

# Chapter 4

## Root Phenomics

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**Abstract** Root systems have several functions that go beyond of plant support. Several metabolic reactions in plant roots are initiated that adjust them to a stress that can be instantaneous or permanent. Thus, many breeders believe that the key to success for obtaining genotypes tolerant to many types of abiotics is situated below the soil surface. Because of this, for decades much efforts have been invested in trying to develop tools that enable to analyze precisely the growth and development of roots under undesirable conditions. In the early 2000s were created some hardwares and softwares that enabled evaluation of several root parameters such as length, volume, surface area, projected area, among others. However, most of them have the disadvantage of destroying the sample to be evaluated. Recently, others methods have been developed which enable large-scale phenotyping, such as computed tomography-based. They are important for breeding programs because they allow evaluation of hundreds of genotypes in an easy and fast way. Moreover, they are not destructive methods and they permit to follow the root development in several phenological phases of the plant and in real- time. Given the above, the aim of this chapter is present the most used methods of root phenotyping for plant breeding. For that, we present some procedures and their computational basis, followed by their advantages and limitations.

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## 4.1 Introduction

In addition to providing support, roots determine a plant's ability to absorb water and nutrients present in the soil, synthesize and provide active biomolecules, and identify/signal stresses, among several other functions that are important for plant establishment in a given environment (Hodge et al. 2009). However, due to high soil resistance, detailed studies on root growth and development were lacking for a long time, especially when compared to other plant compartments, such as the leaves and stem.

The way roots develop in soil may have a critical effect on plant growth and, consequently, crop productivity. Since the 1990s, this has spurred innovative tools that have allowed roots to be studied in more detail. Currently, a wide variety of techniques are available for this purpose, ranging from the invasive (i.e., allowing the genotype to be differentiated by destructive sampling) to the noninvasive (i.e., allowing plant growth and development to be followed under desired conditions). These methods are extensively employed in ecology and physiology, especially in plant breeding programs aiming to select genotypes with efficient water and nutrient use or those tolerant to water and nutrient scarcity (Chun et al. 2005; Fritsche-Neto et al. 2012).

When plants are grown in an environment with a nutritional or water deficit, they usually exhibit greater carbohydrate allocation to the root system (Nielsen et al. 2001). With this modification, there is increased root length and density but reduced root diameter, which allows roots to have more contact with the soil (Ma et al. 2001; López-Bucio et al. 2002). These adaptations also include obtaining nutrients or water with minimal carbon cost, and this is only possible due to increased root growth and changes in branching pattern, total root length, root hair elongation, lateral root formation, and root architecture (Lynch and Brown 2001; Fan et al. 2003). In a quick survey of papers published and indexed in the Web of Science, it appears that most studies addressing the root system, especially those related to stress, have focused on root architecture. Root architecture refers to the spatial configuration of the root system—that is, the geometric arrangement of the root axes within the soil portion.

With the development of modern methods, it has been possible to initiate studies associating root architecture with other root attributes in young plants, thereby providing a basis for rapid phenotypic characterization (Singh et al. 2010). In this context, in addition to better understanding root system development and the mechanisms of tolerance to certain stresses, these modern methods allow breeders to undergo early and efficient selection among the thousands of genotypes that arise during every cycle in breeding programs. In this sense, this chapter aims to address the main methods for large-scale root phenotyping, their applications in plant breeding, and prospects for the future.

## 4.2 Large-Scale Root Phenotyping Methods

Due to technological advances and rapid dissemination of information, much work has been performed to automate plant phenotyping. In this context, several platforms and computer programs for collecting and analyzing root images have been developed in recent years. These platforms are used to accurately characterize root systems regarding both quantitative and qualitative aspects.

Roots are notoriously difficult to phenotype under field conditions. In addition to technical considerations, characterization under these conditions is limited by genotype–environment interactions, which are usually significant (Gregory et al. 2009). Traditional methods employed to study roots have emphasized root excavation techniques in which root system length and density may be determined (Araus and Cairns 2014). However, the excavation process is laborious and slow. Because of this, an Australian industrial research organization recently implemented a high-yield soil sampling system. This system consists of a hydraulic press that compresses up to 200 cm soil depth per day. Currently, this system is used to evaluate the effect of root architecture on water absorption, and especially to characterize more drought-tolerant genotypes (Gregory et al. 2009).

Some noninvasive techniques, such as those based on electrical capacitance, and other more innovative ones, such as magnetic resonance and three-dimensional (3D) computed tomography, have been proposed mainly for annual (herbaceous) crops. Trachsel et al. (2011) proposed a less costly and rapidly executed method designed a priori for grasses. This method was named *Shovelomics*, and it assigns grades (scores) to root architectural traits by visually inspecting the roots of individuals. In maize, for example, the numbers, angles, and patterns of nodulated and adventitious root branches are considered. Thus, at the end of the procedure, each individual has a final score that allows for classifying it as adapted or nonadapted to a marginal condition of cultivation. However, there are still problems related to limited resolution when working with tree species (Wasson et al. 2012).

