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## Review

# Challenges and opportunities for quantifying roots and rhizosphere interactions through imaging and image analysis

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## ABSTRACT

**The morphology of roots and root systems influences the efficiency by which plants acquire nutrients and water, anchor themselves and provide stability to the surrounding soil. Plant genotype and the biotic and abiotic environment significantly influence root morphology, growth and ultimately crop yield. The challenge for researchers interested in phenotyping root systems is, therefore, not just to measure roots and link their phenotype to the plant genotype, but also to understand how the growth of roots is influenced by their environment. This review discusses progress in quantifying root system parameters (e.g. in terms of size, shape and dynamics) using imaging and image analysis technologies and also discusses their potential for providing a better understanding of root:soil interactions. Significant progress has been made in image acquisition techniques, however trade-offs exist between sample throughput, sample size, image resolution and information gained. All of these factors impact on downstream image analysis processes. While there have been significant advances in computation power, limitations still exist in statistical processes involved in image analysis. Utilizing and combining different imaging systems, integrating measurements and image analysis where possible, and amalgamating data will allow researchers to gain a better understanding of root:soil interactions.**

*Key-words*: abiotic interactions; automation; biotic interactions; computed tomography; microscopy; root system architecture (RSA); root:soil interactions; soil.

## INTRODUCTION

An increasing world population that is estimated to reach 9.6 billion by 2050 (United Nations D.o.E.a.S.A., Population Division 2013) and changes in dietary choices, including increased meat consumption, has resulted in unprecedented food, and therefore, crop production demands (Tilman *et al*. 2011; White *et al*. 2013b). In addition many of the crop-producing regions of the world are experiencing unfavourable environmental conditions such as drought or flooding and agricultural land is under pressure because of competition for the production of biofuels (Valentine *et al*. 2012a). Currently, crop production in many regions relies heavily on mineral fertilizers, however, mineral resources for the production of these fertilizers are finite and the production process relies heavily on fossil fuels (White *et al*. 2013a). The global nutrient use efficiency (NUE) for nitrogen, phosphorus and potassium has been estimated at 50, 40 and 75%, respectively, and there is therefore significant scope for improvement in fertilizer use efficiency (Tan *et al*. 2005). In addition, crop production must be maintained for the long term, so crop improvement objectives must either maintain crop yields with reduced inputs or increase yield under intensive agricultural practices while avoiding long-term ecological damage (Gomiero *et al*. 2011). Since roots of crop plants are responsible for the uptake of resources from the soil, an understanding of the processes that are involved in root soil exploration, root nutrient acquisition and yield limitations as a consequence of both biotic and abiotic interactions could enable new strategies for sustainable yield production through better nutrient and water use efficiency, overcoming soil constraints and by improved C sequestration (Kell 2011; White *et al*. 2013b).

Roots have evolved to be extremely adaptable and responsive to their local environment. Their growth, morphology and physiology are intimately linked to both the plant genotype and the properties of the soil or medium in which they grow. For example, root elongation rates and numbers of lateral roots can be reduced by high soil density or high water content with a consequent reduction in shoot growth (Grzesiak *et al*. 2002; Bingham & Bengough 2003; Bengough *et al*. 2011). Similarly, the availability of nutrients such as phosphate can cause alterations in root system architecture (RSA; Lopez-Bucio *et al*. 2002; Hammond & White 2008; Dai *et al*. 2012) and root anatomy (Wu *et al*. 2005; Burton *et al*.

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2013; Hu *et al*. 2014). Ultimately, the abiotic stresses experienced by roots have an impact on the yield of crops (Batey 2009; Wang & Frei 2011). In addition, RSA and root growth are influenced by biotic factors including saprotrophic and pathogenic micro and macro-organisms as well as arbuscular mycorrhizal (AM) symbiotic associations (Osmont *et al*. 2007) and growth-promoting bacteria (Vacheron *et al*. 2013). Increased understanding of the plant responses to both biotic and abiotic soil conditions may therefore assist in the selection of crop varieties that are more resistant to invasion of plant pathogens (Bailey *et al*. 2006) or that are able to take advantage of positive soil biotic interactions and may thus allow the selection of crops that are pre-adapted to the impacts of climate change or particular abiotic soil conditions (Den Herder *et al*. 2010).

Selection of crop varieties often involves the screening of large populations for specific beneficial phenotypes in the search for quantitative trait loci that will enable the development of genetic markers for marker-assisted breeding (Mir *et al*. 2012). Typically, these populations range in size from 80 to 400 lines (Quarrie *et al*. 1994; Lebreton *et al*. 1995; Kreike *et al*. 1996; Ray *et al*. 1996; Loudet *et al*. 2002; Balasubramanian *et al*. 2009); however, in the case of mutant populations, the numbers can run into several thousands (Caldwell *et al*. 2004; Bovina *et al*. 2014). These large populations and the need to understand responses to variable environmental conditions, together with the highly variable nature of root growth, leads to a requirement to phenotype several hundreds of individual plants rapidly, under a range of environments or stress treatments with replication an important consideration (Adu *et al*. 2014). In an ideal world, phenotyping of roots would be achieved by time-lapse imaging of roots *in situ* in undisturbed soil in glasshouses or in the field. Image analysis systems would be developed not only to record the shape of root systems at a specific time point but also to provide information on the mechanisms of root growth and the genetic or physiological responses over time. This would be linked to information on the heterogeneous biological and physical environment of the soil. Unfortunately, limitations to observations in soil are such that to be able to image living roots, scientists must often find a compromise between growth conditions and quality of data (Neumann *et al*. 2009).

Traditional methods for measuring roots grown in soil, such as root washing and root tracing are destructive and slow (Smit *et al*. 2000). However, recent advances in imaging methodologies including cameras, scanners, fluorescence and radiation-based techniques, for example, X-ray imaging, has enabled the non-destructive exploration of root growth processes and plant:soil interactions with the abiotic and biotic environment, including soil pathogens and plant growth-promoting rhizobia (Bloemberg *et al*. 2000; Reddy *et al*. 2007; Valentine *et al*. 2007; Abbas-Zadeh *et al*. 2010; Bengough *et al*. 2010; Wuyts *et al*. 2011; Downie *et al*. 2012; Keyes *et al*. 2013; Bao *et al*. 2014). These various imaging techniques allow visualization of different aspects of soil structure, root growth and physiological processes, microbes and water in soils or growth medium (Fig. 1). The majority of root measurements however are still done *ex situ* by laying the roots on a flat surface, imaging them and later tracing them (Walter & Schurr 2005; Hund *et al*. 2009; Villordon *et al*. 2011; Clark *et al*. 2012, 2013; Wells *et al*. 2012) and therefore, there is still a great deal of scope for improving the collection of data on root:soil interactions using novel imaging and analysis techniques.

Several recent reviews have detailed the progress in phenotyping root systems through imaging and image analysis (Zhu *et al*. 2011; Dhondt *et al*. 2013; Fiorani & Schurr 2013). In this review, we seek to establish that root phenotyping research must focus more on interactions with environment and investigate rhizosphere traits and processes as well as root phenotyping. This could be achieved by bringing together different imaging solutions, thus linking the root phenotyping with quantification of rhizosphere processes. We first discuss techniques for imaging and analysing roots and root growth dynamics. We also review imaging and image analysis of roots within the context of delivering improved understanding of root-genotype × environment interactions (both abiotic and biotic) and give examples of where combinations of technologies have allowed different aspects of the root:environment processes to be explored. As part of this root:environment phenotyping process, scalable methodologies, under conditions similar to those encountered in the environment, must be developed that will allow knowledge to be translated to practical applications through breeding programmes for new crop varieties. This will require pushing the boundaries of both the imaging and computational techniques already available.

## PHENOTYPING ROOT SYSTEM ARCHITECTURE

### Two-dimensional (2D) root imaging

Root systems consist of numerous interconnected roots with different orders of lateral roots and RSA describes the system's morphology. Early studies of root systems date back to the 18th century and mainly involved digging up roots and manually measuring their weight and length. The ecologist J.E. Weaver (1919; Fig. 2a) was one of the pioneers of root research by field excavation, but many others also cultured plants in containers in order to study their root systems (Bohn 1979). Hiltner (1904), Bates (1937) and Kutschera (1960) also quantified root systems in field soil or in pots by observation, sketching or tracing. Most of these historic techniques, including the measuring wheel, rulers or the transect methods employed to determine the length of excavated washed roots, were fraught with inaccuracies and biases (Baldwin *et al*. 1971). More recently, attempts have been made to automate the extraction process (Fig. 2b; Benjamin & Nielsen 2004) but fine roots are often lost during these extraction processes. An alternative high-throughput method was reported by Trachsel *et al*. (2011) who carried out a high-throughput screening study of root traits of mature plants in the field, where many root traits from 218 inbred lines of maize were measured by shovel excavation and visual scoring. The protocol is, however, destructive and laborious.

