# Non-invasive approaches for phenotyping of enhanced performance traits in bean

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**Abstract.** Plant phenotyping is an emerging discipline in plant biology. Quantitative measurements of functional and structural traits help to better understand gene–environment interactions and support breeding for improved resource use efficiency of important crops such as bean (*Phaseolus vulgaris* L.). Here we provide an overview of state-of-the-art phenotyping approaches addressing three aspects of resource use efficiency in plants: belowground roots, aboveground shoots and transport/allocation processes. We demonstrate the capacity of high-precision methods to measure plant function or structural traits non-invasively, stating examples wherever possible. Ideally, high-precision methods are complemented by fast and high-throughput technologies. High-throughput phenotyping can be applied in the laboratory using automated data acquisition, as well as in the field, where imaging spectroscopy opens a new path to understand plant function non-invasively. For example, we demonstrate how magnetic resonance imaging (MRI) can resolve root structure and separate root systems under resource competition, how automated fluorescence imaging (PAM fluorometry) in combination with automated shape detection allows for high-throughput screening of photosynthetic traits and how imaging spectrometers can be used to quantify pigment concentration, sun-induced fluorescence and potentially photosynthetic quantum yield. We propose that these phenotyping techniques, combined with mechanistic knowledge on plant structure–function relationships, will open new research directions in whole-plant ecophysiology and may assist breeding for varieties with enhanced resource use efficiency varieties.

**Additional keywords:** fluorescence, imaging spectroscopy, non-invasive, resource use efficiency.

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## Phenotyping for improved agricultural resource use efficiency in the 21st century

The green revolution of the 1950s resulted in a remarkable increase in average yield of most crops (Evans 1997). For example, roughly 50% of increased corn production can be ascribed to genetic improvements and 50% to improved agricultural management practices (Duvick 2005). Key factors include progress in molecular breeding techniques that reduced the time required for developing new genotypes and the availability of relatively cheap energy justifying larger use of fertiliser and water. Today, the task to further increase yield by these mechanisms faces considerable challenges (Godfray *et al.* 2010). In past decades, increased energy consumption in the agricultural sector has been both economically and socially viable, with few restrictions imposed on increasing resource inputs. In today's climate, agricultural practices must increasingly consider the trade-off of pursuing higher yield against the costs of more intensive management. Present and future plant research objectives must now address optimisation of resource use efficiency and ensure the stability of yield at the regional and global scale (see Ainsworth *et al.* 2011). In this respect, bean and other protein producing plants may become increasingly important in the near future to provide proteins directly for human consumption and to contribute to the increasing demand of protein-rich livestock feed in times of increasing global meat consumption. Plant phenotyping may not only provide possibilities for a total increase of protein production but may also help to direct protein quality and specific protein content.

To pursue these objectives, significant reliance is placed upon plant phenotyping capabilities. Phenotyping is rapidly evolving as a new discipline in plant sciences (see special issue of *Functional Plant Biology* Volume 26, Issue 10–11) using non- or minimally-invasive sensors to measure plant performance and uncover genetic determinants of enhanced agronomic traits. Complex plant physiological traits, such as growth, biomass accumulation and yield, result from the interplay of genetic and environmental determinants throughout the plant (crop) growing season. Environmental factors modulate the expression of genes in space and throughout developmental time and greatly influence the phenotype. As a result, important physiological traits vary along a continuum following a dose–response paradigm. Responses can be generalised for individual trait-environmental factor pairs using a quantitative meta-analytical framework (Poorter *et al*. 2010). A recent overview on the challenges of phenomics and the statistical aspects of the gene–environment interplay was presented by Houle *et al*. (2010).

Phenotyping can take place under laboratory, greenhouse or field conditions. Under laboratory conditions, environmental factors may be controlled and varied as desired, manipulating one or more factors in dedicated experiments. Via this controlled approach, the influence of specific genetic and chemical factors interacting with a limited number of environmental factors can be investigated. In contrast, field conditions are highly variable, fluctuating in time and space (Rascher and Nedbal 2006; Schurr *et al*. 2006; Mittler and Blumwald 2010). Despite this, new technologies capable of high-throughput phenotyping coupled with environmental monitoring at high spatial and temporal resolution can deliver datasets large enough for statistical approaches. For example, imaging spectroscopy can provide high resolution pictures from ground and airborne platforms that contain high resolution spectral data of millions of pixels (Rascher *et al*. 2009). Single spectra can be attributed to single plants or experimental plots and related to plant functional traits (Ustin and Gamon 2010).

In this communication we present state-of-the-art technologies and approaches for plant phenotyping and how these can be applied to populate the trait matrix referring to whole-plant resource use efficiency (Table 1). Plant phenotyping and improved phenological understanding are targeted towards specific traits that influence improved resource use efficiency in plants. Below-ground traits such as root architecture or root function are associated with improved water and nitrogen use efficiency; above-ground traits are mainly linked to photosynthetic energy conversion and, thus, govern light use efficiency; transport and allocation processes link the components of plants and have a great influence on nitrogen and water use efficiency of plants (Table 1). In the following we will give a brief definition of our terms.

### Light use efficiency

Above-ground processes are largely dictated by photosynthesis and the conversion of solar energy to biomass. Photosynthetic light use efficiency (LUE) is defined as the amount of fixed carbon per unit of absorbed (or incident) photons. At low light intensity net $CO_2$ assimilation rate is linearly dependent on irradiance and LUE is maximal as well as constant. Crop productivity can be improved through enhancement of photosynthetic LUE (Long *et al*. 2006; Murchie *et al*. 2009). The strategies are divided into two non-mutually exclusive approaches: optimisation of light harvesting and optimisation of carbon assimilation. Improved light harvesting may be achieved by either modifying the biophysical and biochemical properties of photosynthesis on the leaf level (enhancing quantum efficiency of photosystem or minimising energy loss from photoprotective dissipation; (Long *et al*. 2006) or by modification of canopy architecture and light interception in the canopy (altered leaf angle distribution and canopy closure; Rascher *et al*. 2010*a*). Enhanced carbon assimilation may result from molecular engineering of Rubisco (Parry *et al*. 2003; Whitney *et al*. 2011), or introduction of $C_4$ traits into $C_3$ crops (Hibberd *et al*. 2008; Gowik and Westhoff 2011). Overall, the values of LUE of grain legumes are somewhat lower than in other crop plants, presumably because of relatively high energy costs required for symbiotic N fixation (Sinclair and Muchow 1999).

