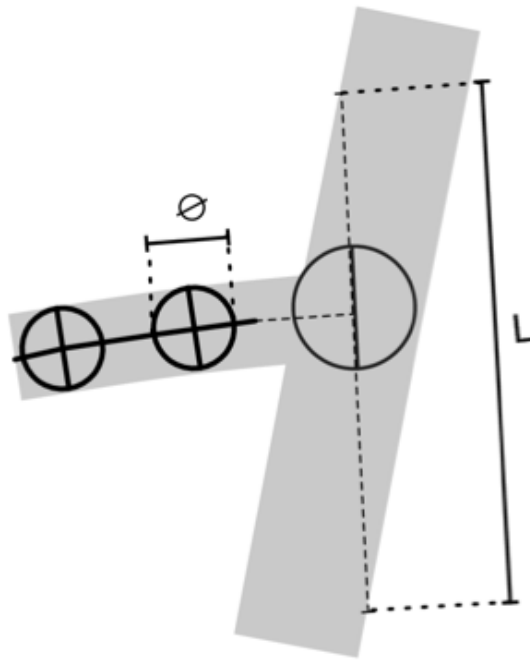


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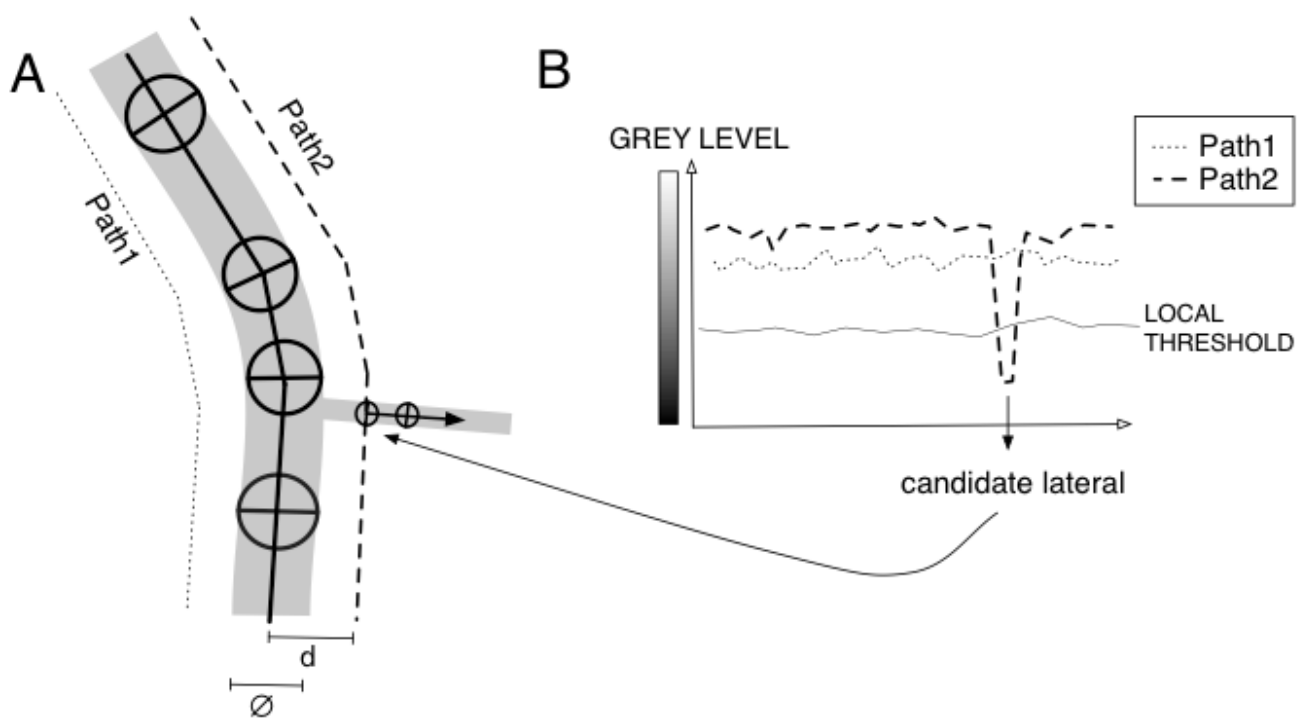


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