

COMMENTARY

Analyzing Lateral Root Development: How to Move Forward

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Roots are important to plants for a wide variety of processes, including nutrient and water uptake, anchoring and mechanical support, storage functions, and as the major interface between the plant and various biotic and abiotic factors in the soil environment. Therefore, understanding the development and architecture of roots holds potential for the manipulation of root traits to improve the productivity and sustainability of agricultural systems and to better understand and manage natural ecosystems. While lateral root development is a traceable process along the primary root and different stages can be found along this longitudinal axis of time and development, root system architecture is complex and difficult to quantify. Here, we comment on assays to describe lateral root phenotypes and propose ways to move forward regarding the description of root system architecture, also considering crops and the environment.

Root system architecture is a key determinant of nutrient and water use efficiency

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and describes, on a macro scale, the organization of the primary root and root- and stem-derived branches where they are present in monocots and dicots (Hochholdinger et al., 2004; De Smet et al., 2006; Hochholdinger

and Zimmermann, 2008; Péret et al., 2009; Coudert et al., 2010). Overall root architecture of dicots, such as *Arabidopsis thaliana*, and monocots, such as maize (*Zea mays*) and rice (*Oryza sativa*), differs. The dependence on the

COMMENTARY

embryonically derived system of a primary root and its lateral roots is a feature typical of dicots (taproot system). Perennial dicot species, for example, *Brassica*, usually have a broadly similar root system in terms of development and morphological layout to *Arabidopsis*, though their comparatively longer life cycles usually result in a larger and denser root system. In maize and rice, the postembryonic shoot-borne root system with many different types of root branches builds the majority of the root system (fibrous root system). On a micro scale, root system architecture includes root hairs that increase the surface area (Gilroy and Jones, 2000; Tominaga-Wada et al., 2011). In addition, adaptive root structures, such as cluster roots (Neumann and Martinoia, 2002; Lambers et al., 2006), nitrogen-fixing nodules (Crespi and Frugier, 2008; Oldroyd and Downie, 2008), and mycorrhizae (Bonfante and Requena, 2011; Smith and Smith, 2011), can improve water and nutrient uptake by the plant root system. Here, we will focus on lateral roots.

In the past, various approaches have been used for lateral root phenotyping, ranging from total numbers of (emerged) lateral roots, (emerged) lateral root density, lateral root index, etc. (Dubrovsky et al., 2009; Dubrovsky and Forde, 2011). Dubrovsky and Forde (2011) suggested a set of methods to analyze lateral root development quantitatively in *Arabidopsis*. While Dubrovsky and Forde (2011) offer useful points for consideration to reduce mistakes describing root branching phenotypes, their solution is restricted in scope to *Arabidopsis* grown on agar. In addition, notwithstanding their parameters can have value for in-depth analyses of *Arabidopsis* root architecture, these approaches should not become the sole standard in phenotyping root branching. Also, with the growing importance of studying root system architecture in crops, including cereals, we require a wider portfolio of assays to address different aspects of root systems. With respect to (cereal) crop species, there are other features, such as volume, surface area, length distribution, etc., that are straightforward to measure and can be taken into account (Iyer-Pascuzzi et al., 2010). Here, we further highlight novel and

original approaches that move from two- to three-dimensional (2D to 3D) image analyses, and we phrase the importance for wider adoption of imaging approaches capturing the dynamic nature of roots, such as root branching kinetics and interactions with the environment. Moreover, we stress the future need to look at lateral root development in more ecologically and agronomically realistic contexts, such as when grown in soil or even under field conditions. All these aspects are important for both small-scale and high-throughput approaches, with the latter also being tackled in several phenotyping facilities.

WHAT MEASUREMENTS CAN WE USE?