### Water use efficiency

Water use efficiency is a complex property that depends on a multitude of above-ground, below-ground and transport processes. In agronomy, water use efficiency (WUE) is defined as the ratio between dry matter accumulation (or yield) and the total amount of water spent by the crop over the same period (Tambussi *et al*. 2007). At a leaf level, WUE is defined as net $CO_2$ assimilation rate ($A$) divided by transpiration rate ($E$); WUE calculated in this way is also called 'instantaneous' WUE ($WUE_{A/E}$). A related parameter is the 'intrinsic' WUE defined as the ratio between $A$ and stomatal conductance ($g_s$) ($WUE_{A/gs}$; Osmond Björkman and Anderson 1980). Compared with $WUE_{A/E}$, $WUE_{A/gs}$ is less influenced by vapour pressure deficit (VPD) between leaf and air, rendering $WUE_{A/gs}$ a better parameter to characterise genotypic differences of leaf

**Table 1. Conceptual overview showing how better understanding or quantification of specific below-ground, above-ground or transport traits will can contribute to improve light-use-efficiency (LUE), water-use-efficiency (WUE) or nitrogen-use-efficiency (NUE)**

|  | Phenotype | Method/approach | Improvement of |
| --- | --- | --- | --- |
| Above ground | Energy conversion in light reaction | Fluorescence, imaging spectroscopy | LUE |
|  | 3-D canopy structure | Stereo imaging | LUE |
| Transport/allocation | Sap flow | MRI, velocity radiotracers | WUE, NUE |
|  | Plant water status | NMR | WUE |
| Below ground | Root architecture | 2-D rhizotrons, 3-D MRI | NUE, WUE |

processes (Gilbert *et al.* 2011). At the plant scale, root architecture and function greatly determine foraging and exploration of water resources below-ground. Recent studies point towards a high importance of root growth and angles for water uptake (Lynch and Jonathan 2007; Hammer *et al.* 2009) and, thus, are an important influence on whole-plant WUE.

### Nitrogen use efficiency

The nitrogen use efficiency (NUE) is determined mainly by below-ground and transport and allocation processes. In agriculture, NUE is defined as a ratio of biomass production to input of nutrient fertiliser. NUE can be expressed as crop yield (biomass or crop harvest) per unit of N available in the soil (Moll *et al.* 1982), dry matter produced per N content in the plant (Good *et al.* 2004), or the proportion of N taken up and metabolised by plants as a fraction of totally available N. Due to their ability to obtain N from symbiotic fixation of atmospheric $N_2$, legumes can accumulate large amounts of N in non-fertilised soil; for example, in well watered conditions ~65 and 85% of the total N content of cowpea and soybean plants, respectively, can be attributed to N fixation (Sinclair *et al.* 1987).

The main source of N changes for plants between the vegetative phase and the reproductive phase (Hirel *et al.* 2007). Young vegetative plants rely on N uptake from the soil or N-fixing symbionts. N remobilisation from the existing structures (e.g. Rubisco degradation) increases at later stages, generally after flowering. N fixation during seed filling varies among different species (Sinclair *et al.* 1987). Breeding strategies to improve NUE are, therefore, directed to optimisation of physiological or structural properties related to N uptake, assimilation or remobilisation (Hirel *et al.* 2007; Masclaux-Daubresse *et al.* 2010; Kant *et al.* 2011).

## Optimised resource use of above-ground energy conversion

In nature, photosynthesis operates under temporally highly fluctuating conditions with the photosynthetic apparatus adapted to an ever changing and highly variable stream of photons (Rascher and Nedbal 2006). Plants compete for above-ground space to support the light reactions of photosynthesis. As a consequence, plants may invest in foliage beyond the need to capture light with plant growth driving photosynthesis, rather the other way round (Körner 2011). In intensive agriculture, however, conditions are different. Nutrient limitation is often avoided and plants are planted in monocultures with high planting densities. Under these conditions, light energy may become a limiting factor suggesting improvement of photosynthetic efficiency may be a valid longer term focus for breeders and farmers (Long *et al.* 2006; Zhu *et al.* 2010). More specifically, it has been argued that three main processes may address improvements in photosynthetic efficiency: (i) waste of energy by non-photochemical dissipation may be optimised on the cellular level (Zhu *et al.* 2004); (ii) the balance between transpiration and $CO_2$ uptake may be engineered at the leaf level (with implications for plant growth under global change) (Long and Ort 2010); and (iii) the penetration and interception of light within the dense canopy of crops could be improved by optimised leaf, plant and canopy structure (Dermody *et al.* 2008; Rascher *et al.* 2010).

### High-throughput screening for optimised photosynthesis by automated fluorescence techniques

Rapid and non-invasive measurements of photosynthetic parameters are essential in screening for plants with optimised photosynthetic capacity and may facilitate the selection process in plant breeding. Chlorophyll fluorescence measurements are highly attractive because they allow fast and non-invasive determination of relevant photosynthetic parameters such as quantum yield, electron transport rate, or non-photochemical quenching (Maxwell and Johnson 2000; Baker 2008).

GROWSCREEN FLUORO is an automated screening device that combines a fluorescence imager (Maxi PAM, Walz GmbH, Germany) with computer controlled moving stages and automated data analyses routines for high-throughput measurement of relevant photosynthetic parameters. This set-up enables an automated screening of the model species *Arabidopsis thaliana* (L.) Heynh. at a throughput of 60 plants per hour (Fig. 1; Jansen *et al.* 2009). Moreover, projected leaf area and morphological parameters for describing the shoot architecture can be extracted automatically from the chlorophyll fluorescence images (Fig. 1).

Simultaneous measurement of photosynthesis, growth and morphology delivers multiple phenotypic data and helps in understanding how environmental factors modulate the plant phenotype. By acquiring time courses of phenotypic factors (for example, see Fig. 1 for growth and quantum yield), we can investigate how and when differences between plants start developing. Concomitantly, the considerable throughput of 60 plants per hour enables comparison of populations (Fig. 1), for example, for the detection of mutants that react differently to given environments. GROWSCREEN FLUORO analyses can reveal influences of mutations, transgenes or environmental factors on diverse phenotypic properties of the plants. Such high-throughput phenotyping facilitates functional genomics by linking phenotypes to given genotypes to help select plants with enhanced resource use efficiency or stress tolerance. Whereas GROWSCREEN FLUORO itself was developed for the model species *A. thaliana*, the system can be adapted to a range of canopy architectures across a diversity of crop plants.