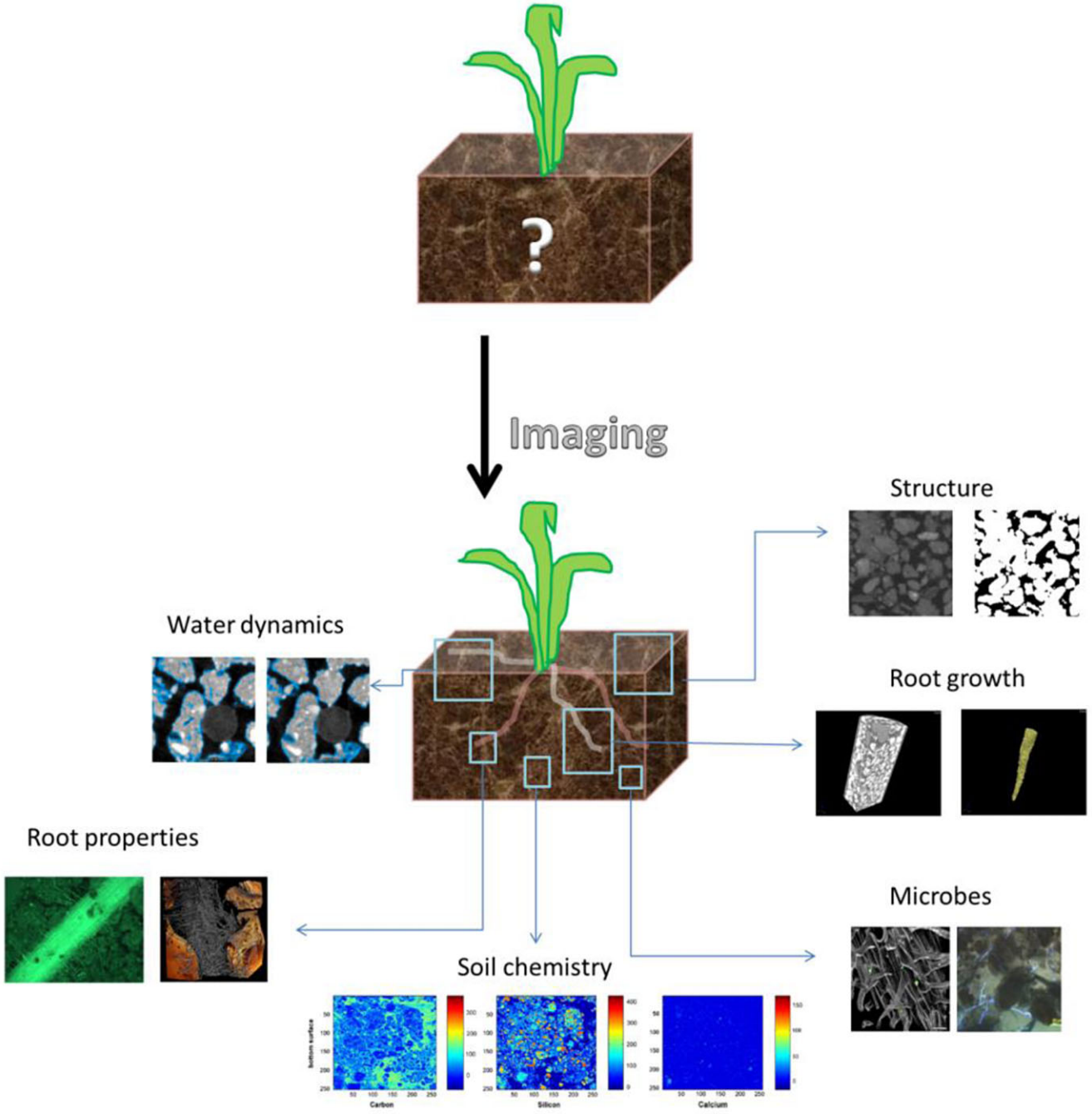
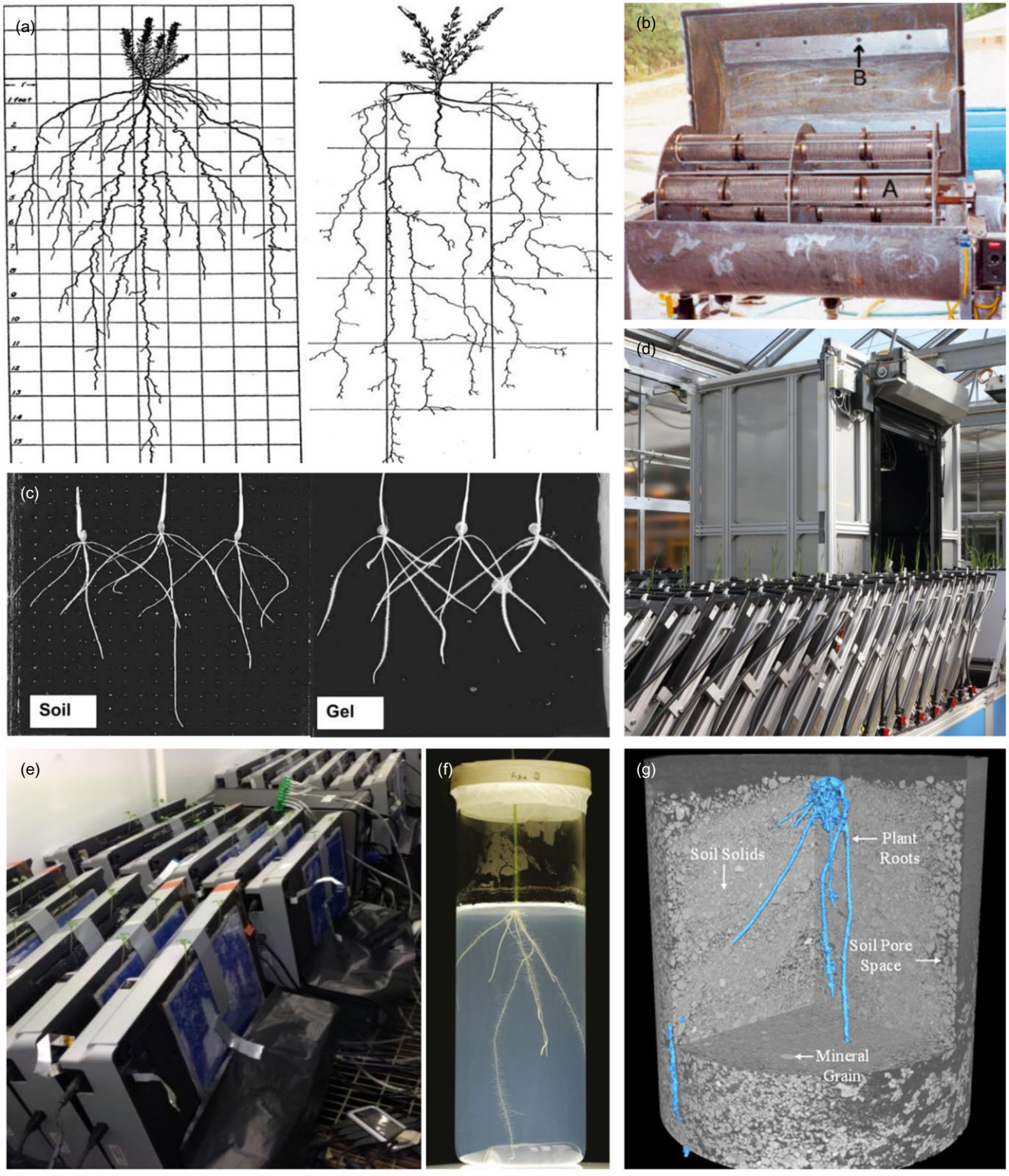



**Figure 1.** Visualization of rhizosphere abiotic and biotic interactions. Interactions in the rhizosphere involve many different physical, chemical and biotic processes. This requires a range of imaging and image analysis solutions. Soil chemistry images courtesy of Simona Hapca. Microbes, (left) Downie *et al.* (2012), (right) with kind permission of Elsevier Limited, reproduced from Harris *et al.* (2002).

Recently, the study of RSA has benefitted greatly from the introduction of relatively inexpensive imaging facilities including flatbed scanners and digital video cameras (Ortiz-Ribbing & Eastburn 2003). Simple camera set-ups can be used to capture images of root systems both *in situ* (Dannoura *et al.* 2012) and *ex situ* (Clark *et al.* 2011). Image acquisition with these systems is technically simple, cheap, readily accessible and can frequently offer resolutions of up to 1600 dpi (scanners) or 8 MP for cameras (Pierret *et al.* 2003). Scanners and cameras facilitate high-throughput experiments because of their image acquisition speed and low cost (Dong *et al.* 2003). For example, Bengough *et al.* (2004) used flatbed scanner-based 2D gel chambers to predict which barley seedlings in landraces would develop shallow or deep root distributions (Fig. 2c) and Shi *et al.* (2013) utilized a high-throughput 2D growth system and flatbed scanners to quantify root architectural traits enabling the identification of Chromosomal Quantitative Trait Loci




(QTLs) associated with responses to phosphate availability. 2D imaging is also suitable for imaging roots growing in soil with flatbed scanner rhizotron systems (Dong *et al*. 2003). These are often angled such that roots grow along the glass surface but are in contact with soil (Dechamps *et al*. 2008). The advantage of the rhizotron system is that roots can be imaged without disturbance and they have proved useful in assessing root growth dynamics in many crops including apple, maize and barley as well as for studying the effects of changes in water content during plant growth (Kuchenbuch