Typically, these phenotyping platforms are divided into two main groups: *ex situ* analysis-based (using samples or the entire root system outside of the growth environment) and *in situ* analysis-based, which are also named noninvasive (evaluating the entire root system and *in situ*). Next, some of the most widely used methods in plant breeding programs for phenotyping roots of genotypes are presented.

### 4.2.1 *Ex Situ* Evaluations

In many situations, it is only possible to observe the effect of a given phenomenon on crop growth and developmental dynamics under controlled conditions (i.e., artificial environments). In fact, this requires avoiding the action of other factors not considered in the experiment, and it facilitates visualizing and capturing images.



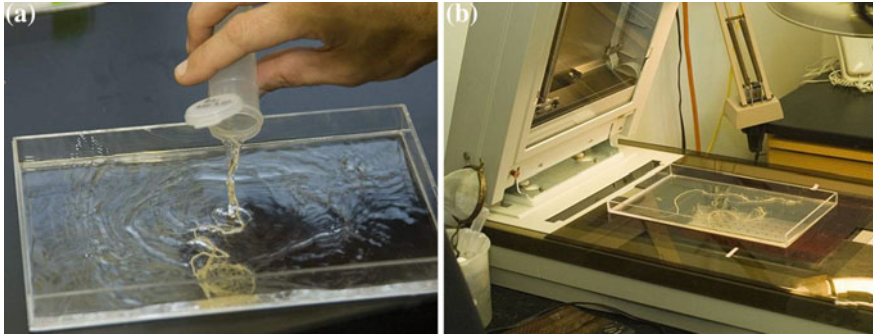
**Fig. 4.1** Planting of maize strains in PVC pipes wrapped in plastic bags under high and low nitrogen availability (a); root washing process (b, c, and d); and the root system obtained using this technique (e)

The most commonly used techniques for this purpose are hydroponics, aeroponics, culture medium in agar, pots, and even polyvinyl chloride (PVC) pipes. Alternatively, rhizotrons and minirhizotrons have been employed, which allow roots to be studied while still within soil. However, they artificially restrict the direction of root system growth to two dimensions. Moreover, they do not allow for phenotyping a large number of individuals, which slows the selection step in breeding programs.

Planting in pots or PVC pipes (Fig. 4.1a) are methods that require washing the root system, which often leads to underestimating fine roots due to breakage during the washing process. To minimize errors from losing these roots, it is recommended that the containers used in the experiment (pots or pipes) be wrapped in a plastic bag (Fig. 4.1b). However, in addition to being a very laborious process, the spatial configuration of the roots can be lost and the inferences about root architecture can be limited (Mairhofer et al. 2013). However, there are methods that allow for phenotyping a reasonable number of individuals, and they are currently the most employed.

#### **4.2.2 Scanning or Digital Scanning**

Scanning combined with computerized image analysis is a fast method of evaluating root morphological patterns, such as length, diameter, topology, and branching. Computerized scanning complements manual estimates and those obtained using cameras. Scanning can be performed on small root samples or whole root systems obtained from hydroponic crops. Digital output from an image is stored on a computer as a TIFF file and then analyzed with the appropriate software. However, accurate image scanning and analysis depends not only on the software used, but also on the sample preparation and the scanning protocol (Polomsky and Kuhn 2002).



**Fig. 4.2** Root samples placed in a transparent acrylic box with a predefined volume of water (a). The box is placed on the scanner table to start the scanning process (b)