### Mapping crops by imaging spectroscopy

Imaging spectroscopy describes a novel discipline within optical remote sensing and is driven by the rapid development in sensor technologies. Until recently, imaging techniques relied on the use of a few spectral bands. Nowadays it is technically feasible to measure a continuous spectrum of light using imaging sensors (see Ustin *et al.* 2004; Rascher *et al.* 2007). How can this technology be applied to plant tissues? In brief, solar radiation that interacts with plant tissues or plant canopies is reflected, absorbed or transmitted. Spectral characteristics of the three components at leaf or canopy scale are a function of the optical properties of leaves, stems and other canopy components, canopy architecture and effects such as observation geometry. Imaging spectroscopy focuses on the reflected part of radiation to derive information about the biochemical and structural
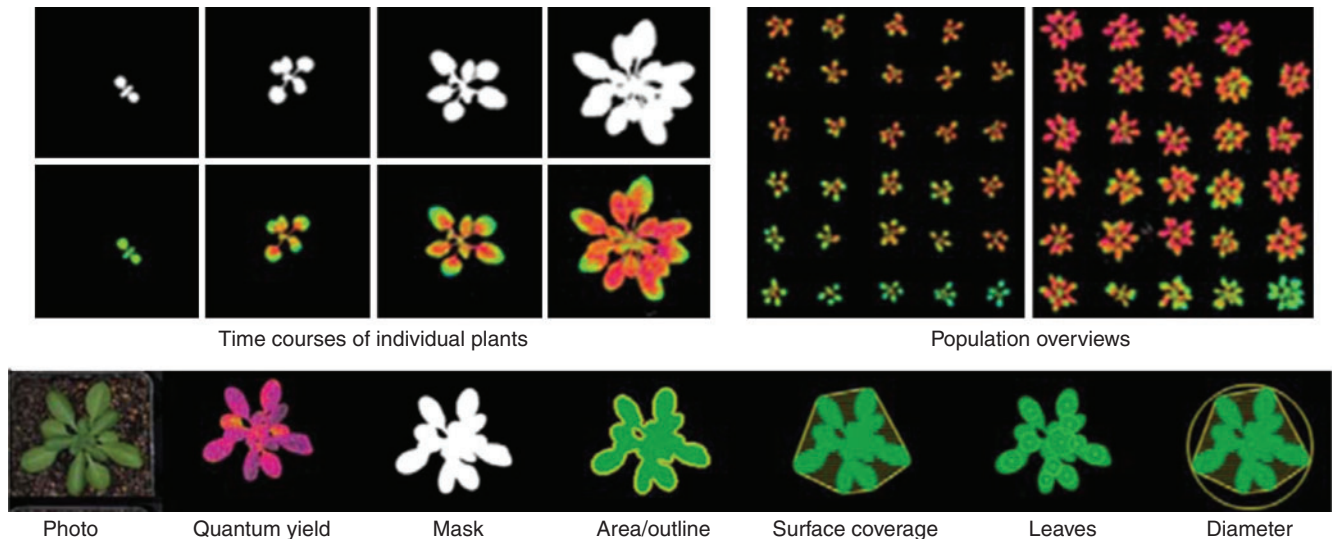
**Fig. 1.**  High-throughput automated screening of photosynthesis and growth using GROWSCREEN FLUORO. Illustration of representative phenotypic parameters measured with GROWSCREEN FLUORO: time courses of projected leaf area and quantum yield of an individual *Arabidopsis thaliana* plant; colour-coded overview of quantum yield of a population at two different growth stages; phenotypic parameters, such as quantum yield, projected leaf area, area-to-outline-ratio, surface coverage, leaf count and rosette diameter.

properties of plants at leaf and canopy level. For instance, the low reflectance of plant leaves in the visible (400–700 nm) range of the light spectrum results from the strong absorbance of the photosynthetic foliar pigments, whereas the high reflectance in the near infrared (700–1100 nm) is due to low absorption of light by the internal leaf mesophyll tissues. Reflectance in the shortwave infrared (1100–2500 nm) is strongly affected by the amount of water in plant tissues (Curran 1989; Rascher *et al.* 2010*b*).

During the phenological cycle, and in response to environmental conditions, the biochemical components of plants and canopies change, resulting in a changing interaction with solar radiation. Using knowledge of the specific absorption and reflection properties of these components, one can characterise single leaf and canopy components from the reflected light. For practical reasons, vegetation sciences often concentrate on a few relevant spectral regions and often use normalised difference indices, such as the normalised difference vegetation index (NDVI) that is correlated with the chlorophyll content and leaf area index (LAI). However, methods in spectral feature extraction have greatly improved and it can be expected that advanced quantification methods will be used in plant sciences in the near future. For example, principle component analyses in combination with decision tree algorithms can be used for early detection of leaf rust (Franke *et al.* 2005) or inversion of leaf optical models can map invasive species (Asner and Vitousek 2005). Recently it even became feasible to extract sun-induced fluorescence from high-performance imaging spectroscopy data which will open a new path of remote sensing of plant functionality (Malenovsky *et al.* 2009; Meroni *et al.* 2009; Rascher *et al.* 2009).

Imaging spectroscopy can be used for plant phenotyping in the field. The Soy-FACE (free-air carbon dioxide enrichment) facility provides an ideal test bed for such a case study as several varieties of soy bean cultivars are grown under elevated $CO_2$ and $O_3$ conditions (Rogers *et al.* 2004). Hyperspectral image cubes of the Soy-FACE facility of the University of Illinois, Urbana-Champaign, were acquired in July 2004 with the SOC-700 (Surface Optics Corp, San Diego, CA, USA). The instrument is a line scanner that can be operated from a wide range of distances and acquires 12 bit radiance images between 440 and 880 nm with ~4 nm spectral resolutions yielding hyperspectral cubes consisting of 120 spectral bands (for detailed description of the instrument see Rascher *et al.* 2007). Images were taken around mid-day from a height of ~20 m at an angle of ~45° using an elevated platform. About 10 single images were taken to cover the experimental setup. A reflectance standard (50% Spectralon, Labsphere, North Sutton, NH, USA) was positioned for each image and relative reflectances were calculated for each image. Single images were later registered to one another to provide a full image of the experiment. Reflectance images were filtered using the filtering procedure in 'principal component space' as described in detail by Rascher *et al.* (2007). From the filtered images, selected vegetation indices were calculated (formulae see legend in Fig. 2).