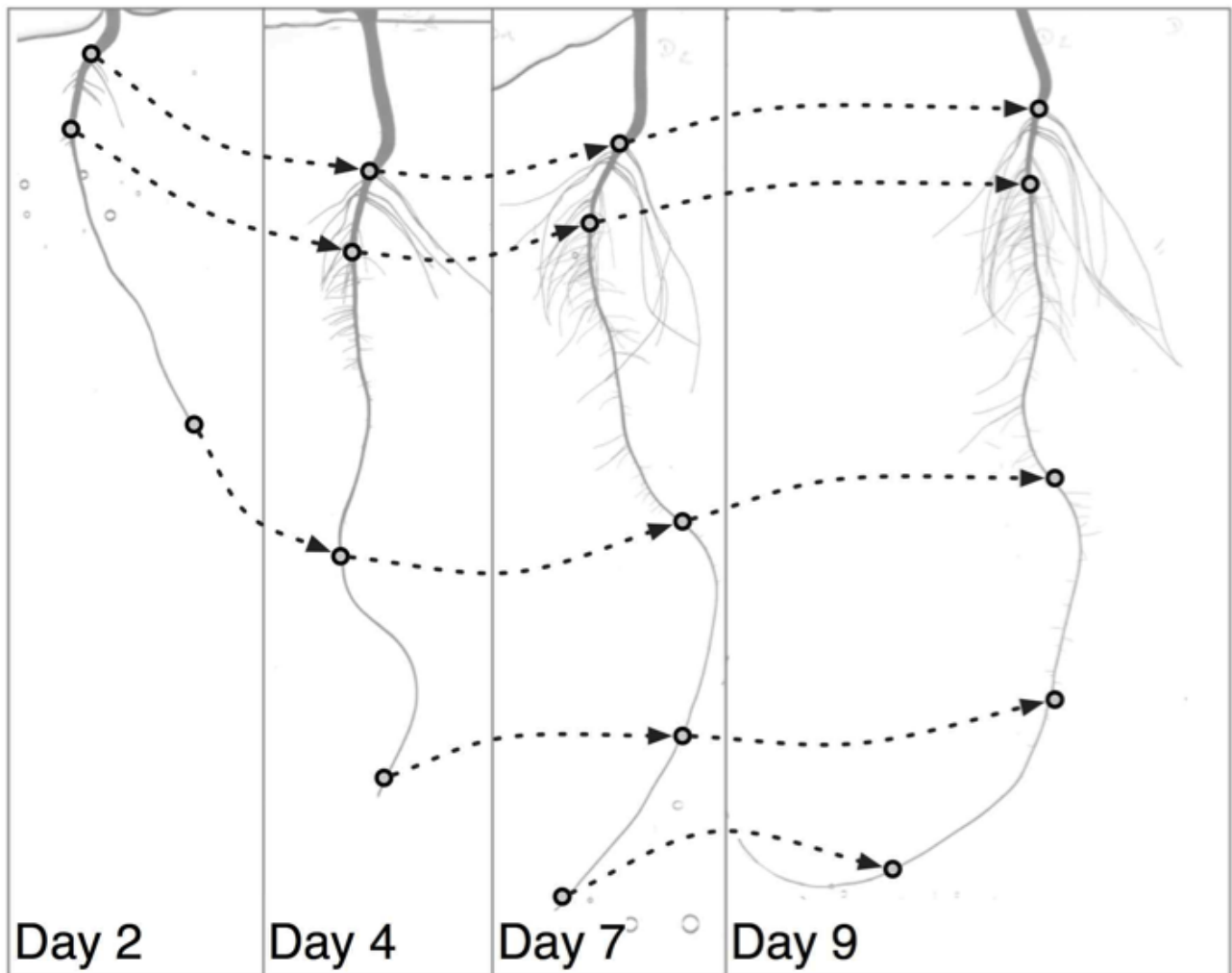


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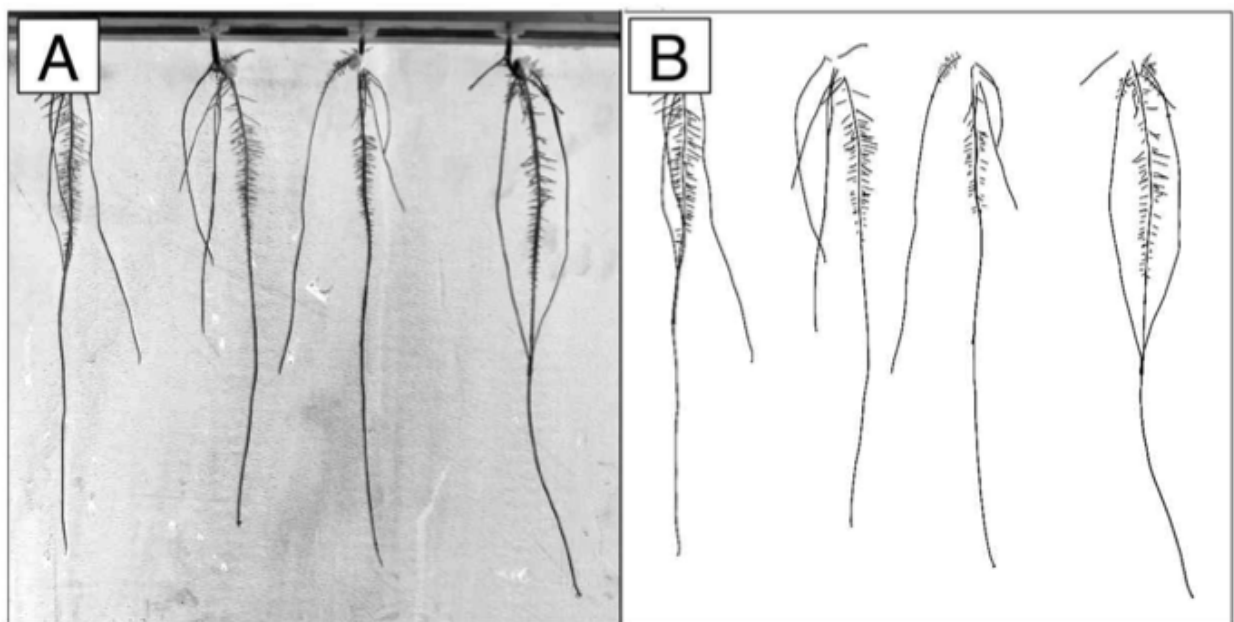