**Figure 2.** Root imaging from destructive harvests to 2D automated imaging systems and 3D phenotyping of roots in soil. Imaging systems have progressed from manual tracing of roots extracted from soil through to *in situ* analysis of roots growing in soil. Roots were initially manually extracted from soil and an image produced by tracing the roots (a). Some automated systems for extracting root from soil have been developed (b). Scanners can be used to assist in analysis and quantification of extracted roots or for capturing of root data *in situ* in both gel and soil systems (c, d, e). These scanner systems are conducive to automated image capture of root growth of multiple plants due to either multiple scanning points (e) or by automated movement of plant growth boxes (d). 3D analysis of roots growing in gel systems for optical imaging or in soil using for example, X-ray–$\mu$Ct imaging (f, g). (a) Manually traced root systems (Weaver 1919). (b) Automated extraction of roots from soil (Benjamin & Nielsen 2004) . (c) Barley seedlings grown in 2D soil and gel system imaged by scanner illustrating root growth patterns (Bengough *et al*. 2004). (d) Automated robotic phenotyping system, GROWSCREEN-Rhizo (Nagel *et al*. 2012). (e) Automated scanner bank for automated time-lapse imaging of roots growing on filter paper (Adu *et al*. 2014). (f) Roots growing in a gel-based system used for 3D tomography optical imaging (Clark *et al*. 2011). (g) Roots *in situ* in soil imaged using X-ray–$\mu$Ct (Zappala *et al*. 2013). (a) Reproduced under open licence from DigitalCommons@University of Nebraska. (b, c, g) Reproduced with kind permission from Springer Science and Business media. (d) Reproduced with kind permission from CSIRO Publishing. (f) Reproduced with kind permission from the American Society of Plant Biologists.

& Ingram 2002; Dong *et al*. 2003; Nagel *et al*. 2012). The main disadvantage of 2D systems, such as flatbed scanners, is that they often restrict root growth to a thin layer, which could potentially obscure the complex three-dimensional (3D) orientations of many root systems and could induce thigmotropic responses from the roots because of the continuous root to glass contact. Further, most use plant culturing systems that do not truly represent an undisturbed soil system in terms of mechanical impedance, temperature, moisture distribution, solute concentrations and redox reactions (Herrera *et al*. 2012), and thus, the results obtained may not be applicable to field conditions (Bengough *et al*. 2004; Gregory *et al*. 2009a,b; Wojciechowski *et al*. 2009; Wells *et al*. 2012; Watt *et al*. 2013). Automated systems utilizing scanners or cameras to take time-lapse images of root systems during development have recently been developed using either filter paper or soil based systems (Fig. 2d,e; Nagel *et al*. 2012; Adu *et al*. 2014). These systems generate large datasets of images with their own individual image analysis challenges. These will be discussed in detail later in this review.

Some phenotyping systems allow roots to grow in 3D space but also enable imaging of roots in 2D. These include some aeroponics systems, which produce roots that are more anatomically similar to roots grown in soil than is achievable with hydroponics (Redjala *et al*. 2011). These root systems are imaged using 2D acquisition tools, thereby losing information on 3D root orientation. The data can nevertheless prove useful for high-throughput phenotyping.

## 3D root imaging

At the cellular scale, 3D imaging of roots employs both destructive and non-destructive methodologies. Imaging has utilized both fixed samples and transgenic plants expressing fluorescent protein such as GFP to build 3D images (Bougourd *et al*. 2000). One destructive method recently developed by Burton *et al*. (2012) for imaging root cellular structure uses laser ablation of the root and gives a complex segmentable 3D image of the root cell structure. Rapid screens such as this can be used to quantify the numbers of a particular cell type such as aerenchyma that have been implicated in 'cheaper' roots (i.e. ones that require a lower resource input by the plant per produced length). This is potentially a beneficial phenotype in drought regions where plants have to access deeper water resources (Lynch 2013). This latter method however is destructive.

There has also been a drive towards imaging roots *in situ* in 3D, through two separate approaches, by either growing plants in soil and imaging using various forms of radiation-based imaging or through the development of artificial transparent growth media that allows the visualization of the root without disturbance using optical imaging, including confocal and fluorescence-based imaging (Fig. 2f & Fig. 4h). Within this latter category, artificial media have been developed for optical imaging of 3D RSA using plants grown in phytagel systems (Fig. 2f; Fang *et al*. 2009, 2011; Clark *et al*. 2011). Phytagel is similar to agar and is homogeneous and water saturated. It is however, very dissimilar to common soils in relation to soil strength, and therefore, great care should be taken when interpreting the results of experiments using different gel strengths to impose physical impedance on roots (Clark *et al*. 1999). Recently, developments have been made to incorporate the physical heterogeneity of soils into transparent substrates for culturing plants. This 'transparent soil' (TS) made from the particles of the ionic polymer (ionomer) Nafion allows control of moisture content during plant growth in a granular, unsaturated substrate, thus allowing higher oxygen transfer to the root system and interactions with a complex pore structure. To allow optical imaging of roots, the substrate is saturated with a solution that is refractive index-matched to the Nafion particles just prior to imaging (Fig. 4h; Downie *et al*. 2012).

Both phytagel and TS can be used in combination with a number of imaging systems such as confocal laser scanning microscopy (CLSM), optical projection tomography (OPT) and light sheet microscopy (LSM) including the use of fluorescence to produce 3D images (Downie *et al*. 2012; Yang *et al*. 2013). OPT is a 3D imaging system that can be used for samples up to several millimetres in size and was developed for imaging animal embryos (Sharpe *et al*. 2002). It has also been used to image plant shoots and roots (Lee *et al*. 2006). The method involves projecting light through the sample and collecting transmission images while the sample is rotated through 360°. Fluorescence can also be captured using an

**Figure 3.** Analysis of Root system architecture dynamics. Analysis of root growth dynamics from cellular through to architectural scale using time-lapse snap shots (a-c, f,g) and motion analysis (d, e). (a) Repeated imaging of Rice roots *in situ* in soil using X-ray $\mu$-CT imaging (Zappala *et al*., 2013) allowing analysis of 3D architectural dynamics in soil. (b) Automated scanner bank (see Fig. 2e) based architectural analysis (previously unpublished image; Adu *et al*. 2014). (c) 3D visualization of root architecture changes over time (Basu & Pal 2012). (d) Motion analysis of individual cell boundaries to analyse cell expansion utilizing *PlantVis-R* [*Arabidopsis* expressing GFP:LTI in the plasma membrane imaged using confocal laser scanning microscopy (CLSM)] (Wuyts *et al*. 2011). (e) Kinetic analysis of root elongation at the meristem scale using IR imaging (van der Weele *et al*. 2003). (f) Automated camera-based high-throughput imaging and image analysis of root elongation and curvature (French *et al*. 2009). (g) Analysis of C sequestration using a combination of magnetic resonance imaging (MRI) and positron emission tomography (PET) imaging (Jahnke *et al*. 2009). (a, d) Reproduced with kind permission from Springer Science and Business media. (b) Previously unpublished image (e, f) Reproduced with kind permission from the American Society of Plant Biologists. (c, g) Reproduced with kind permission from John Wiley & Sons.

ultraviolet (UV) light source to illuminate the sample and emitted light can be captured as well as the transmission images (Fisher *et al*. 2008).

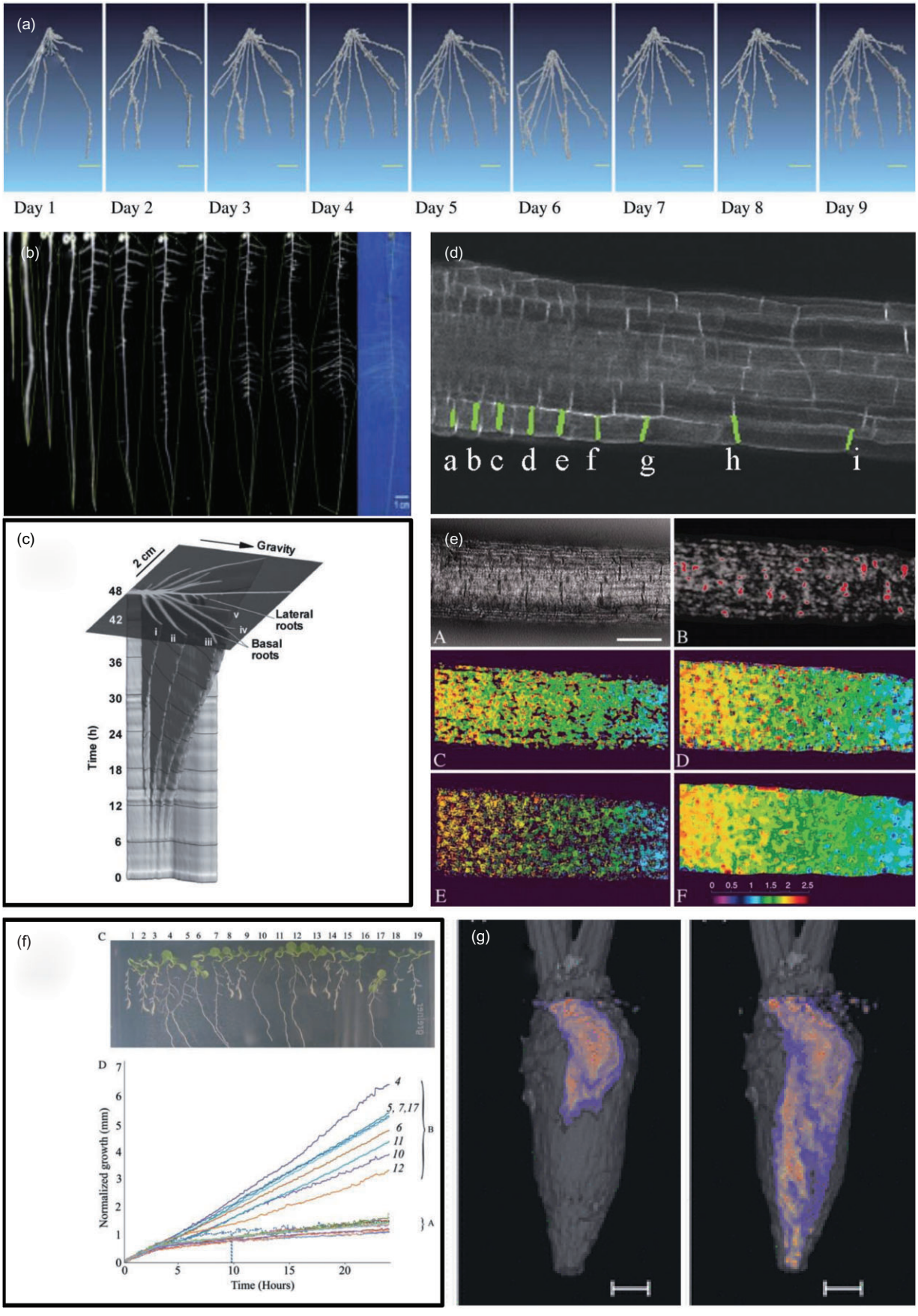
Another useful recent development in microscope optics is the 'mesolens', which is a lens 0.5 m in length, with 4× magnification and a numerical aperture of 0.47 (Amos *et al*. 2010; Saini 2012). It allows imaging of samples of up to 6 mm but with subcellular resolution without the need to reconstruct the final image from a series of images. The developers aim to integrate it into CLSM and light sheet microscopes for 3D imaging. The mesolens would allow the imaging of the whole seedling root at high resolution, thereby, it would be potentially possible to relate the root morphology and growth to cellular processes within one image dataset.