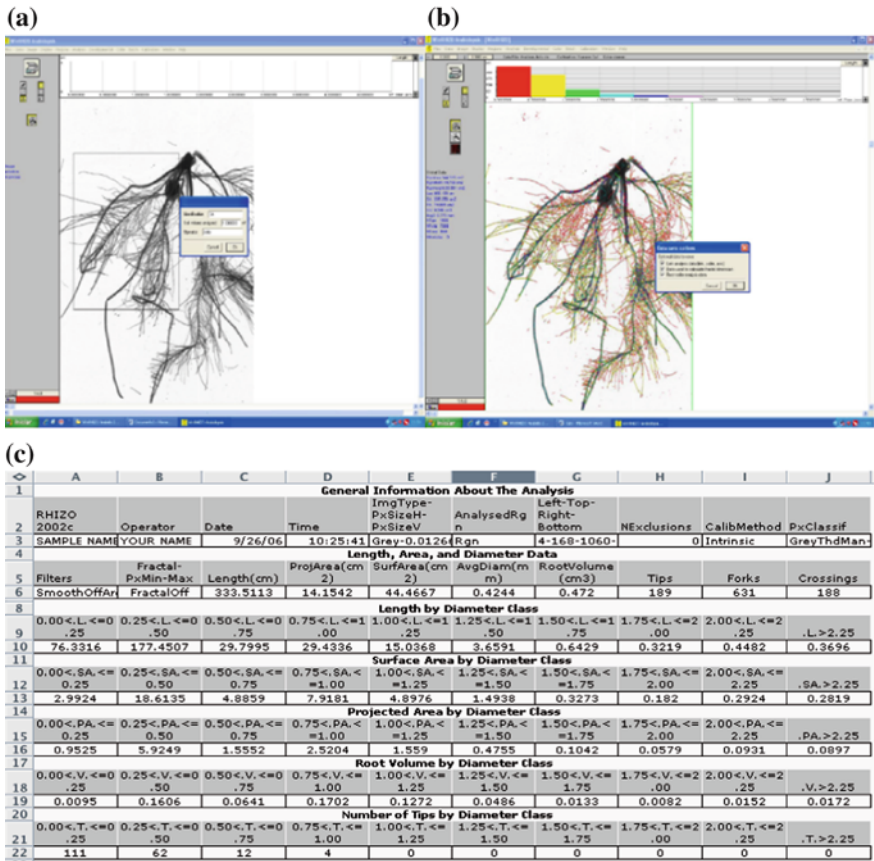
#### 4.2.2.1 Sample Preparation

Whole root systems or samples with root segments are washed to remove soil particles from their surface. Next, they are placed and spread onto an acrylic box (transparent) with a predefined volume of water (usually covered with 2–5 mm of water; Fig. 4.2). The box is then placed on the table of the scanner and the roots are scanned. Samples with large volumes of roots should be divided into subsamples to minimize overlay, which is one of the main sources of error in such estimates (Bouma et al. 2000).

#### 4.2.2.2 Scanning Protocol

Scanning resolution and initial transformations are important parameters that should be detailed in the study methods because they allow for possible comparisons of the results (Polomsky and Kuhn 2002). Software such as WinRHIZO and Delta T-Scan recommend a resolution of 400 dpi (Bouma et al. 2000).

The original images, which are obtained in grayscale in most of the scanning procedures, are transformed into binary versions (black and white). The highest pixel values in grayscale from the initial procedure are considered in only a portion of the image and are defined as black (value of 1). Conversely, the lowest pixel values of the gray scale represent the background of the image and are defined as white (value of zero; Polomsky and Kuhn 2002). According to these authors, the subsequent step is the skeletonization process of the axial roots (larger diameter), which is obtained by repeatedly removing pixels from the edge of the image until only a single chain of pixels represents a line in the center of the sample.

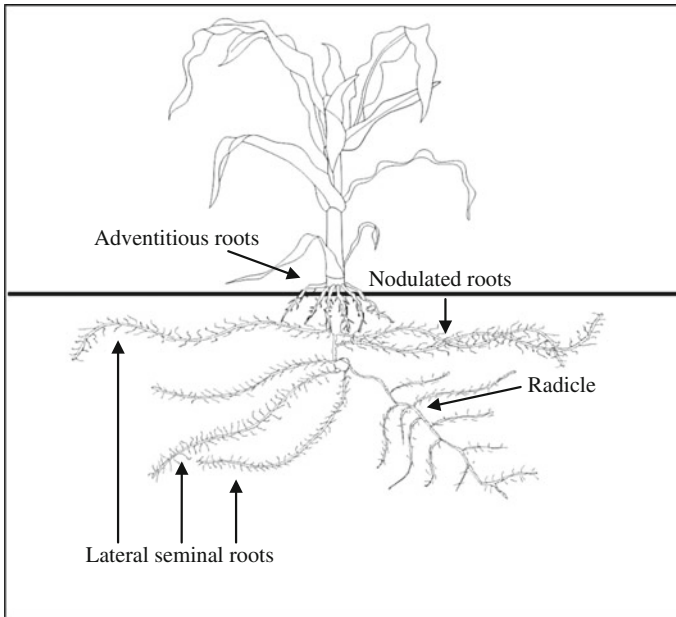


**Fig. 4.3** **a** Acquisition (scanning) of an image of a maize sample using an EPSON Expression 10000 XL scanner equipped with an additional light (TPU). **b** Image analyzed using WinRHIZO Pro 2009c software. **c** Spreadsheet with data from each sample generated using this software. *Source* Basic, Reg, Pro & Arabidopsis for Root Measurement

### 4.2.2.3 Evaluations Using WinRHIZO Software

WinRHIZO software allows for more flexible and automatic selection of variation generated using the initial image capturing procedure. Estimating root diameter combined with different colors and configurations makes this procedure very precise (Fig. 4.3a, b).