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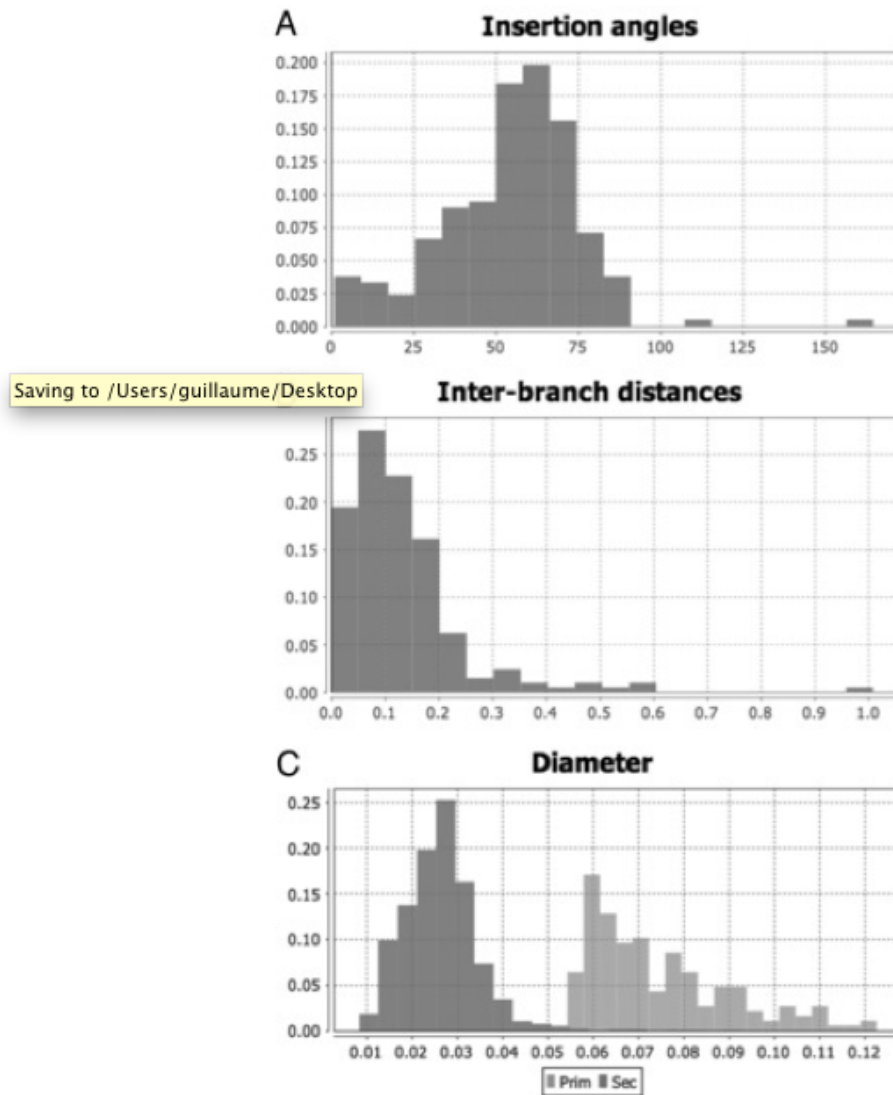


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Figure 12: Analysis of a 16-day old lupin root system