Simple measurements of lateral root density (lateral root primordia and/or lateral roots per unit of total primary root length or of branching zone) remain useful statistics when interpreted appropriately and should be sufficient to characterize root branching phenotypes in the first instance. In addition, the *Arabidopsis* model system can be used to gain further insight into the developmental basis for an altered number of emerged lateral roots in given mutant backgrounds, and the total number and distribution of stages of lateral root primordia can be determined in wild-type versus mutant roots (Swarup et al., 2008). Nevertheless, such analyses are commonly performed in a static way (i.e., only taking one time point into account). While single time point assays can provide informative answers, the age of the plant is important as emergence of lateral roots varies considerably with time as suggested by the variable densities reported (Dubrovsky and Forde, 2011). In *Arabidopsis*, lateral root primordia emerge at ~7 d after germination, soon after the first pairs of leaves are visible as they represent the source of auxin to promote root outgrowth (Bhalerao et al., 2002). Hence, small delays in (leaf) development can provoke major temporal differences in lateral root density that disappear in older plants. In addition, a naturally heterogeneous environment affects root system architecture at a specific point in time and space. As such, the root system also reflects previous local environmental situations (Füllner et al., 2011). Therefore, we should be mindful that time

is an important parameter when considering development of primordia and root systems.

In addition, the importance of events occurring before the stage I primordia are visible has lately become more apparent in *Arabidopsis* (De Smet et al., 2007; Moreno-Risueno et al., 2010). In this respect, the production of prebranch sites and founder cells are features that could also be taken into account when describing lateral root phenotypes. In *Arabidopsis*, this can be addressed using available markers, such as *pDR5:LUCIFERASE* (Moreno-Risueno et al., 2010), *pDR5:β-glucuronidase* (De Smet et al., 2007), *pDR5rev:green fluorescent protein* (Dubrovsky et al., 2008), and *pGATA23:nuclear localization signal:green fluorescent protein* (De Rybel et al., 2010). Besides how many lateral roots are being produced, it is also important to determine how fast they grow and how they are positioned along the primary root axis.

As current approaches neglect the fact that lateral root density changes as a function of the rate of root growth (Dubrovsky and Forde, 2011) and to avoid drawing incorrect conclusions based on variation in lateral root densities at one time point, we need to monitor the dynamics of lateral root development. To zoom in on a potential alteration of the process of lateral development and/or emergence, focused, reliable, and reproducible quantitative phenotyping based on manually curved roots (J-hooks) (Laskowski et al., 2008) or the gravistimulation of lateral roots in a well-defined zone where the root apex has been reorientating its growth toward the new gravity vector are useful approaches (Lucas et al., 2008; Guyomarc'h et al., 2012). In the latter approach, subsequent stages of lateral root development occur at regular time points following gravistimulation, making this an ideal system to record specific deviations from the normal lateral root organogenesis process and for analyzing the timing of gene expression (De Smet et al., 2010).

WHICH GROWTH MEDIA AND CONDITIONS TO STUDY ROOTS?

To study roots, we need ways to culture and observe them in the lab. There is

COMMENTARY

a large diversity of growth media reported in the literature, and Dubrovsky and Forde (2011) highlighted that this as a serious limitation to the robustness and repeatability of results across laboratories. However, when significant differences are reported on lateral root densities, and both mutants and the wild type were properly compared on the same medium, there should not be a problem interpreting the data. In many cases, the medium has little interaction with root responses to experimental treatments, and many mutants conserve their phenotypes across a range of environmental conditions. It is difficult to imagine how the scientific community could benefit from a standardization of growth media that would hinder our understanding of root plasticity. Especially since growth conditions are quite different between labs (temperature, light intensity, photoperiod, humidity, etc.) and since standardizing media would not be sufficient to create homogenous conditions. However, as also suggested by Dubrovsky and Forde (2011), a greater level of detail about growth conditions employed in a research article would certainly aid attempts to replicate experiments.