In Fig. 2, different maps were calculated from the hyperspectral reflectance data cubes. Fig. 2*a* shows a red, green blue (RGB) colour composite of the study site for visual orientation. Using the red and near-infrared spectrum of reflectance, the widely used NDVI can be calculated (Fig. 2*b*). Fig. 2*c* shows the photochemical reflectance index (PRI) that is reported to be related to the light use efficiency of photosynthesis (Gamon *et al.* 1992). Further indices are the carotenoid reflectance index (CRI) and the anthocyanin reflectance index (ACI) that measure the carotenoid and anthocyanin content, respectively (Gitelson *et al.* 2001, 2002) (Fig. 2*d, e*). Figure 2*f, g* shows the principal component bands 7 and 9 that were calculated according to the mean noise fraction
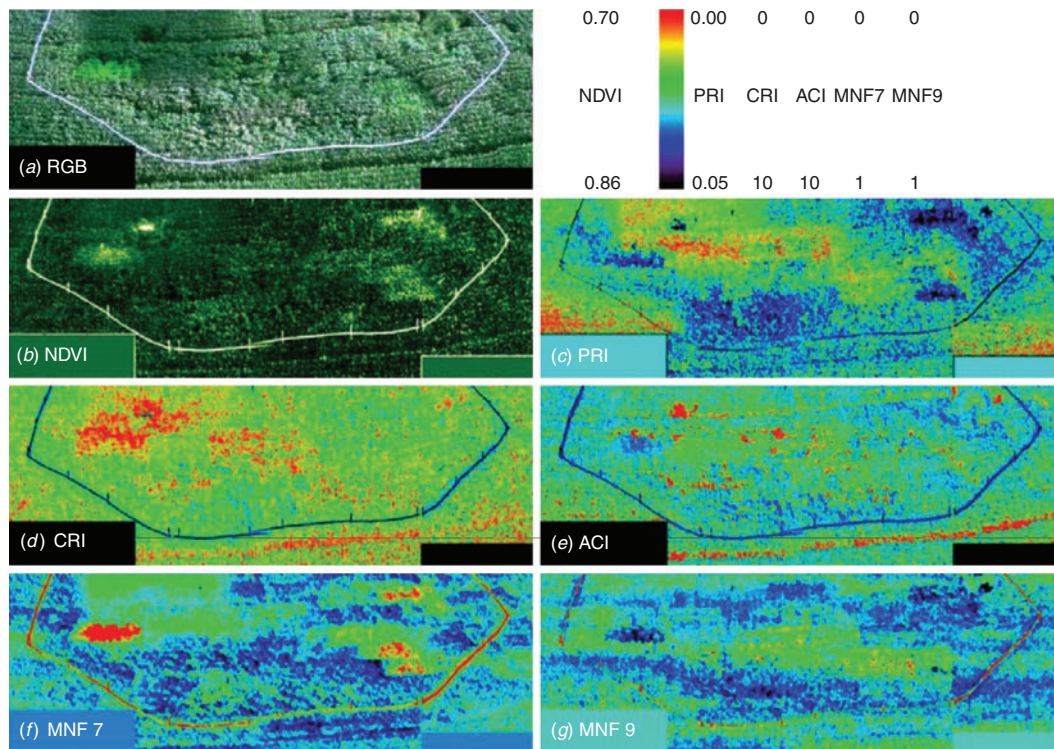
**Fig. 2.** Maps of different vegetation indices derived from hyperspectral images at the soy-FACE (free-air carbon dioxide enrichment) facility. Several hyperspectral image cubes were acquired from a cherry picker above the rings of soy-FACE (University of Illinois, Urbana-Champaign, IL, USA). Single pictures were geo-referenced and stitched to provide an overview of the facility. Different varieties of soy bean are planted within the rings and are tested for the reaction to elevated $CO_2$ and ozone. The different maps are derived using different spectral regions of the hyperspectral data cube, where spectral bands had a spectral resolution of app. 4 nm. For the red, green and blue (RGB) image the reflectance bands of 660, 550 and 440 nm are used for the red, green and blue channel of the image. The vegetation indices are calculated as $NDVI = R_{780}-R_{670}/R_{780}+R_{670}$, $PRI = R_{531}-R_{570}/R_{531}-R_{570}$, $CRI = 1/R_{510}-1/R_{550}$ and $ARI = 1/R_{550}-1/R_{700}$; where $R_x$ denotes for the reflectance at this specific wavelength. The MNF bands were calculated using the ENVI software package (Research Systems Inc., Boulder, CO, USA).

(MNF). MNF analyses rotate the spectral data cube according to their main information content and thus reveal subtle differences in the reflectance properties that are however hard to describe analytically (see Rascher *et al.* 2007 for details on this advanced approach).

In each map, the different variety of the soybean cultivars shows its specific properties and the cultivars can be distinguished from each other by their functional and structural characteristics. For separation, classification methods such as support vector machines can be used. Expanding this approach for different crop species is currently under way.

*Mapping of structure and function of natural canopies by hyperspectral, sun-induced fluorescence and stereo imaging techniques*

Canopies are complex, three dimensional (3-D) assemblages of leaves with various orientations and have an unpredictable surface structure. Light intensity and quality change quickly within natural canopies at different time scales exposing single leaf elements to a variable stream of photons (Frak *et al.* 2002; Yamashita *et al.* 2002). Dynamic acclimation of photosynthetic efficiency to these changes in environmental light conditions

are still not well understood (Rascher and Nedbal 2006) and novel approaches to measure canopy structure and function simultaneously are urgently needed.