grown in aeroponics. A, High resolution (600 DPI) source image from a flat-bed scanner. B, Selected root midlines representation after tracing.

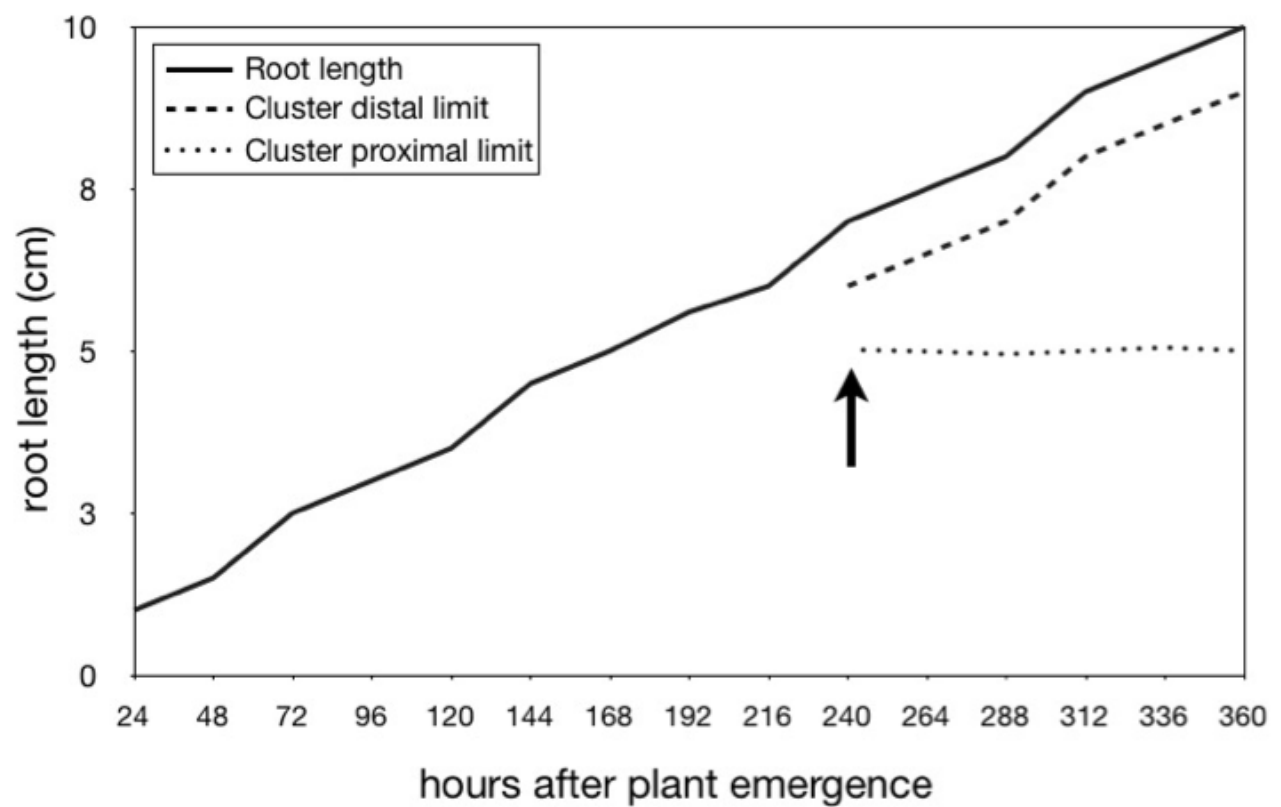