Adaptation of root system architecture to variable environments is highly plastic, and exploring the diversity of these developmental adaptations is one of our key challenges for the future. Root developmental strategies strongly depend on both local concentrations of nutrients as well as on the nutrient status of the whole plant (Gojon et al., 2009) and on soil temperatures (Füllner et al., 2011). We believe that the scientific community might not necessarily profit from the general use of one single growth medium, given that substantial progress has been realized using different media and different growth conditions, instead of one standardized medium. We cannot understand roots without knowing how they adapt to their environment. Experimental studies must therefore expose roots to heterogeneous physical or chemical environments. Split root systems (Remans et al., 2006; Girin et al., 2010; Ruffel et al., 2011) or artificial granular media (Bengough et al., 2010) are examples of systems available to understand the

effects of environments on root development. However, ensuring concentrations of nutrients in growth media are within the levels recorded in soil would help to guarantee that root responses observed are physiologically relevant.

HOW TO IMAGE ROOT SYSTEMS?

The 2D agar-based culture of *Arabidopsis* discussed by Dubrovsky and Forde (2011) is an appealing simple solution to obtain descriptions of the root system at low cost and high throughput. In this respect, aspects of root system phenotypes (root elongation, root angular spread, numbers of root axes, etc.) measured for crop seedlings in simplified 2D systems have been shown to be closely correlated with root properties in 3D and in 2D for mature plants (Liao et al., 2001; Manschadi et al., 2006; Hargreaves et al., 2009).

Nevertheless, several other valuable methods have been developed for describing root architecture of crop species in 2D and 3D. These include growth on moistened germination paper rolls or pouches, sand rhizotrons, rhizoboxes, in compost followed by washing, soil columns and gel-based systems where phenotypic effects can be imaged using flatbed scanners, digital cameras, lasers, or even x-ray computed tomography (CT) (Hetz et al., 1996; Whiting et al., 2000; Bengough et al., 2004; Fang et al., 2009; French et al., 2009; Gregory et al., 2009; Hammond et al., 2009; Iyer-Pascuzzi et al., 2010; Trachsel et al., 2010; Tracy et al., 2010, 2011; Chapman et al., 2011; Lobet et al., 2011; Lucas et al., 2011). Magnetic resonance imaging (for noninvasive analysis of root structures) and positron emission tomography (for analysis of carbon transport and accumulation) can be combined to study the dynamics of structure-function relationships of roots in real soils in a noninvasive manner (Jahnke et al., 2009). In all these techniques, a compromise must be made between the disturbance required to allow observations and the resolution and throughput provided by the imaging device. To obtain relevant high-throughput phenotyping data, new (automated) methods and com-

binations are still required to provide more natural and nondestructive environmental conditions (Nagel et al., 2009; Zhu et al., 2011).

Rice root system architecture can be imaged in gel columns in 3D using optical projection tomography or laser scanning (Fang et al., 2009; Iyer-Pascuzzi et al., 2010). For plants grown in soil, x-ray CT techniques and magnetic resonance imaging (Heeraman et al., 1997; Nagel et al., 2009; Tracy et al., 2010; Lucas et al., 2011; Zhu et al., 2011) have recently increased our capabilities to visualize root system architecture in situ nondestructively. For example, the x-ray CT technique can be used to study root architectures under varying nutrient, moisture, temperature, and soil density conditions in a physiologically relevant way over time. Drawbacks of the system, such as imaging time, imaging area, and a 3D image reconstruction approach, are being overcome with improvements in instrumentation (scan time and image quality) and development of new software (Mairhofer et al., 2011; Mooney et al., 2011).

At present, there are various methods for imaging root system architecture as described above, and each approach has its own merit. A transition from 2D to 3D is essential to fully grasp how root system architecture colonizes its environment, but this involves a large increase in expense and data analysis that needs to be taken into account.

ROOT SYSTEM ARCHITECTURE IN THE SOIL

Some biological questions can be sufficiently addressed using agar-based approaches. However, with the importance of the root system contributing to a new green revolution (Lynch, 2007; Den Herder et al., 2010), we should not neglect the key ecological and agronomical aspects.