The measurements involve total root length, mean root diameter, root projection and surface areas, root volume, and number of root types as a function of ten diameter classes. These classes vary from roots with diameter smaller than or equal to 0.5 mm up to roots with diameter larger than or equal to 4.5 mm. All of the information can be saved to an XLS file and then worked on in Excel (Fig. 4.3c).



**Fig. 4.4** Shoot and root system of a maize plant with six fully expanded leaves (V6)

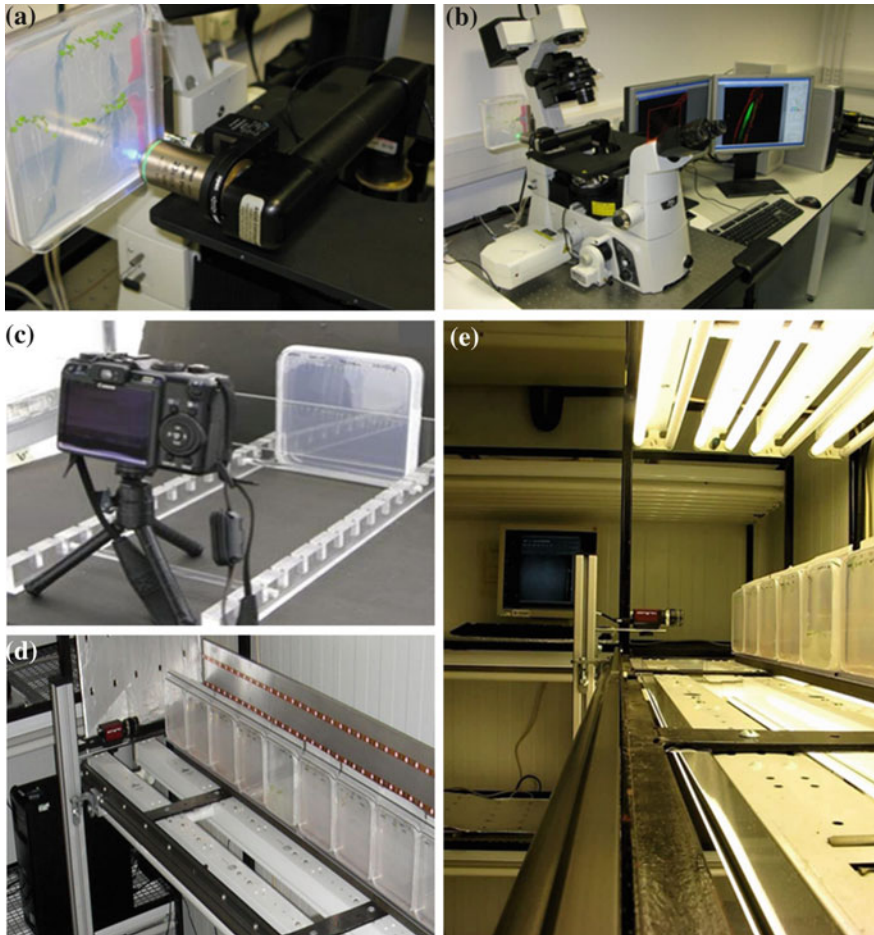
Additionally, the software detects portions of overlapping roots and considers them in estimating the root parameters (Himmelbauer et al. 2004).

Aiming to facilitate the process of characterizing genotypes in plant breeding programs for abiotic stress conditions, some authors have suggested simplifying the ten diameter classes in maize provided by WinRHIZO into only two. Thus, fragments with diameter smaller than or equal to 0.5 mm are considered for parameters related to lateral roots and fragments with diameter larger than 0.5 mm are considered for parameters related to axial roots (Hund et al. 2009; Trachsel et al. 2009).

One of the goals of the study conducted by DoVale and Fritsche-Neto (2013) was to determine the role of the root system in efficient use of phosphorus in maize. For this, experimental hybrids were used at V6 stage—that is, with six fully expanded leaves (Fig. 4.4). The root systems of all the individuals were simplified as mentioned above. These authors found significant positive correlation coefficients ( $p < 0.01$ ) between axial roots and phosphorus absorption efficiency both under high and low phosphorus availability conditions. This study allowed for the conclusion that this simplification is valid in the process of identifying genotypes with more efficient phosphorus use.

Even though root scanning allows for a large number of genotypes to be phenotyped in a breeding program, it usually has low yield. This is because one sample is evaluated at a time. Due to the slowness of this procedure, techniques that allow for high-yield phenotyping have been developed.