Despite these advances in transparent growth media and optical imaging, 3D imaging in soil remains central to root research. Soils have a great impact on root function and RSA development (Wojciechowski *et al*. 2009) and there are still significant gaps in understanding the reasons for the differences in plants grown in artificial systems versus soil grown plants. Radiation tomography, such as X-ray tomography, neutron tomography, positron emission tomography (PET) and magnetic resonance imaging (MRI) have proven to be useful methods to visualize roots in opaque growth media (Fig. 2g, Fig. 3a, Fig 3g, Fig. 4c & Fig. 4d; Asseng *et al*. 1998; Perret *et al*. 2007; Jahnke *et al*. 2009; Moradi *et al*. 2009; Tracy *et al*. 2010; Zappala *et al*. 2013).

Bois & Couchat (1983), Willatt & Struss (1979a), Willatt & Struss (1979b) and Willatt *et al*. (1978) were pioneers in using radiation for studying roots and gained information about germination time and root and shoot growth rates using neutron radiation. Medical scanners were first used to visualize roots in soil and sand with X-ray tomography (Hainsworth & Aylmore 1983; Hamza & Aylmore 1992; Hamza *et al*. 2001). The resolution that could be achieved with medical scanners was >1 $mm^3$ voxel size, and therefore, only coarse roots could be detected. Higher resolutions were achieved using industrial scanners (Heeraman *et al*. 1997; Gregory *et al*. 2003; Kaestner *et al*. 2006; Lontoc-Roy *et al*. 2006; Perret *et al*. 2007; Tracy *et al*. 2010) and presently, it is possible to achieve resolutions <0.5 $\mu$m, with scanners developed for material research (Tracy *et al*. 2010). The scan resolution is influenced by sample size, focal spot size and detector. The highest resolutions can be obtained by X-ray microtomography. In a recent study by Tracy *et al*. (2010), soil samples of 7 cm in height and 3 cm in diameter were scanned at a resolution of 24 $\mu$m, whereas resolutions obtained using neutron tomography for similar sample sizes were >50 $\mu$m (Moradi *et al*. 2011). The resolution that can be obtained with MRI is >100 $\mu$m (Segal *et al*. 2008). More recently, images of root hairs in soil were obtained using synchrotron-based X-ray tomography and while the sample size at this resolution is at present extremely limited, the results were used to enhance models of phosphate uptake by roots (Keyes *et al*. 2013; Fig. 4g). The quality of the images obtained with X-ray tomography can be adjusted with the number of angular projections and the signal acquisition time per projection (Ketcham & Carlson 2001). With more angular projections images with less noise can be produced, but scanning duration is longer. For screening purposes, it is important to keep the scan time as short as possible. Although scanning times are rapidly improving, it may be some time before these are at speeds sufficiently fast for screening purposes. This raises the question of whether screening processes and analysis pipelines should be considered that comprise multiple methods.

## Towards imaging and image analysis of root system dynamics – time-lapse 2D and 3D imaging

Root systems do not grow at the same rate throughout the life cycle of the plant; therefore, it is important to understand both the process of growth and the life cycle dynamics of root systems. Water uptake and nutrient demand also depend on growth stage and season. Imaging and quantification of root growth and functional dynamics has benefited greatly from the introduction of time-lapse imaging, but clearly, this increases the quantity of data for processing. Challenges for this area of research include the utilization of computational image analysis to increase accuracy, throughput and resolution (Baldwin *et al*. 1971). At the acquisition stage, the length of time necessary to capture the image needs to be taken into consideration particularly when dealing with 3D images. For analysis, high-throughput but accurate methods of extracting the relevant geometric features from the captured images must be developed. Features of interest include RSA traits, such as root lengths, their relationships (primary, seminal, first, second, third, etc. order laterals), spatial distribution and cellular traits such as root hairs and their dynamic behaviour. Simple techniques used to measure these traits have been very informative. For example, Darwin investigated root