The study of root architecture has always been compromised by the inherent difficulties in studying a system that necessarily operates in an opaque belowground environment. Some approaches cover Petri dishes with foil, cloth, or wavelength selective filters; the latter allows infrared imaging

COMMENTARY

of root growth (Wells et al., 2012). However, the majority of the root phenotyping assays at present are plate based and expose the roots to light, implying artificial conditions in any case. Nevertheless, this has allowed substantial progress over the years. However, depending on the biological question, we need to validate any apparent differences on the level of lateral root density, root angle, etc., by studying root system phenotypes not just in soil, but across a range of varying soil types in terms of texture, structure, and organic matter.

Root architecture is influenced by many soil properties, including mechanical strength and dry bulk density of the soil, the presence and connectivity of air and water-filled pores, soil pH and temperature, and a variety of nutrient and biotic interaction factors that cannot be easily reproduced in highly artificial growth systems. Mechanical impedance in particular is a major physical limitation to root growth that is very difficult to simulate using a gel system, even for the roots of rice (Clark et al., 1998).

Extracting an entire root system from soil, while maintaining its integrity and avoiding damage to the finer elements of the root system, is an almost impossible challenge. Some promising results have been generated via “shovelomics,” a semi-quantitative method of excavating, washing, and phenotyping roots, but these have focused mainly on crown root systems (Trachsel et al., 2010; Lynch, 2011). In addition, there are conventional soil coring and underground observation chambers (Neill, 1992; LeCain et al., 2006). As mentioned above, this can be downscaled using x-ray CT, although the low-throughput and sample size:resolution trade-off remain a constraint (Mooney et al., 2011). Less extensively, magnetic resonance imaging has been used in a similar way to study root architecture in situ, as has neutron tomography (Heeraman et al., 1997). In all these approaches, however, cost, effort, the limited throughput, and accessibility (e.g., limited availability of synchrotron beam time for neutron tomography) are still major drawbacks. However, recent technical developments promise increased throughput in the near future.

USING MATHEMATICAL SIMULATION AND MODELING

Despite recent advances in imaging, there are still many processes that cannot be observed experimentally. For these reasons, lateral root research is making increasing use of simulation and mathematical models to understand the way roots function (Lynch, 2007; Laskowski et al., 2008; Lucas et al., 2008). Simple models are useful tools to interpret experiments. Models can test and validate biological hypotheses, and models can be inverted to determine the hidden parameters of a system (Dupuy et al., 2010). Models can also integrate complex environmental and developmental variables, including, for example, response to water and nutrient supply (Dunbabin et al., 2002; Draye et al., 2010), various phosphorus concentrations (Fang et al., 2009), root adaptation to low nitrogen soil under carbon flux modifications (Brun et al., 2010), and the formation of root cortical aerenchyma in response to soil nutrient status (Postma and Lynch, 2011). Software packages are also available to facilitate the (re)construction of root systems, such as SimRoot (Lynch et al., 1997). Recent progress in elucidating the biological, chemical, and physical processes affecting root growth in soil allows models to be constructed that integrate fundamental regulatory mechanisms into powerful mathematical frameworks incorporating both variability and plasticity (de Dorlodot et al., 2007; Draye et al., 2010). Taken together, these tools will help understanding the system and knowledge gaps and/or capture and predict the relevant properties of a root system.

CONCLUSION

We agree with Dubrovsky and Forde (2011) that the use of standardized definitions and standard protocols can help to compare data in the scientific community. However, we also think it is important to avoid uniform rules and growth conditions that may be unnecessarily restrictive. To investigate root branching, we should take advantage of the variation that provides unexpected insights. In addition, the value of a screening platform is completely

dependent on the trait of interest, and as such, there is no single ideal platform for all root characteristics. Using several approaches to uncover the hidden half of plants is the best way to move forward. These approaches, as described here and by Dubrovsky and Forde (2011), will, when put together, balance out the weaknesses any single approach has.

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COMMENTARY

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AUTHOR CONTRIBUTIONS

All authors contributed equally to this article.

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COMMENTARY

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