**Fig. 4.5** Microscope for acquiring images in vertical plates (a and b), digital camera (c), and hardware for automated capture of root images (d and e). *Source* French et al. (2012)

#### 4.2.2.4 Other Hardware and Software

In addition to scanners, images of roots can be captured using other devices that have higher yield. Researchers from the Center for Plant Integrative Biology, University of Nottingham, United Kingdom currently acquire information for their studies using microscopes with vertical plates (Fig. 4.5a, b), digital cameras (Fig. 4.5c), and hardware for acquiring automated images (Fig. 4.5d, e). The latter are able to phenotype up to 500 genotypes at a time at a maximum speed of 60 mm/s with an accuracy of approximately 187  $\mu\text{m}$  (French et al. 2012). The major limitation of these devices is that evaluation must occur at a very early stage of



development (seedling stage). However, they appear to be useful enough for characterizing genotypes tolerant to the presence of aluminum in soil.

Images captured using these devices are stored in an image database and can then be evaluated using different software, similarly to WinRHIZO. RootTrace version 1 (RT1), RootTrace version 2 (RT2), and RootNav are some examples of software compatible with the automated devices most commonly used by research groups that conduct studies involving root systems.

The RootTrace application analyzes the image from top to bottom based on a starting point predefined by the user. Thus, it is possible to follow plant growth and monitor the changes that occur in the root system using the growth rate of primary roots, angulation, and branching, among other parameters. RT1 considers root growth in the direction of gravity. The model developed for following these growth dynamics moves one pixel ( $\sim 0.05$  mm) every step, reflecting the effect of gravity on root growth. However, this monitoring is only possible if the roots exhibit curvature less than or equal to  $90^\circ$  at the root tip (Naeem et al. 2011).

The user can adjust the model with a type of multidirectional bar. When this bar is configured further to the left, the gravity-dependent RT1 model is employed. In contrast, when the bar is moved to the right, more or less points are fitted to predict the model (Fig. 4.6a, b). This procedure allows for more reliable monitoring of root curvature (Fig. 4.6g–j). RT2 users are able to calculate (predict) a monitoring model to analyze the data referring to root growth without needing to input a numerical parameter (Naeem et al. 2011).

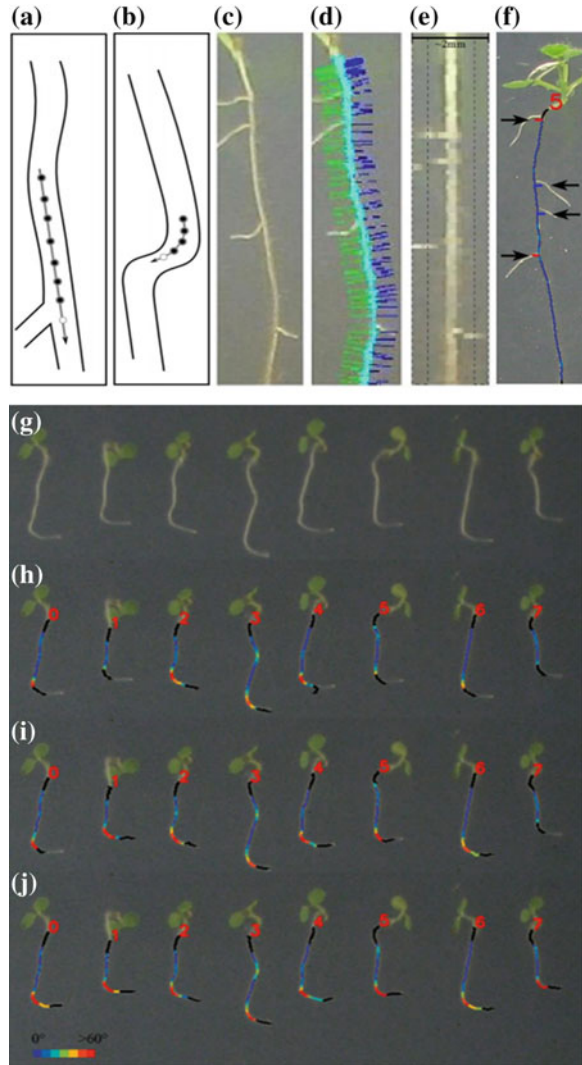
The RootNav application is another new tool that allows root system architecture to be quantified for a range of crop species. An automatic component of this software is also based on a top-down approach, and it uses a powerful algorithm of maximum classification to analyze regions of the input image and then calculate the probability that certain pixels correspond to roots (Pound et al. 2013). According to these authors, this information is used as the basis for optimized approximation in detecting and quantifying roots.

Thus, like RootTrace, RootNav makes an optimized estimate from the seed to the root apices (Fig. 4.7). However, it also allows the user to easily and intuitively refine the results by visual inspection. Moreover, it provides supporting information necessary for extracting a variety of biologically relevant measurements. This is because there is a separate viewer tool in the center of the application that allows a rich set of traits related to root architecture to be retrieved from the original image.