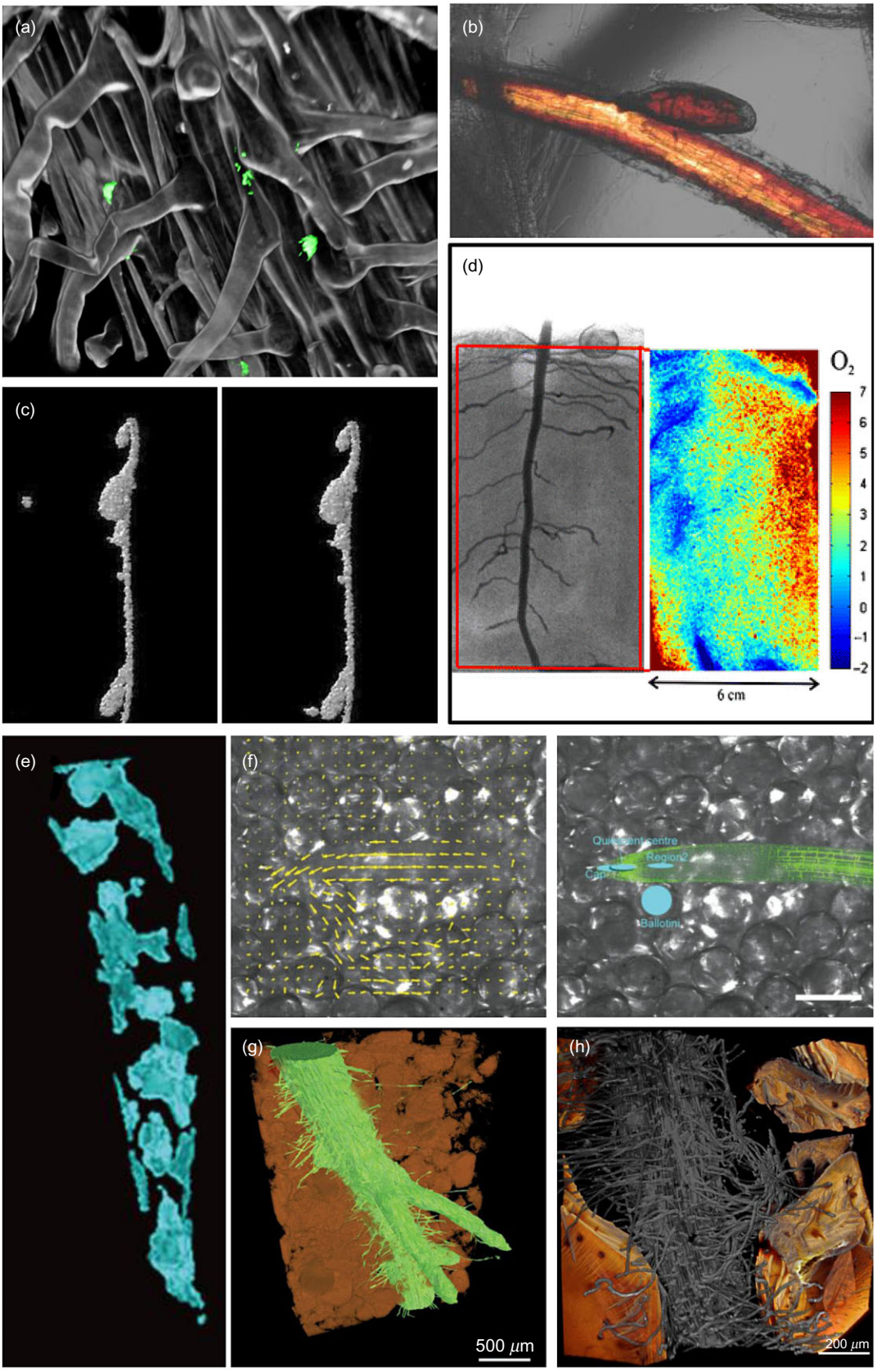


**Figure 4.** Imaging and image analysis of biotic and abiotic interactions at the root:rhizosphere interface. Imaging and image analysis of biotic and abiotic interactions at the root:rhizosphere interface. Visualization of biotic interactions (a–c), chemical (d) and physical interactions (e–h). (a) GFP-expressing bacterial colonies forming on roots of plants grown in transparent soil (Downie *et al*. 2012). (b) *Heterodera schactii* feeding on roots infected with tobacco rattle virus expressing mRFP protein to visualize the uptake of mRFP by the nematode during feeding (unpublished image – Valentine *et al*. 2007). X-ray CT utilized to image Setona seeking out root nodules in an intact root:soil sample (Johnson *et al*. 2004). (d) Physical interactions: neutron radiography image of roots (left) with image of oxygen gradients (right) obtained using oxygen sensitive foil (Rudolph *et al*. 2012). (e) Analysis of root soil contact, blue represents areas of root surface in contact with soil particles (Schmidt *et al*. 2012). (f) Dynamic root growth analysis using particle image velocimetry (PIV) showing movement of surrounding constraining growth medium in response to root penetration (Bengough *et al*. 2010). (g) Synchrotron data enabling visualization of root hair contact in intact soil samples (Keyes *et al*. 2013). (h) Fluorescence-based (confocal laser scanning microscopy; CLSM) imaging to visualize root hair particle interactions in transparent soil (previously unpublished image – Downie *et al*. 2012). (a) Reproduced under Creative Commons Attribution License. (b) Previously unpublished image. (c, e, f, g) Reproduced with kind permission from John Wiley & Sons. (d) Reproduced with kind permission from Springer Science and Business media.

growth dynamics in crops including *Brassica oleracea* and *Vicia faba*. By growing plants in wet sponges fastened to transparent plates and manually tracing root growth with pencils, he was able to reveal growth dynamics such as circumnutation and geotropic root growth (Darwin 1880; King 1883). Manual root sketches and traces are still useful, but not only are these methods painstakingly time consuming, they are also subjective. Root growth has also been captured using other fairly simple imaging techniques, such as cameras and scanners (Dannoura *et al*. 2012; Wells *et al*. 2012; Clark *et al*. 2013; Adu *et al*. 2014). For detailed studies involving the cells of root tissues, magnification is required using microscopes. For example, CLSM and other modern light microscopes connected to software controlled cameras to capture time-lapse images of root growth (van der Weele *et al*. 2003; Bengough *et al*. 2010; Wuyts *et al*. 2011).

Methods for the analysis of time-lapse images can be performed at an individual image level using many of the methods described in the section above or by analysing the sequence of images as an integral part of the analysis (Fig. 3). In the former, each individual image can be analysed to study the cell structure or RSA at scales from confocal images showing root cell structure through to 3D architecture, and then each individual structural description is joined together to visualize the time-lapse dynamics of each quantified parameter (Fig. 3a,b; Federici *et al*. 2012; Galkovskyi *et al*. 2012; Zappala *et al*. 2013; Adu *et al*. 2014). Recently, an interesting alternative approach has been taken by Basu & Pal (2012). They have developed the concept of turning 2D time-lapse images into 3D topologies that describe the changing root over time (Fig. 3c). Alternative methods use more than one image for each data 'time point' and the 'motion' or 'change between images' is analysed often using optical flow algorithms. These techniques are more commonly used for cell growth or single meristem analyses (Fig. 3d,e). Beemster & Baskin (1998) and van der Weele *et al*. (2003; Fig. 3e), for example, studied living plants and analysed the relationship between root cell division and expansion. Root gravitropic dynamics have also been studied using video recording (Mullen *et al*. 2000; Brooks *et al*. 2010). The production of plants with a range of spectral variants of fluorescent proteins marking cell membranes and nuclei has enabled automated image analysis of the dynamics of root cells during root elongation of *Arabidopsis*, using newly developed image analysis tools (Roberts *et al*. 2010; Wuyts *et al*. 2011; Federici *et al*. 2012; Fig. 3d). Functional information can be recorded through direct linking of imaging, with image analysis and temporal expression of fluorescent markers linked to cell development or physiological status of the root (Brady *et al*. 2007).

Time-lapse imaging and analysis in 3D has been limited partly because of the length of time it takes to acquire 3D datasets. To reduce image acquisition time and light exposure of samples, there has been a recent trend towards LSM techniques for 3D imaging of biological samples. This technique uses a thin sheet of laser light that illuminates an optical section of the sample. An objective lens is positioned at an orthogonal angle to the illumination plane and the illuminated section of the sample is focused on. 3D images are created by moving the sample through the illumination plane while a sequence of 2D images is captured (Huisken *et al*. 2004). This technique has advantages over CLSM because of an improvement in the axial resolution and also because the excitation light illuminates a much smaller section of the sample for each image, thereby reducing potential problems of photodamage to the sample. This is particularly important when imaging live specimens at multiple time points. Sena *et al*. (2011) used light sheet fluorescence microscopy to image cell divisions and the nuclear dynamics of *Arabidopsis* roots grown in a small hydroponics system over several days. Similarly the *Arabidopsis* primary root tip growth and lateral root primordial growth has been imaged using a light sheet-based system (Maizel *et al*. 2011). These modern microscopes improve acquisition speed, sample exposure and field of view, facilitating imaging over time or studying large numbers of samples. In addition, numerous research groups have custom-built their own systems at relatively low cost to suit a particular application rather than relying on commercially available systems (Sharpe *et al*. 2002; Huisken *et al*. 2004; Santi *et al*. 2009; Clark *et al*. 2011, 2013).

At the root system scale, scanner banks, conveyors and standard cameras have been employed to generate high-throughput and time-lapse datasets (French *et al*. 2009, 2012; Adu *et al*. 2014). For example, a high-throughput 2D system of two cameras fixed to a conveyor was used to image root systems of up to 20 genotypes of *Arabidopsis* plants and the images were analysed automatically using customized software to extract quantitative information about root growth

dynamics (Fig. 3f; French *et al*. 2009, 2012). Similarly, Nagel *et al*. (2012) described a prototype for automatically analysing RSA in 2D for plants grown in rhizotrons (Fig. 2d). This system has increased throughput, allowing simultaneous camera imaging of root and shoot growth from up to 72 rhizotrons per hour.

The utilization of X-ray computed tomography (CT) imaging for time-lapse growth studies has also been restricted, partially because of the length of time required for each image scan. However, recent reductions in scan time to less than 20 min while maintaining the necessary resolution for segmentation of roots from the collected images has allowed Tracy *et al*. (2012a,b) and Zappala *et al*. (2013) to compare root growth and development in 3D images of tomato and rice plants imaged over 9 consecutive days and to compare the roots of three varieties of wheat by rescanning seedlings at 2, 5 and 12 d after germination (Fig. 3a). Despite the decrease in scan time, time-lapse–X-ray CT is still limited to tens rather than hundreds of scans per day.

Combinations of techniques can also reveal functional processes within plant roots using time-lapse imaging. These include methodologies such as PET and MRI, where, for example, carbon allocation can be tracked by following tracer molecules using PET, and placed in a plant context by imaging of the plant structure using MRI (Fig. 3g). These combined methodologies may also prove useful in understanding root:rhizosphere interactions.

## IMAGING ROOT:RHIZOSPHERE INTERACTIONS

The soil environment and the rhizosphere significantly influence the overall shape and size of root systems. Roots can also influence each other, affecting root growth, lateral root production and, ultimately, root architecture. Utilization of fluorescence technology has started to allow us to separate the different influences on root growth through labelling of roots to separate individual plants (Faget *et al*. 2009, 2013a,b), and labelling of roots and rhizophere bacteria and fungi to study colonization (Gage *et al*. 1996; Genre & Bonfante 2005; Downie *et al*. 2012, 2014). Further, the physiological responses of plant roots to their environment can be visualized utilizing the multitude of reporter proteins now becoming available (Chapman *et al*. 2005; Dixit *et al*. 2006; Okumoto *et al*. 2012). One of the major advances of non-destructive imaging of root systems is that it offers opportunities to quantify root interactions with the biotic and abiotic environment.