*Canopy structure*

Spatial and temporal distribution of leaf angles are important indicators for the canopy function and plant state. Diurnal heliotropic leaf movements, which were described for 16 different plant families (Ehleringer and Forseth 1980), contribute to high LUE by optimising light distribution within the canopy over the course of day (Kao and Forseth 1992). Furthermore, under drought stress and N limitation, paraheliotropic leaf movement (i.e. direction of leaf lamina parallel to the sun's ray) enhances WUE and NUE (Kao and Forseth 1991). The canopy structure within plant populations is important to estimate the cultivation density avoiding, for instance, excessive neighbour competition. The separation and analysis of each of these levels as well as applications like the extraction of particular population parameters, e.g. height, LAI or the density of pods, can be addressed by further image processing of the 3-D stereos.

Over the past few years, computer stereo vision has been extensively studied in systems where 3-D information of objects in their environment is of crucial importance, e.g. in automatic processing of autonomous systems and robotics (e.g. van der Mark and Gavrila 2006; Olson *et al.* 2007). Taking advantage of these dramatic improvements in stereo vision, we developed a stereo approach that is specifically suited for the 3-D mapping of natural plant canopies in the field (Biskup *et al.* 2007, 2009). The use of two synchronised digital SLR cameras allows 3-D mapping that is robust against canopy movements and varying illumination and that permits the monitoring of plant architecture on different scales in the field. In a first case study, we were able to use this stereo system to quantify diurnal changes in leaf orientations in soybean. Highly resolved data on leaf angle distribution, together with forward modelling of sun movement and light penetration in a complex 3-D canopy, leads to a better understanding of the interplay of

structural and functional properties in radiation use in natural canopies (Rascher *et al.* 2010*a*).

### Canopy function

A robust approach to measure the light use efficiency of photosynthesis is the quantification of chlorophyll (Chl) a fluorescence (Maxwell and Johnson 2000; Baker 2008). However, in conventional fluorometry the photosynthetic apparatus needs to be excited actively, reducing applicability to large spatial scales under natural light. One active, scanning approach is LIFT, which was successfully used to measure fluorescence parameters and to detect stress response from a distance of 50 m (Ananyev *et al.* 2005; Kolber *et al.* 2005; Rascher and Pieruschka 2008; Pieruschka *et al.* 2010). Recently, the passive non-invasive detection of the sun-induced Chl fluorescence ($F_s$) retrieved from the spectral signature of the canopy by using the Fraunhofer line depth (FLD) principle, has become a promising approach to overcome this problem (Plascyk and Gabriel 1975; Moya *et al.* 2004; Liu *et al.* 2005; Alonso *et al.* 2008; Malenovsky *et al.* 2009; Meroni *et al.* 2009). There is experimental and theoretical evidence that $F_s$ can also be correlated with photosynthetic efficiency and the stress induced limitation of photosynthetic electron transport and thus may serve as a proxy to quantify photosynthetic LUE (Rosema *et al.* 1998; Flexas *et al.* 2000; Meroni and Colombo 2006; Damm *et al.* 2010). It has been established, for example, that at low light, when the non-photochemical protection mechanism is not activated, there is a negative correlation between the photochemistry and the $F_s$ signal (van der Tol *et al.* 2009).
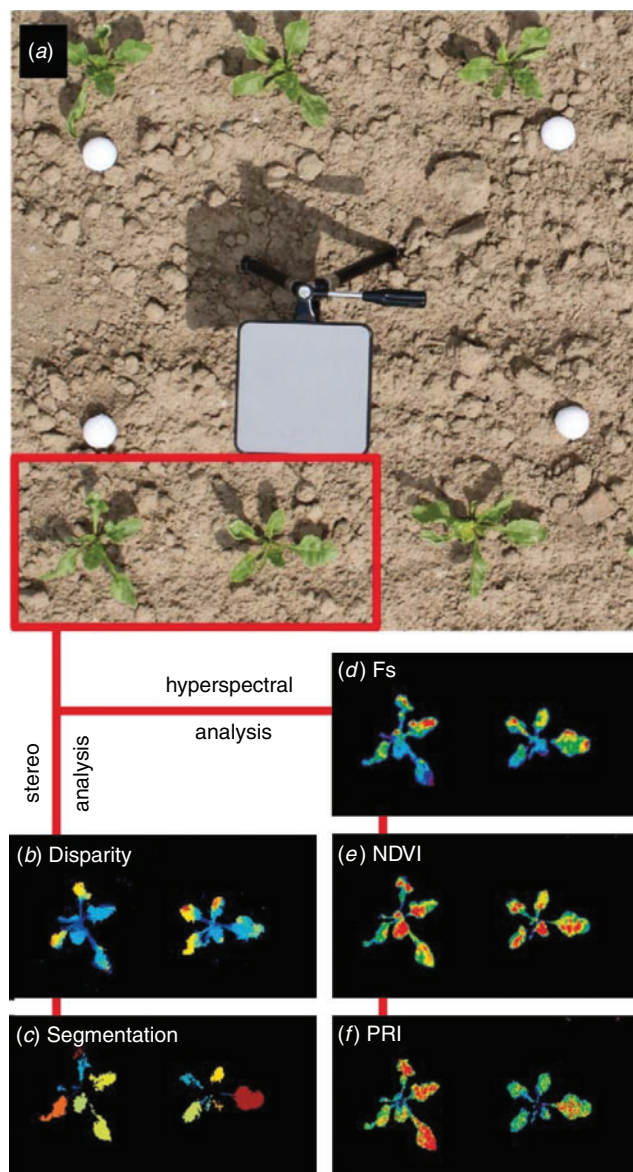
**Fig. 3.** Maps of co-registered images giving the 3-D structure and the spatial distribution of sun-induced fluorescence of young sugar beet plants in the field. (*a*) Original left stereo image from sugar beet (*Beta vulgaris* var. Pauletta); the white marker balls are used for co-registration, the reflection standard is used in hyperspectral imaging for the reflectance calculation. (*b*) Disparity map representing the depth information of each image pixel in (*a*); the disparity values are computed from the horizontal distance of corresponding points in both stereo images after target-calibrated rectification (modified after Bouguet 2005; for a general overview see, for example, Hartley and Zisserman 2004); the detection of corresponding points is based on a block method using normalised sum of squared differences (NSSD) matching metrics (for details on block methods see Brown *et al.* 2003). Noisy and non-plant image parts have been previously removed by occlusion (Fua 1993) and colour segmentation filters. (*c*) Leaf-segmented image displaying colour-coded single leaves detected with a graph-based segmentation method; this variant of the Felzenszwalb-Huttenlocher method uses image saturation, intensity and hue from (*a*) and 3-D information from (*b*) to separate image elements (Felzenszwalb and Huttenlocher 1998). (*d*) Sun-induced fluorescence ($F_s$) obtained using the 3-FLD method (Maier *et al.* 2003). The hyperspectral images were acquired with a PS V10E camera (SPECIM, Spectral Imaging Ltd, Oulu, Finland). This camera acquires spectral information for the range of 400 to 1000 nm and has a spectral sampling of 0.63 nm and a FWHM of 2.8 nm. The camera works as a push broom system and was mounted on a linear scanning bar fixed in a platform able to reach 4–8 m height. (*e*) Normalised difference vegetation index (NDVI) calculated as $NDVI = R_{780} - R_{670} / R_{780} + R_{670}$; were $R_x$ is the reflectance at the specific wavelength. (*f*) Photochemical reflectance index (PRI) calculated as $PRI = R_{531} - R_{570} / R_{531} - R_{570}$.