Figure 13: Evolution of a single proteoid root containing a single, long-lived root cluster (lupin). Length of the proteoid root (plain line), emergence of the root cluster (arrow) and extension of the cluster boundaries (dashed lines).

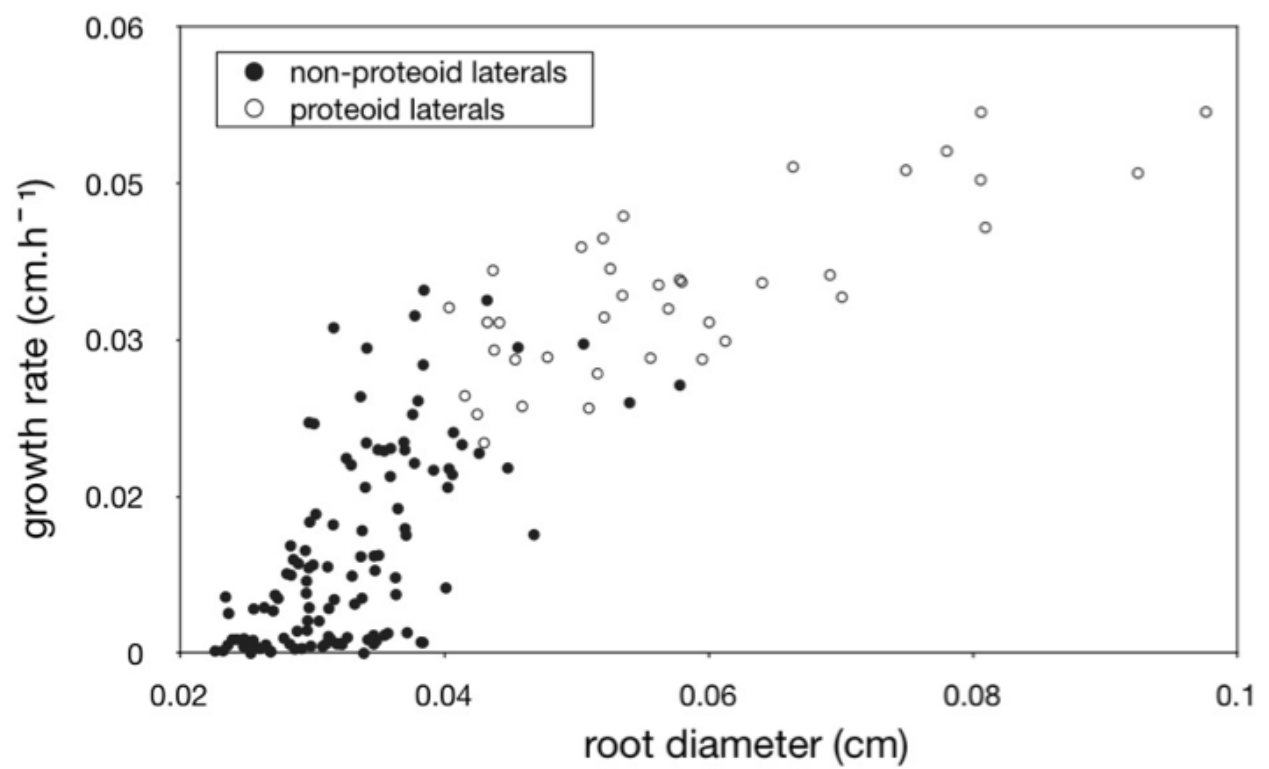


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