### ***4.2.3 In Situ or Nondestructive Evaluation***

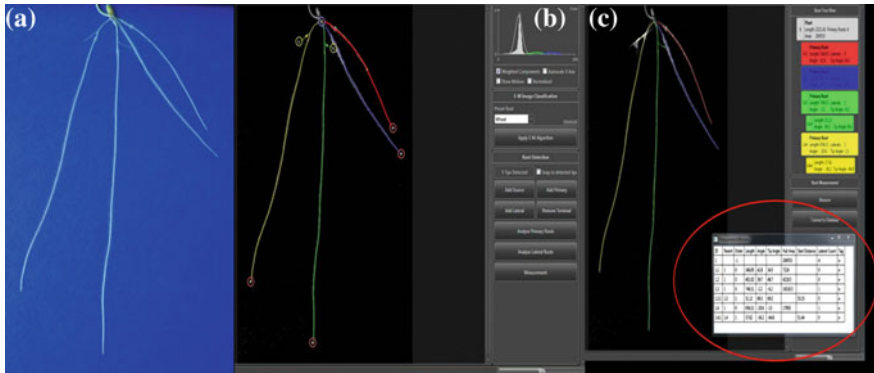
When growing plants in pots, the roots quickly fill the container, bending, distorting, and consequently substantially modifying their growth and development compared to what would usually be observed in the field. Therefore, to observe a more realistic root distribution, pots with volume much greater than the estimated volume of the roots should be used for each plant (which usually makes the study

**Fig. 4.6** Models for monitoring growth in roots with low curvature **(a)** and high curvature **(b)** obtained using RootTrace. **(c)** Original root image. **(d)** 40 pixels based on growth angles. **(e)** Area selected to detect the marked lateral regions. **(f)** Lateral detection. **(g)** Original image showing a pronounced gravitropic response. **(h–j)** Results of applying monitoring models using the multidirectional bar; colors indicate root curvature intensity (*red* for high and *blue* for low). *Source* Naeem et al. (2011)



infeasible), or the plants should be evaluated in their natural environment (in the field). Under these conditions, roots can grow deeper without their spatial distribution being affected. However, evaluation in these “open systems” has drawbacks, such as the sheer volume of the soil to be analyzed and the difficulty of sampling roots for further analysis.

The analysis of root images using samples or even whole roots is an arduous task. Additionally, as aforementioned, in the process of obtaining the samples, significant information regarding root system distribution in the soil can be lost. In this context, many researchers have sought to develop faster and more reliable methods for analyzing root images. Among these, the following two are the most innovative.



**Fig. 4.7** a Original image of the wheat root system. b Partial analysis using RootNav software with the option of redirecting the analysis to selected areas. c Output of the application with data stored in spreadsheets. *Source* French et al. (2012)

#### 4.2.3.1 “CI-600 RootSnap Scanner and Software” System

This system is used to analyze root growth, development, and function in adapting to a given environment. In this, the CI-600 scanner nondestructively captures high-resolution digital images (Fig. 4.8).

**Fig. 4.8** CI-600 root scanner and acrylic tubes used in capturing the images. *Source* CID Bio-Science (2014) <http://www.cid-inc.com/>

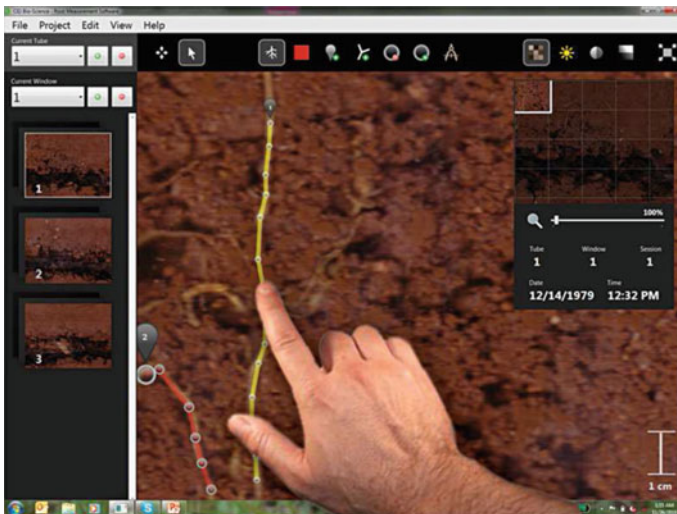


This phenotyping system is designed for long-term studies on living plants in the field where each plant can be evaluated several times during their growth cycle. For this, before or during planting, acrylic tubes are installed within the study area (in the plots). When the plants begin to construct their root “networks” around the tube, images of the structure and behavior of the roots can be obtained with the scanner and analyzed using CI-690 RootSnap software.