### Interactions with biota

There is growing evidence to indicate that the microbiome associated with plants roots is highly important for plant health, where the plant is able to shape the community of microorganisms it associates with, for example, by recruiting bacteria that can protect it from pathogens (Berendsen *et al*. 2012). Soil microorganisms can have a significant effect on root growth both indirectly because of nutrient turnover and also directly because of mechanisms such as nodulation, perception of bacterial quorum sensing signals or the production of plant hormones such as auxin by the bacterial population (Bauer & Mathesius 2004; Goh *et al*. 2013). The interaction between soil biota and roots is of interest for a number of applications including biological pest and disease control, plant growth promotion through enhanced nutrient supply from bacterial processes and rhizoremediation to improve soil quality. A greater understanding of these complex interactions could lead to new opportunities for protecting plants from diseases while limiting the use of agrochemical control products (Chaparro *et al*. 2012). Imaging and image analysis of thin embedded sections of soil cores have revealed soil stabilization processes involving roots and bacteria (Bruand *et al*. 1996). Fluorescence *in situ* hybridization (FISH) can also be carried out on soil samples in order to label microorganisms so that they can be detected using microscopy techniques after sectioning the soil sample (Moter & Gobel 2000; Eickhorst & Tippkoetter 2008). Further, FISH has been used to detect and quantify bacteria colonizing wheat roots after extraction of the roots from soil (Watt *et al*. 2006). However, while there has been a great development in imaging techniques to visualize roots in 3D *in situ* in soil, resolution currently limits the direct visualization of bacteria and individual fungal hyphae in soil. In contrast, utilization of fluorescent reporter proteins such as GFP expressed by fungi and bacteria (e.g. *Fusarium oxysporum*, *Pseudomonas fluorescens* and *Escherichia coli*) has enabled the exploration of root colonization by bacteria in 2D or 3D, gel or TS media (Fig. 4a; Nonomura *et al*. 2003; Gamalero *et al*. 2005; Humphris *et al*. 2005; Czymmek *et al*. 2007; Martino *et al*. 2007; Downie *et al*. 2012, 2014). Similarly, Haynes *et al*. (2004) developed a system for observing different stages of nodule formations in legumes. This enabled rapid screening and isolation of plant nodulation mutants with phenotypic differences in thread growth and cellular invasion. Recently, the TS system was used to quantify bacterial distribution after imaging bacteria and roots live and *in situ* (Downie *et al*. 2014). Similarly, CLSM imaging has been used to study the interactions of viruses and parasitic nematodes with plant roots *in situ*, *in vitro* (Fig. 4b; Valentine *et al*. 2004, 2007) and developments in plant growth substrates such as TS may facilitate a better understanding of how root morphology impacts biotic interactions (Downie *et al*. 2012, 2014). While in many of these studies, the fluorescent tag is used as a tool for imaging where the roots or bacteria or viruses are present, the development of dynamic reporters has also enabled the exploration of the dynamic communications and interactive processes such as bacterial responses to specific plant exudates via utilization of LUX reporters or fast-folding forms of GFP-based fluorescent proteins (Rochat *et al*. 2010).

In soil, X-ray microtomography has also been useful to help understand macrobiotic interactions with roots as it was used to track the movements of the pest *Sitona lepidus* larva towards clover roots nodules (Fig. 4c; Johnson *et al*. 2004). For many of these areas of study, the challenge is now to increase the throughput of these techniques, to extend and enable high-throughput screening by automation of the tech-

niques and also to enable the use of 3D and 4D (3D × time) imaging of processes where appropriate.

## Interactions with abiotic aspects of soil

Changes in soil pH, water content, oxygen availability, strength, macropore availability, bulk density, aggregate size and root:soil contact can affect root elongation and impact on water and nutrient uptake rates of roots (Veen *et al*. 1992; Schmidt *et al*. 2012; Tracy *et al*. 2012a,b, 2013; Valentine *et al*. 2012b). Further, roots forage for nutrients in variable nutrient patches within the soil while elemental toxicity and effects such as salinity can cause significant changes in root elongation rates and architecture (White *et al*. 2013a,b). Equally, as roots penetrate through the soil, they influence the physical and chemical structure and composition around them (Czarnes *et al*. 2000; Lambers *et al*. 2009). Our limited understanding of how roots can overcome and adapt to abiotic conditions is potentially one of the major limitations in translating results from laboratory and glasshouse studies of root behaviour to field conditions (Bengough *et al*. 2004; Gregory *et al*. 2009a; Valentine *et al*. 2012b). Field soil is far more physically heterogeneous than laboratory conditions and roots can exploit the high variability in soil strength, soil pore structure including biopores and macropores and water availability (Ehlers *et al*. 1983; McKenzie *et al*. 2009; White & Kirkegaard 2010; Bengough *et al*. 2011; Valentine *et al*. 2012b).

Recently, time-lapse, CLSM, X-ray CT and neutron radiography techniques have all been used to explore the relationship of roots with their physical environment. Bengough *et al*. (2010) grew *Arabidopsis* plants in a mixture of gel and glass ballotini and imaged the growing roots using CLSM. Using particle image velocimetry (PIV), they showed root growth kinematics at the cell and meristem scale, and additionally, quantified the displacement of the external granular media (Fig. 4f). The root cap and mucilage had a considerable impact on this interaction for maize seedlings in sand (Vollsnes *et al*. 2010). Application of this type of analysis to root growth and dynamics of the environment is limited currently by the requirement to obtain data with the right resolution and within short time scales. The TS in combination with optical tomography (Downie *et al*. 2012) is also a suitable system for this type of research because of the particulate nature of the medium and the ability to control the substrate particle size as well as the water content. In real soil systems, X-ray tomography is especially suited to imaging the soil structure and its relationship with root architecture. Using X-ray CT, Tracy *et al*. (2012a,b) showed that effects of bulk density on root growth were in agreement with destructive studies, and they were able to quantify the decrease in root length with increasing bulk density. Perhaps more striking, and not achievable with other destructive methods mentioned previously, a method for estimating root:soil contact from 3D volumetric images (X-ray–CT) was developed by Schmidt *et al*. (2012) and the effects of growth material and matric potential on root:soil contact and root elongation rate has been investigated (Fig. 4e). Root:soil contact dynamics from 3D microtomographs were also studied by Carminati & Fluehler (2009) by determining the gap around roots after wetting and drying cycles, but actual root:soil contact was not quantified. High-resolution imaging has also allowed the visualization of the interaction of root hairs and particles in artificial media (TS) and soil (Downie *et al*. 2012; Keyes *et al*. 2013; Fig. 4g,h). Root hairs are important features involved in soil contact, and are affected by the soil physical and chemical conditions and are integral to the development of potentially important agricultural traits such as the rhizosheath (Watt *et al*. 1994; Brown *et al*. 2012; Delhaize *et al*. 2012; George *et al*. 2014; Haling *et al*. 2014). Root hairs, root:soil contact and rhizosheath development are thus important parameters in understanding uptake of water and nutrients by roots and the ability to image these and follow changes dynamically will be a huge step forward in understanding root function.

In addition to the soil–structure relationships discussed above, the spatial distribution of water around roots has been a topic of extensive investigation with 3D imaging techniques (Bottomley *et al*. 1986; Macfall *et al*. 1990, 1991; Hamza & Aylmore 1992; Hamza *et al*. 2001; Oswald *et al*. 2008; Pohlmeier *et al*. 2008; Segal *et al*. 2008; Tumlinson *et al*. 2008; Carminati *et al*. 2010; Moradi *et al*. 2011). Using a whole-body X-ray CT system, Grose *et al*. (1996) showed how wheat seedlings were surrounded by a heterogeneous landscape of water content and derived from that their susceptibility to infection. As root material and soil water solution show similar attenuation coefficients, contrast enhancers are often used before the water content can be determined from changes in greyscale values (Hainsworth & Aylmore 1983; Wildenschild *et al*. 2005; Carminati & Fluehler 2009). MRI and neutron radiography are, in contrast, very sensitive to changes in water content because of the interaction with H-atoms. Studies using MRI to measure water uptake and dynamics around individual roots showed that fine roots of loblolly pine (*Pinus taeda* L.) were more efficient than tap or lateral roots at water uptake (based on weight; Macfall *et al*. 1990; Pohlmeier *et al*. 2008; Segal *et al*. 2008). In more recent studies, neutron radiation has been used to visualize and quantify water distribution in close proximity to roots in 3D (Oswald *et al*. 2008; Carminati *et al*. 2010; Moradi *et al*. 2011). It is worth noting that these techniques are limited in their application to soils of intermediate water content and with a content of ferromagnetic particles <4%, as both high and low water content can lead to low contrast and ferromagnetic particles cause artefacts (Bottomley *et al*. 1986; Rogers & Bottomley 1987; Macfall *et al*. 1990, 1991; Pohlmeier *et al*. 2008).

Of the chemical characteristics of the root:soil environment, pH has received the most attention. Most recently, rhizosphere pH has been explored using videodensometry and planar optode imaging (Blossfeld & Gansert 2007; Blossfeld *et al*. 2010, 2013; Rudolph *et al*. 2012, 2013). This technique allows for detailed, dynamic 2D imaging of pH gradients with the plants growing in soil and the roots growing along a flat surface with a planar optode. By imaging roots at 15 min intervals, daily variations in pH and overall

acidification were revealed. The application of optodes is not limited to studying pH. For example, Blossfeld *et al*. (2011, 2013) and Rudolph *et al*. (2012) carried out studies on the dynamics of rhizosphere pH and soil oxygen and $CO_2$, which have important implications in the survival of rhizosphere bacteria and rates of inhibition of root growth due to hypoxia (Fig. 4d). The technique has also been used to study the depletion of ammonium around roots (Stromberg 2008) and in bulk soil (Delin & Stromberg 2011). Further, dissolved P distribution and depletion zones around roots have been imaged by Santner *et al*. (2012) using diffusive gradient films, and laser ablation inductively coupled plasma mass spectrometry. These techniques, currently applicable to 2D imaging can be combined with techniques such as neutron imaging to investigate the integral links between plant architecture and the chemical dynamics. The quantification of rhizosphere processes made possible with these techniques makes it likely that these adaptable approaches will become more popular and available to root researchers as an imaging tool in the future.