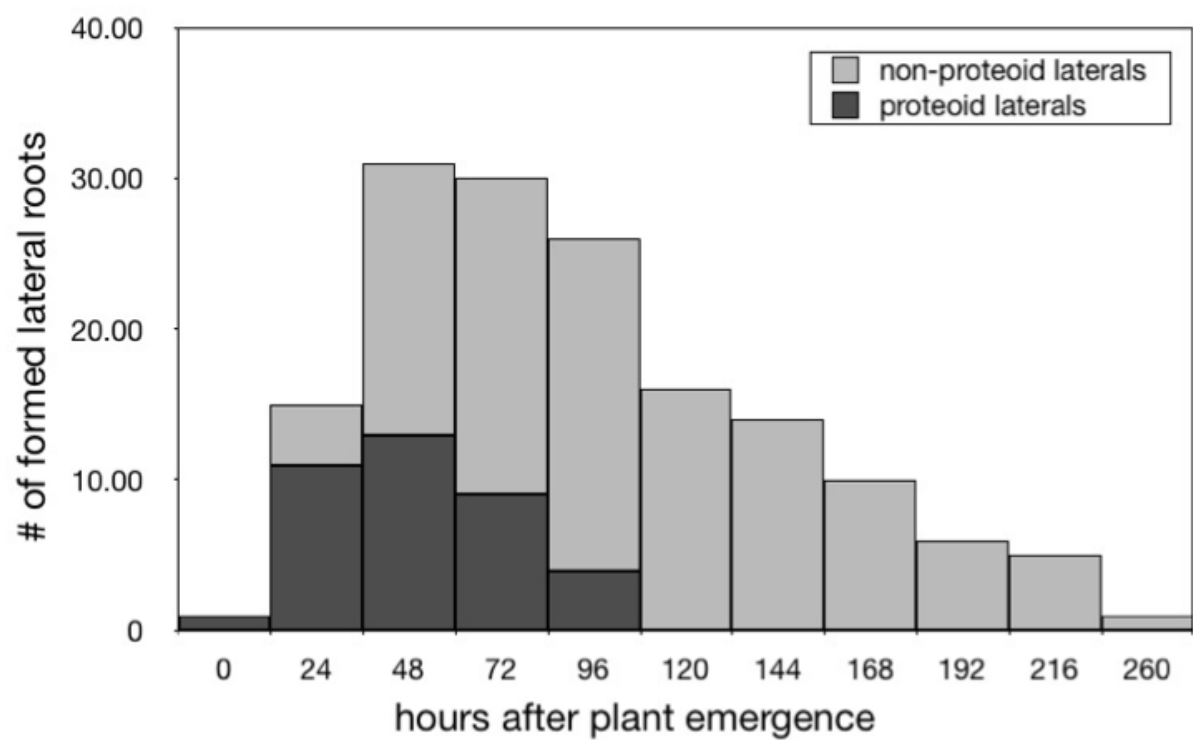


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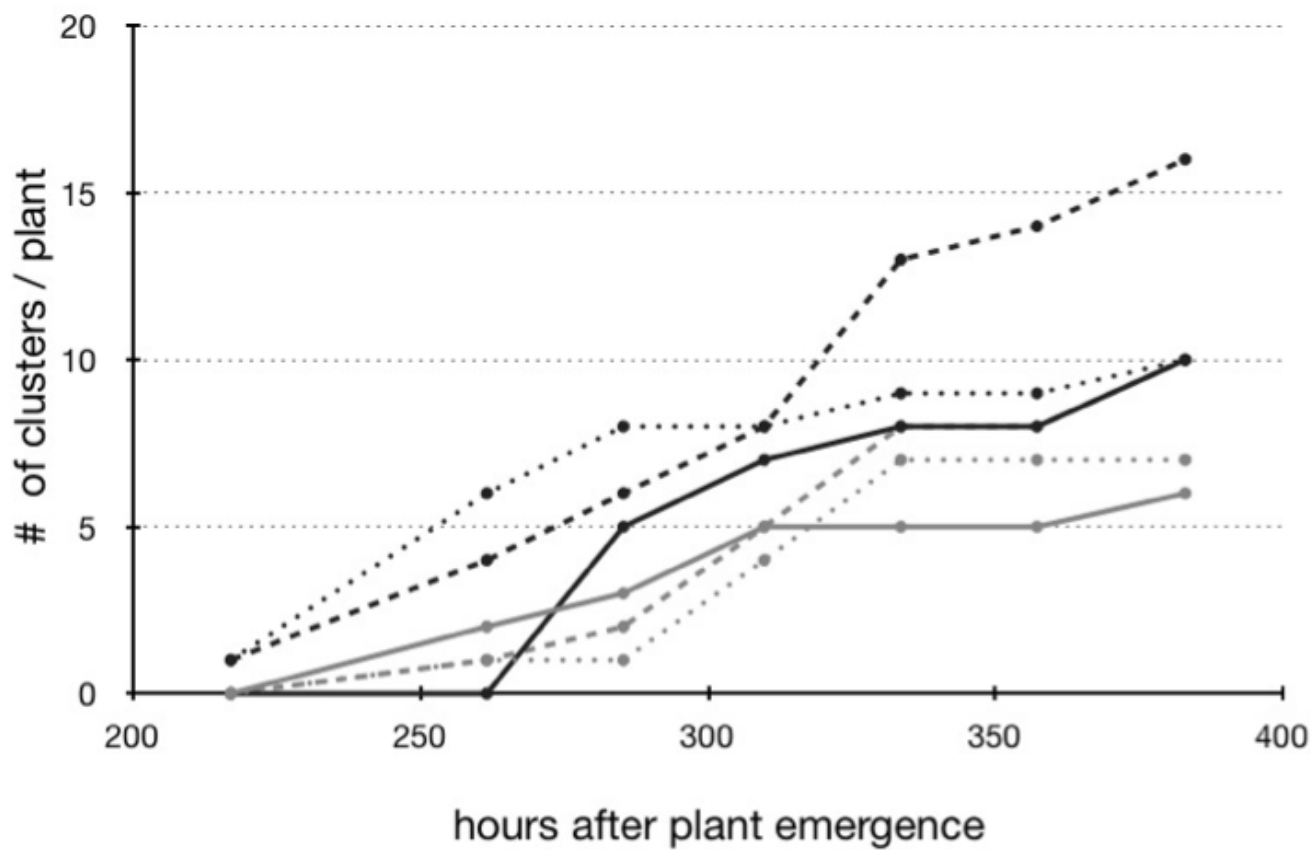


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