To evaluate images of the roots in the plots, it is necessary to insert the CI-600 reading device into a transparent acrylic tube preinstalled underground and start the scanning program on a computer (Fig. 4.8). The reading device automatically rotates approximately 360°, creating images of the soil and roots of approximately  $21.59 \times 19.56$  cm, in color, and in high resolution (188 million pixels). Regarding the reading depth, it is possible to easily move the device to different depths, and from tube to tube, choosing an ideal image according to the goal of the study and the species.

The equipment is extremely portable (750 g) and fast handling (5–15 s per reading depending on the resolution). Additionally, it allows for viewing root growth and behavior during an entire growth season or for even longer periods.

To interpret the images and store them, the equipment has a USB interface that allows for connection to mobile devices, such as tablets and laptops. However, it is necessary to have software that processes the images and estimates the phenotypic values of the individuals such as length, volume, and surface area of the roots. RootSnap is such a root image analysis package. When installed on equipment with a multi-touch LCD screen, the software allows users to quickly and easily track roots using their fingers (Fig. 4.9).



**Fig. 4.9** Output of the RootSnap software, an automated root image analyzer. *Source* CID Bio-Science (2014) <http://www.cid-inc.com/>

The software also automatically overlays different tracing points on the root system. Additionally, the files are stored in the common open XML format and data export to applications such as Excel, WinRHIZO, RootTrace, and RootNav is supported.

#### 4.2.3.2 X-ray Computed Tomography for Obtaining Noninvasive 3D Images

Methods based on this technology are well described in the review by Mooney et al. (2012). Usually, such methods seek to observe the roots in their natural state in the soil, both in space and time, maintaining their complex 3D morphology throughout their growth and development (four-dimensional, 4D). Several energy sources may be used to generate tomographic images. The X-ray technique is the most widely adopted because it is noninvasive and allows viewing inside objects in 2D or 3D based on the principle of attenuation of electromagnetic waves.

In this context, medical scanners have been used the most to investigate macroscopic characteristics of roots (Heeraman et al. 1997). These scanners are advantageous because several images can be easily obtained in a relatively short period of time. However, their resolution is usually limited to a slice thickness of 0.5 mm. Thus, if the goal of the study is to analyze fine roots, industrial X-ray devices are necessary such as synchrotron scanners or others specific for this purpose. There are already advanced systems of this type for animals, such as in vivo *X-treme* used for analyses in mice, and they can be adapted to plants. This system captures images in 3D and with high sensitivity for luminescence, fluorescence, X-rays, and radioisotopes (Bruker 2014; <http://www.bruker.com/>).

Although many studies have successfully visualized roots in situ, few have been able to extract the volumetric descriptions of material necessary to produce 3D models of their architecture. In this sense, two automated root tracing approaches were recently proposed, one based on assigning probability functions named RootViz (Tracy et al. 2012; [www.rootviz3d.org](http://www.rootviz3d.org)) and another based on level set methods named Rootrak (Mairhofer et al. 2011).

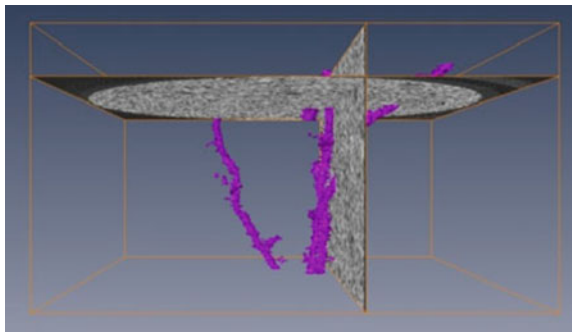
The first assigns a probability function to determine which specific pixels of an image represent root material and if they can be used to provide a 3D view of root distribution in the soil. Kaestner et al. (2006) used this technique and successfully characterized the root architecture of speckled alder (*Alnus incana*) (Fig. 4.10).

Another example of RootViz application was presented by Tracy et al. (2012) in wheat (*Triticum aestivum*), where it was possible to view the root architecture at the initial growth phase (Fig. 4.11).

The second approach processes a set of “slices” of 2D images to construct a gray scale related to the known root structure. These initial grayscale values allow a simple model to be constructed. Thus, a search for connectivity between the images obtained is initiated to construct overall and 3D images of the root system. This technique detects both thick roots and fine roots that grow vertically. However, any disconnected roots or those that grow in irregular directions are not recorded.