## RESOURCES FOR IMAGE ANALYSIS

There are a growing number of resources for image analysis available and these have recently been assembled in an online database that can be found at www.plant-image-analysis.org (Lobet *et al*. 2013). Computed image analysis encompasses a cascade of processes including image acquisition, enhancement, storage and quantification (Duncan & Ayache 2000). Image analysis of roots frequently involves digitally separating or segmenting them from non-root objects within the image and is often fundamental and challenging (Zhang *et al*. 2008). Utilizing transparent growing systems (e.g. gels and TS) along with fluorescent markers or stains can facilitate the image segmentation during root functional studies (Wuyts *et al*. 2011; Downie *et al*. 2012; Federici *et al*. 2012; Faget *et al*. 2013b). However, root images, 2D or 3D, colorimetric or grayscale, often include artefacts that complicate the processing and extraction of information (Lobet *et al*. 2011). While developments in computer capabilities mean that segmentation of digital images could be automated and accelerated, there is no off the shelf solution for all data sets (Sezgin & Sankur 2004). Different images require different segmentation procedures resulting in potential subjectivity (Zhang *et al*. 2008).

Software dedicated to root system analysis should be capable of discriminating roots from non-roots based on simple shape descriptors other than pixel or voxel intensity gradients alone. When imaging in soil using X-ray scanners, some soil particles, water and roots have overlapping distributions in the histograms of image intensity. These cause problems in segmenting the different phases of the sample (Tracy *et al*. 2010; Mairhofer *et al*. 2012). Recently, Mooney *et al*. (2012) summarized in detail the developments in image segmentation when studying roots. Two approaches have primarily been used: separation of the image parts by their position on a histogram of the entire image (i.e. clustering by global thresholding) or identifying a region by growing the region of interest from a seed point (i.e. co-opting parts of the image around an initial seed point depending on its value relative to a local threshold; Pierret *et al*. 1999a,b; Gregory *et al*. 2003; Mooney *et al*. 2012). The global threshold can overestimate the root volume by 10-fold (Mairhofer *et al*. 2012). RootViz3D® and Rootrak, have been developed from these segmentation techniques using automated tracking approaches (Kaestner *et al*. 2006; Perret *et al*. 2007; Jassogne *et al*. 2009; Tracy *et al*. 2010; Mairhofer *et al*. 2012). Segmentation of roots in RootViz3D® is based on applying a probability function to determine whether a specific voxel represents root material. Rootrak employs multiple models of the appearance of root material, where models built from root sections are identified and used to search for root material in another section (Mairhofer *et al*. 2012). RootViz3D® overestimated segmented root volumes compared with data obtained on washed roots using WinRHIZO® (Tracy *et al*. 2012a). Improvements in segmentation techniques for roots over the past 15 years have reduced the error in root length and volume measurements from between 21 and 42% (Heeraman *et al*. 1997) to 10% (Gregory *et al*. 2003; Perret *et al*. 2007). This error is expected to be reduced further with developments in scanning resolution and segmentation algorithms.

Root research would also benefit from a greater integration of the numerous existing algorithms employed in clinical image analysis. Objects such as vascular networks or neural network share many similarities with root systems in their intricacies, complexities and structure. Accordingly, the integration of pre-processing algorithms common in medical image analyses such as vesselness, hessian-based filters and livewire segmentation into root image analysis programs could be applicable (Frangi *et al*. 1998; Poon *et al*. 2007). These shape descriptor-based filters are capable of searching for geometrical structures, which can be regarded as tubular and would be less affected by the presence of noises of different shape orientations. For example, livewire-assisted semiautomatic segmentation was recently employed to analyse root growth dynamics of *Phaseolus vulgaris* and *Cicer arietinum* from 2D time series images, from which spatio-temporal 3D structures were constructed to reveal multimodal transient growth zone in basal roots (Basu & Pal 2012).

Recently, there has been a trend in root system analysis software to facilitate the quantification of traits more complex than number and lengths of root axes, lateral root length and density, which are most commonly measured (Draye *et al*. 2010; Dubrovsky & Forde 2012). Analysing images of roots in soil from rhizotron and minirhizotron systems can be more complicated (Neumann *et al*. 2009; Wells *et al*. 2012). Gasch *et al*. (2011) proposed the use of geographic information systems (GIS)-based image analysis technology for these types of images where the operator selects a few target features within an image to serve as 'learning sets' to train the software in locating additional similar features within the image. Once validated, the feature analyst approach of classifying pixels based on spectral characteristics could enhance rhizotron image analysis.

**Table 1.** Root phenotyping facilities

| Location | Facility | Link |
|---|---|---|
| The James Hutton Institute | Scanner bank | http://www.archiroot.org.uk |
| Aberystwyth University | Plant Phenomics Centre | http://www.phenomics.org.uk/ |
| University of Nottingham | X-ray computed tomography ($\mu$CT) | http://www.cpib.ac.uk |
| The Australian Plant Phenomics | The Plant Accelerator® | http://www.plantaccelerator.org.au/ |
| Jülich , Germany | Jülich Plant Phenotyping Centre | http://www.fz-juelich.de/ibg/ibg-2/EN/organisation/JPPC/JPPC_node.html |
| Montpellier, France | | http://www.montpellier.inra.fr/ |
| LemnaTec, GmgH, Aachen, Germany | Developer and provider of phenotyping sensors and analysis software | http://www.lemnatec.com |

## LIMITATIONS

Efforts are increasingly being made throughout the scientific community to develop solutions to some of the current limitations in imaging root systems (Mooney *et al*. 2012; Dhondt *et al*. 2013; Fiorani & Schurr 2013). Each of the imaging and analysis systems described above has advantages and disadvantages. While fluorescence techniques, for example, can offer real-time gene expression analysis, X-ray and MRI offer root images *in situ* in soil and PET offers metabolite tracing. It is possible that a greater level of understanding could be gained from addressing some of the limitations, and where possible, combining methodologies. Recently, for example, staining techniques have been developed in animal research that allow protein expression patterns to be visualized using $\mu$CT (Metscher & Mueller 2011) and efforts are also being made to combine different methodologies harnessing the power of each. Jahnke *et al*. (2009) have combined PET and MRI imaging to track the allocation of C over time in sugar beet tubers (Fig. 3g), radish and maize roots, the latter of which were imaged *in situ* in soil over time. Since several short- and long-lived positron-emitting radiotracers are becoming available for tracing a variety of metabolites and some elements (Kiser *et al*. 2008; Ishikawa *et al*. 2011), there is much scope for further developments in this area. Rhizosphere interactions are also accessible to this combined approach. Faget *et al*. (2013a) have combined the use of planar optodes to measure soil pH dynamics with GFP-expressing plants to differentiate root identity in soil, enabling examination of the different species interactions and the effect of this interaction on soil acidification. Rhizosphere microbial and root phosphatase co-activity have also been mapped using soil zymography and $^{14}C$ imaging revealing spatial differentiation of activity and activity groups (Spohn & Kuzyakov 2013). These few examples show the potential gains obtainable by combining the power of different methodologies to understand not only the behaviour of plants but also in some cases to gain an understanding of the influence of the rhizosphere on the processes studied.

To increase throughput, many systems are employing robotics and conveyor belts to move plants automatically and position them in front of the imaging devices (see examples, Table 1). Many, however, are limited by their proprietary software, complexity and large investments needed for their infrastructure. The cost of imaging technologies is therefore a major barrier to broad availability and in addition to the 'high investment' phenotyping systems, there is a need to develop root imaging technologies and applications that are cost-effective and thus are readily accessible (Tsaftaris & Noutsos 2009). Cheaper systems may also have the benefit of replication and high throughput (Reynolds *et al*. 2012); a recent example is Adu *et al*. (2014). Cheaper high-throughput root phenotyping will also aid reverse genetic approaches, where the screening of many genotypes is needed (Walter *et al*. 2012). Some of the boundaries of cost of access to high-cost facilities are being overcome by initiatives such as the IPPN (International Plant Phenotyping Network www.plant-phenotyping.org) and EPPN (European Plant Phenotyping Network www.plant-phenotyping-network.eu), which can assist in making the larger automated platforms available for researchers around the globe. Examples of some of the automated systems focused on roots are included in Table 1. These initiatives also bring together experts in the different phenotyping technologies, so these have the potential to facilitate combinations of techniques.

Currently, there are severe limitations in the size of samples, which can be imaged (Herrera *et al*. 2012). For many 2D imaging systems, plant growth is restricted to the seedling stage because of the size of rhizoboxes, making translation of results to mature plants challenging. 3D images from gel and TS samples published so far mostly range in the region of less than 5 cm diameter, and the most common volume of X-ray CT images are also in the region of 5 cm diameter (Tracy *et al*. 2010; Downie *et al*. 2012; Lind *et al*. 2014). Some of the recently developed systems are pushing the sample size boundaries: with some automated systems using 18 L soil volume, and allowing a root depth of 90 cm (Nagel *et al*. 2012). The system at the University of Nottingham Hounsfield Facility will facilitate phenotyping roots in samples with soil volumes of 25 cm diameter × 100 cm length (http://www.cpib.ac.uk).