*Structure–function relation*

Figure 3 displays co-registered 3-D and hyperspectral images and further processing steps using an example of sugar beet (*Beta vulgaris* L. var. Pauletta). Two steps of processing stereo images (Fig. 3*a* only left stereo image shown) are depicted, including the 3-D reconstruction (Fig. 3*e* the disparity denotes the depth information) and the automatic selection of particular plant structures (Fig. 3*f* segmentation of single leaves). Further steps include the quantification of leaf angles with respect to species- and variety-specific leaf models and the derivation of light absorption properties.

In Fig. 4*d*, the spatial distribution of the sun-induced fluorescence signal ($F_s$) is presented. The interpretation of the $F_s$ signal might depend not only on the physiological status of the plants, but also on the light-leaf interaction determined by the orientation of the different canopy elements. Thus, the combination of $F_s$ with the 3-D parameterisation of this data offers a unique opportunity for a better understanding of the structural and functional changes of photosynthesis adjustments under changing light conditions within the canopy. Hyperspectral information is also used to obtain other physiological parameters commonly used in remote sensing. In Fig. 3*e* and 3*f* the NDVI
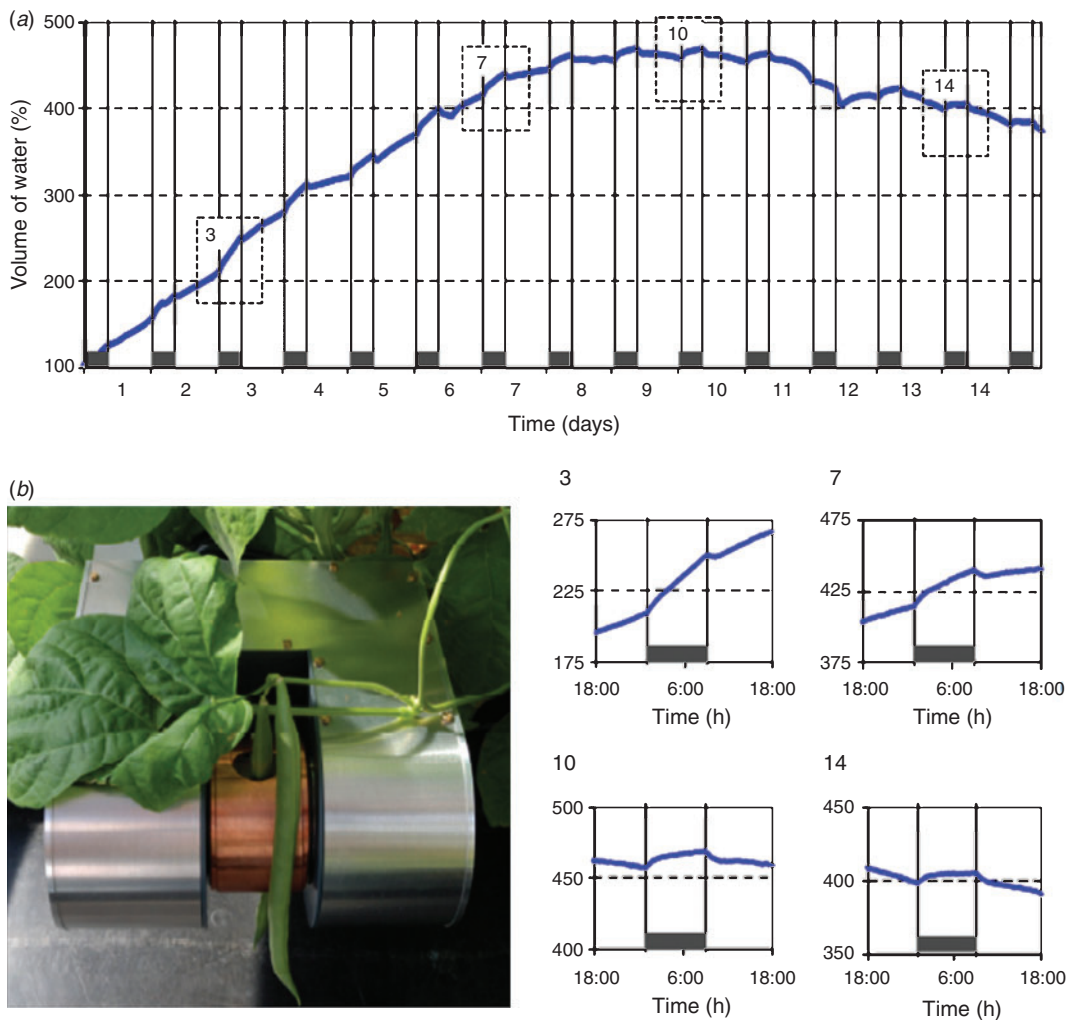