**Fig. 4.10** A 3D view of the *Alnus incana* root system, with resolution of 36  $\mu\text{m}$  and sample spatial dimensions of  $36.9 \times 36.9 \times 59.15 \text{ mm}^3$ . *Source* Kaestner et al. (2006)



**Fig. 4.11** A 3D view of roots of wheat grown in sandy soil with resolution of 18  $\mu\text{m}$ , obtained using RootViz. Sample dimensions = 91 mm high  $\times$  29 mm wide. *Source* Mooney et al. (2012)

A risk in applying this type of segmentation is its imprecision, which may introduce attenuation values into the appearance model that do not derive from the root. Subsequent segmentations can thus undergo greater imprecision, leading to higher distortion of the model. To avoid this situation, the shapes of root sections extracted from adjacent images are compared, and if they differ significantly then



**Fig. 4.12** A 3D view of maize roots grown in sandy soil with 30- $\mu$ m resolution obtained using Roottrak, with sample dimensions of 50  $\times$  120 mm. *Source* Mooney et al. (2012)



the model is discarded. Despite some limitations, the method has been successfully applied in tomography of maize (Fig. 4.12), wheat, and tomato grown in a variety of contrasting soil textures.

The use of relatively small samples (e.g., 25 mm wide) and higher resolution (e.g., voxel size of 100  $\mu$ m) has been suggested for obtaining images, aiming to ensure that the fine roots can be accurately viewed (Jenneson et al. 2003). When the goal is to analyze thick roots (or main), larger samples (e.g., 150 mm wide; 500 mm tall) and relatively low resolution (e.g., >1 mm) may be used (Johnson et al. 2004).

Image quality is also strongly affected by the type of container for the samples. In this sense, thinner (<3 mm) pots made of low-density plastic material are preferred compared to metal cylinders (Lontoc-Roy et al. 2006).

The soil moisture content of the sample is another key issue. Soil moisture conditions below field capacity produce better quality images than those obtained from soil closer to saturation. This is most likely due to the moisture content in the roots (Mooney et al. 2012).

Other points that still deserve more studies are related to obtaining 4D images—that is, repeated images of the same plant over time to evaluate root growth and development. Repeated exposure to X-rays potentially has deleterious effects on the plant, which can lead to errors in obtaining and interpreting results. Additionally, researchers should be aware of the position of the sample inside the scanner, always seeking to put it in the same position as for the previous readings, because this prevents errors in reading the dimensional axes (Mooney et al. 2012).

### 4.2.3.3 3D Views of Roots Using Other Nondestructive Methods

A series of other imaging techniques has been developed to view and quantify root properties in situ, such as nuclear magnetic resonance (Jennette et al. 2001), magnetic resonance (Pohlmeier et al. 2008), thermal neutron tomography (Tumlinson et al. 2008), and neutron radiography (Carminati et al. 2010). Similarly to X-ray tomography, each of these approaches has a series of advantages and some limitations when used to view root system architecture directly in soil.

Magnetic resonance can be used alone or together with other techniques to view root morphology, volume, and length. However, this technique is particularly sensitive to the moisture content of the samples. Additionally, the use of this technique is limited to studies in soil of root diameters greater than 1 mm due to the presence of paramagnetic ions, such as  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ , and  $\text{Mn}^{2+}$ .

This technique has been combined with positron emission tomography to quantify carbon allocation and storage as sugars in beet and maize. The other aforementioned techniques, and even X-ray tomography, when combined with magnetic resonance allow for performing other types of studies, such as identifying the water status of roots throughout the growth cycle. However, in contrast to computed X-ray tomography, for which benchtop systems have become extensively available, limited access to magnetic resonance facilities limits its use. Additionally, there are other significant disadvantages compared to X-ray techniques regarding most soil containing iron and/or manganese ions in large quantities, which negatively affects image quality (Heeraman et al. 1997).

## 4.3 Prospects

Despite major advances in root phenotyping, there are still large drawbacks to be resolved, especially related to the quality of data collection, adequate image resolution, and accurate analysis.

Regarding collection, it is noteworthy that the medium (substrate) and the cultivation container (pot or natural soil) have significant effects on image quality obtained and on root growth and development. This may lead to serious experimental errors and, consequently, misleading results and conclusions. In this sense, it is still necessary to improve and standardize protocols for conducting and evaluating experiments with this goal so that the real growth conditions reliably represent the site for which the new genotypes to be developed will be recommended. Additionally, it is necessary to improve the image capturing equipment to maximize reproducibility and minimize interference of the medium in image quality and resolution.

Regarding aspects after obtaining the images, it is necessary to develop statistical-mathematical algorithms and models that better describe the 4D structure of roots and transform the image data into quantitative variables that can be analyzed as such. This will facilitate the use not only of simple variables such as length,

diameter, and volume, but also of routinely employing complex traits, such as angles between roots, growth rate, and their spatial distribution as a function of changes in soil factors.

Finally, the study of roots is a relatively new subject among geneticists and breeders. However, the results already observed and the equipment and techniques developed (or under development) give this field a very exciting outlook.

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