Development of field-based imaging systems is also essential for validation of data obtained from laboratory-based experiments. With adequate development in terms of throughput, applicability to all soil types and to crop plants of varying developmental stages, geophysical imaging techniques hold potential in field-based root and rhizosphere research (Luster *et al*. 2009). Ultimately, the target is to achieve high-throughput screening of root traits under field conditions but most current soil and field-based methods including soil cores (Herrera, *et al*. 2012) and CT methods (Tracy *et al*. 2010) are yet to realize this objective. Geophysical methods including electrical resistivity, capacitance and ground penetrating radar (Barton & Montagu 2004; Amato *et al*. 2009) could offer fast and automated field measurements, but care must be taken to validate methods as accurate root detection has not been achieved so far (Dietrich *et al*. 2013). Geophysical methods can be 2D or 3D, and have been used to produce images of root systems *in situ* in the field using information on soil moisture distribution (al Hagrey 2007), and there is also the potential to monitor changes and processes in 4D.

Further development in phenotyping must consider the implications of using commercial versus homemade systems. While commercial systems come with full pretesting, which may put them at an advantage over homemade systems, many homemade systems are built on open-source software and are therefore cheaper and potentially more easily manipulated for specific situations. Progress in the development of robust and faster computer hardware and software for image analysis must be concurrent with proper experimental designs and statistical power of analyses. Further, mathematical modelling approaches should be integral in analysing resulting data in order to reveal temporal and spatial variation that might be inherent in the data as a result of local environmental effects. Moreover, for optimal exploitation of emergent and scaled-up phenotyping approaches, it is imperative that suitable databases and bioinformatics tools are developed to manage the large, complex datasets. Central databases and automated management of data flows and retrieval will aid cross-laboratory communication and lead to the creation of a powerful knowledge environment for linking genotype–phenotype root system information (Thorisson *et al*. 2009). The possibility of combining or creating a universal platform that integrates multiple platforms will represent, potentially, a tremendous breakthrough. Hapca *et al*. (2011) have developed a method of sequential sectioning to align 2D chemical maps with 3D volumetric images. This method offers the potential to link information obtained with 2D image techniques to spatial data obtained with radiation techniques that can operate in 3D such as combining X-ray tomography and PET to study changes in soil chemistry and assimilate allocation in the rhizosphere (Jahnke *et al*. 2009; Garbout *et al*. 2012). Further progress is also likely to be made by combining synchrotron techniques with both modelling and plant molecular biology (Donner *et al*. 2012; Keyes *et al*. 2013).

## SUMMARY AND FUTURE DIRECTIONS

Generating robust, reliable and relevant root and rhizosphere trait information is the key to understanding root:soil interactions and to ensure enhanced and sustainable crop production in a changing climate. Currently, selection and breeding of crop genotypes based on root traits is extremely limited. Variability and stochasticity of root traits is such that the number of replicates required to detect differences is very high. It is made more challenging by the high genotype × environmental interactions that are implicit in root plasticity. The need to incorporate the diversity of soil in which crops are grown, the strong heterogeneity of soil conditions and the biotic and abiotic intereactions, add a further level of complexity. Optimization of statistical power of collected data must therefore be considered in order to provide reliable estimates of phenotypes and G × E effects (Walter *et al*. 2012). For root imaging to make an impact in agriculture, it will have to enable detailed analysis of root systems and rhizosphere status at spatial and temporal scales that have not been achieved before (Houle *et al*. 2010). Increasing pixel or voxel resolution and faster image acquisition techniques and time-lapse studies have greatly increased the amount of image data available for root analyses. The present need for high-throughput screening and data aggregation across many different sites for genetic and QTL studies will further compound issues of image capture, image processing speed and complexities of the image analysis process.

**Table 2.** Applicability of imaging techniques to root:rhizosphere interactions (x, low usage to xxx, highly suitable)

| | X-ray tomography | MRI | Neutron tomography | PET | Optodes | Flatbed scanners | Cameras | Fluorescence microscopes | CLSM | Light sheet microscopes | OPT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Soil structure (2D) | xxx | xx | – | – | x | x | x | x | x | – | – |
| Soil structure (3D) | xxx | x | – | – | – | – | – | – | – | – | – |
| Root system architecture | xxx | x | x | – | – | xxx | xxx | x | – | x | xxx |
| Root cellular structure | – | – | – | – | – | – | – | xxx | xxx | xxx | – |
| Root cellular processes | – | – | – | – | – | – | – | x | xxx | xxx | – |
| Root–microbe interactions | – | – | – | – | x | – | x | x | xxx | xxx | x |
| Water | x | xxx | xxx | – | – | – | – | – | – | – | – |
| Chemicals | – | – | – | xxx | xxx | xxx | x | x | xxx | x | x |

CLSM, confocal laser scanning microscopy; MRI, magnetic resonance imaging; OPT, optical projection tomography.

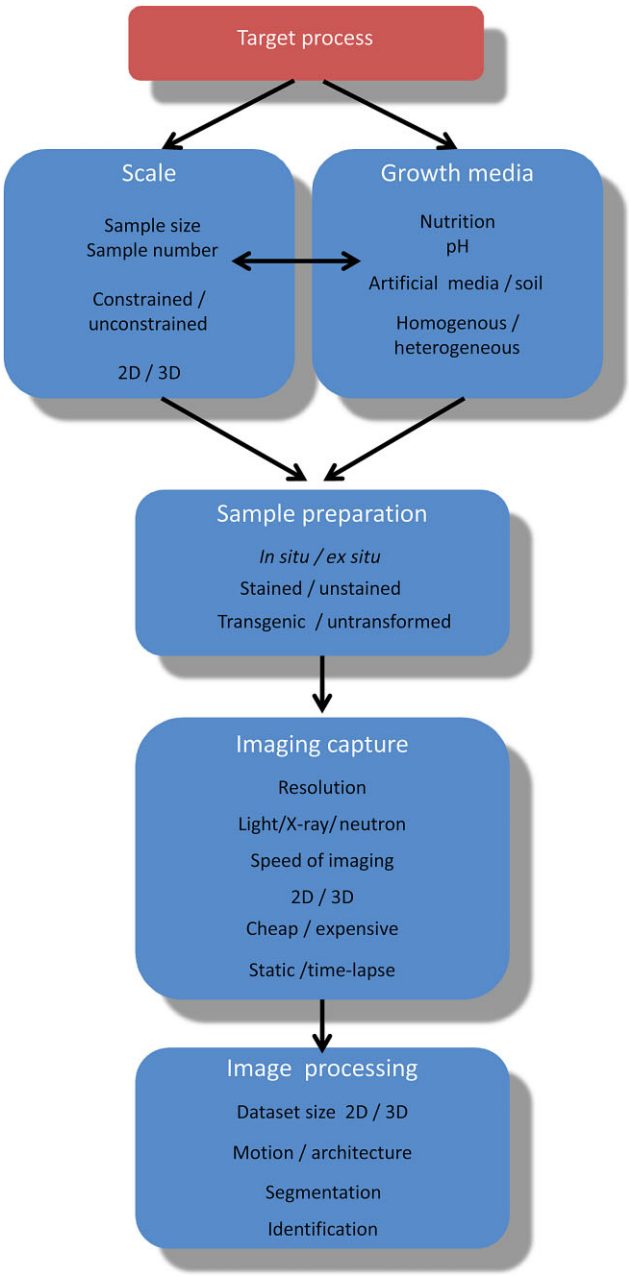



**Figure 5.** Decision process for root phenotyping pipeline. Phenotyping the rhizosphere via image analysis requires several inter connecting steps, each with many parameters that need to be considered. Each parameter may impact on the downstream processing of the images or may alter the number of images and the type of images that it is necessary to acquired earlier in the analysis pipeline.

However, efforts are being made to produce more integrated and high-throughput systems (Armengaud *et al*. 2009; Wells *et al*. 2012).

There is the possibility to link genetics to our understanding of both root growth and physiological processes. Recent increased resolution of radiation-based techniques and developments in optical techniques such as fluorescence OPT, LSM and the mesolens allow analysis of larger samples and give significant scale overlap between the methodologies. Each technique has advantages in visualization of specific processes and specific imaging and analysis methods are required to extract the biologically relevant information. Table 2 summarizes the root:soil processes that have been examined using the different imaging techniques. Imaging techniques to study roots and soil have proven to be useful tools to gain knowledge about root architecture, water transport and uptake, effects of soil structure on root growth, root:soil contact and interactions with the biotic environment but it is important to consider the choices in methodology at all stages of the imaging pipeline. Figure 5 illustrates several options to be considered at each stage of the phenotying pipeline, such as size of sample or growth substrate. Many of the variables will affect the image analysis process and the ability to automatically extract the root:rhizosphere traits from the images later in the phenotyping process (Fig. 5). We can now (1) image and quantify root and rhizosphere dynamics over time; (2) obtain data on density and clustering of roots and link this with plant nutrient uptake and biological interactions; (3) establish links between root hierarchy and age and response to environmental stimuli; (4) demonstrate interactions with the environment, both local and global; and (5) integrate understanding of the effect of the environment over time and space. Because of the reduction in cost of many imaging technologies, and the development of new analytical algorithms and hardware with increased computation power, it is now possible and beneficial to combine or link the different system to gain an integrated understanding of root growth, root physiology and rhizosphere interactions using the benefits of the different systems.

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