**Fig. 4.** (*a*) Quantifying shoot transport by portable nuclear magnetic resonance (NMR). Growth of a bean pod during a period of 2 weeks. Shown is the relative change in the volume of water in a 15 mm section in the middle of the bean pod, as placed within the coil of a custom built portable NMR scanner. The bean pod was 7 days old at the start of the measurement. Four day-night-day transitions are shown in detail, at night number 3, 7, 10 and 14 (indicated with dashed lines in the main graph). The night periods are indicated with dark grey bars. (*b*) Image of the C-shaped NMR magnet is shown, fitted with a sample holder containing a bean pod. During pod growth the environmental conditions were as follows (climate chamber): day 23°C, RH 65%, 300 µE, 16 h; night 23°C, 65% RH, 8 h. NMR hardware and settings: custom built portable C-shaped magnet, 10.35 MHz, air gap 30 mm, temperature kept constant at 25°C ± 0.1°C by means of an electronically controlled heater. The magnet was insulated to protect it against sudden temperature changes. For the RF coil a solenoid with an inner diameter of 15 mm and 13 windings was used. Magnet and coil were used in conjunction with a Magritek Kea 2 spectrometer with integrated RF amplifier. For each cycle a CPMG type measurement was run, with a repetition time of 7.5 s, 3000 echoes, eight complex points per echo, 32 averages and a spectral width of 100 KHz; total scan time for each point was 4 min.

and PRI are shown respectively. We are currently working to adapt these approaches to a variety of canopies and we expect that robust approaches for field phenotyping will be available soon.

## Transport and allocation processes

Allocation, transport and communication between above- and below-ground components have long been recognised as fundamental processes underpinning plant function. Traditional approaches are largely based upon invasive sampling of plant components with little regard for direct measures of resource flux. For most agricultural crops the final product is the seed or fruit. In agronomy there are several ways to relate crop biomass or carbon budget to seed yield through the 'harvest index'. However, increasing total biomass *per se* may not always result in higher final yield. It is intuitive that transport and allocation processes are a strong pre-determinant of yield.

### Visualising transport and growth processes by nuclear magnetic resonance techniques

The use of nuclear magnetic resonance (NMR) to monitor plant processes non-invasively can take several forms. For example, sap flow can be measured by magnetic resonance imaging (MRI) techniques without the need to spatially resolve the conducting tissue. MRI can also be used, even without imaging, to assess non-invasively the dynamics of growth and water status. In the latter case the technique is no longer called nuclear magnetic resonance imaging (NMRI), but simply magnetic resonance (MR) or, more formally, NMR. A positive side effect of needing no, or only low resolution imaging, is, that the NMR equipment can become simpler and smaller. This even makes it possible to use portable scanner-like instrumentation, suitable for the application in the field.

### Measuring sap flow using MRI velocimetry

An especially appealing application of MRI is sap flow imaging. Xylem and phloem sap flows are difficult to study because of their extreme sensitivity to invasive experimentation (Knoblauch *et al.* 2001). Consequently, little is known regarding the dynamics of sap flow in an intact plant. Considering the pivotal role of the xylem and phloem as all-connecting superhighways in plants' water and carbohydrate economy (Tyree and Ewers 1991; van Bel 2003), this is surprising. MRI flow imaging is uniquely suited to fill this methodological gap. It is non-invasive and does not require contrast agents or marker fluids; it merely records the displacement of water inside the plant.

Although the use of MRI velocimetry in the botanical sciences is still in its infancy, its potential in studying transport towards fruit has already been demonstrated. Recently it was used to quantify xylem and phloem translocation towards a growing tomato truss (Windt *et al.* 2009). Phloem and xylem translocation in the truss stalk were measured, with the objective to quantify their relative contributions. The results were surprising. It had been commonly believed that the overwhelming majority of water reaches the fruit by means of the phloem and that the xylem becomes dysfunctional at an early

stage of fruit development. The results of this study suggest the opposite, a finding that now has been confirmed by other studies (e.g. Hossain and Nonami 2010).

### Measuring growth and plant water status: the portable NMR sensor

One of the most basic applications of magnetic resonance and one that is most easy to implement in a portable NMR device, is to use it as a non-spatially resolved water sensor (Windt *et al.* 2011). The amplitude of the NMR signal is linearly and quantitatively related to the total amount of liquid water in the scanner. In plants such a simple measurement can be surprisingly useful. It provides a unique way to measure growth non-invasively and to assess dynamic changes in the plant water status.

For proof-of-concept we present an experiment in which a 1-week-old bean pod was inserted into a portable NMR setup (Fig. 4*b*) and allowed to grow for 2 weeks during an automated measurement sequence (Fig. 4*a*). It should be noted that during the experiment the bean pod not only grew in thickness, but also in length. It rapidly grew from a length of 9.5 cm at $t = 0$ to 20 cm after 2 weeks, whereas the measurement only took place in the centre 15 mm. Thus, the graph does not represent the growth of the whole pod, but shows only the growth of a 15 mm section near the pod centre. Additionally, the rapid expansion of the pod caused it to move slightly during the experiment, giving a shift around day 12. For this reason, we here focus on timeframes in the growth curve, in which lengthening of the pod can be assumed to be negligible (Fig. 4*a*, detail graphs of day-night-day transitions 3, 7, 10 and 14). Towards the end of the second week, the amount of water in the pod was slowly decreasing. The amount of dry matter at that point, however, was still increasing (data not shown).

The day-night-day graphs exhibit several interesting features. Throughout the growth period the bean pod water content was affected strongly by light–dark cycle and during the night the growth rate always was larger than during the day. Towards the end of week 2, the pod even began losing water during the day and exhibited growth rates close to zero at night. The pod is known to transpire, but that alone is unlikely to cause such large changes in pod water content. Therefore, we conclude that throughout the whole growth period the pod remained apoplastically connected to the rest of the plant, thus, instantly experiencing the same diel changes in water potential as the vegetative part of the plant.

### Quantifying transport processes with radiotracers

As described above, NMR techniques are suitable for studying water transport and giving structural information. Radiotracer technologies on the other hand help to better understand and quantify processes involved in long-distance transport and binding of specific compounds (e.g. recent photoassimilates). In particular, positron emitting radionuclides such as $^{11}C$ ($t_{1/2} = 20$ min) or $^{13}N$ ($t_{1/2} = 10$ min) are used for labelling C- or N-containing compounds, so that their spatial and temporal distribution in plants can be followed. The radioisotope $^{11}C$ is especially suitable for plant studies since it provides traceable photoassimilates when administered to a leaf as $^{11}CO_2$ in the light. Owing to the short half-life, $^{11}C$-experiments can be repeatedly

performed on individual plants to show both diurnal and longer-term changes, for example in the distribution of photoassimilates as a plant develops (e.g. pea, Jahnke *et al.* 1989; wheat, Roeb and Britz 1991). By combining MRI with PET technology, it is possible to reveal both structural traits and transport functionality of bulky organs such as sugar beets and roots hidden in soil (Jahnke *et al.* 2009). Phloem and xylem transport of other short-lived radionuclides can be measured as well, such as $^{13}$N (Kiyomiya *et al.* 2001; Ohtake *et al.* 2001; Gómez *et al.* 2010), $^{15}$O (Nakanishi *et al.* 2002; Ohya *et al.* 2008) or $^{52}$Fe (Tsukamoto *et al.* 2009). Quantitative information and physiological parameters about metabolite fluxes, however, require robust analysis that allows for both the tracer's short half-life and also its lack of equilibrium (e.g. Bühler *et al.* 2011).

## Optimised usage of below-ground resources

On a global scale 50% of living plant material is found below ground where plant roots compete for spatially and temporally distributed resources (Walter *et al.* 2009). Plant roots obtain heterogeneously distributed resources via directed root growth, local promotion of rhizospheric metabolism to liberate resources, adjustment of uptake mechanisms, formation of mycorrhizal associations and by sensing neighbours and distributing their roots accordingly. Root phenotyping clearly has great potential in advancing our knowledge to clarify functional root responses to the environment. For example, a root system with an increased root growth, branching rate and root hair production is one option to optimise the uptake of water and nutrients. This may lead to an increased yield production provided that an acceptable balance in resource allocation between root and shoot is ensured (Lynch and Jonathan 2007). Additionally, water and nutrient capture and therefore yield can also be strongly affected not only by enhanced root growth but also by modifications in root angles (Hammer *et al.* 2009). Due to their hidden nature, root systems are less explored than the above-ground plant parts and the relevance of

roots for enhancing yield seem to be underestimated as recent simulations show (Herder *et al.* 2010).

## *Quantifying the spatio-temporal dynamics of root growth and automated screening for optimised root system architecture*

Approaches for studying the dynamics of root penetration into soil with a high spatial and temporal resolution are essential to achieve the goal of optimised resource use efficiency. Root growth takes place only at the root tip within a zone of a few millimetres in length. The permanent production and stream of new cells through the growth zone leads to a spatial distribution of cell expansion growth along the root tip (Silk 1992; Walter and Schurr 2005). The cellular expansion rate and, therefore, the temporal and spatial distribution of root growth are strongly affected by changes of environmental conditions, like temperature, water and nutrient availability, but also by light intensity or $CO_2$ concentration at the shoot level (e.g. Muller *et al.* 1998; Sharp *et al.* 1988; Walter *et al.* 2002; Nagel *et al.* 2009; Walter *et al.* 2009). In order to quantify root growth adaptations we have developed a method with a high spatial (μm) and temporal (minutes) resolution based on digital image sequence processing (GROW MAP; Schmundt *et al.* 1998). The cell expansion growth is calculated from sequences of grey value images of root tips captured by digital cameras. Plants can be grown in conditions in which root tips are visible and accessible for the cameras, like in transparent agarose gels (Nagel *et al.* 2006), hydroponics (Walter *et al.* 2002), growth pouches (Hund *et al.* 2009) or in transparent rhizoboxes (Watt *et al.* 2006). In the acquired image sequences, cellular growth results in changes of local grey value structures. The first step of the analysis tool is the calculation of displacement vector fields for each image by optical flow. The resulting velocity vector fields are interpolated to fill in missing information and local growth rates are calculated by taking the divergence of the velocities of neighbouring pixels
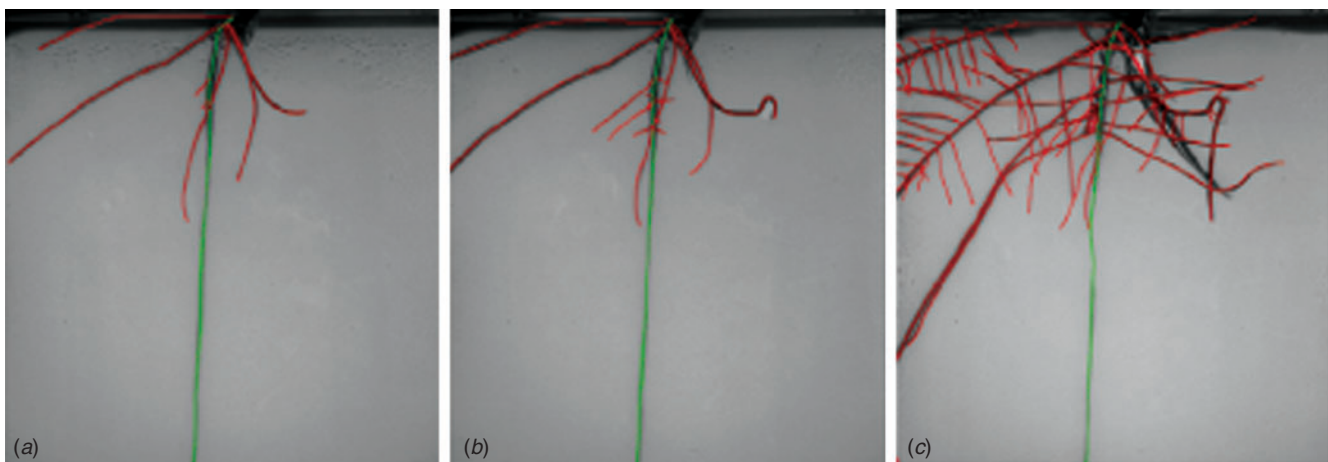


**Fig. 5.** Phenotyping the root system architecture using automated image-based analysis. Dynamic establishment of a *Phaseolus vulgaris* root system grown in transparent nutrient-agar, shown at (*a*) 6, (*b*) 7 and (*c*) 10 days after sowing. Plants were germinated on filter paper for 3 days and then transferred to Petri dishes (12 × 12 cm) filled with nutrient agar. The roots were pushed slightly into the agar and the shoots grew outside the Petri dish (for more details see Nagel *et al.* 2006). Cultivation in such a system enables roots and shoots to be analysed separately. Images of the root systems were analysed with novel image-based software, GROWSCREEN-Root and depicted in false colour (main root in green and lateral roots in red).

(Schmundt *et al.* 1998; Scharr 2007). This method has been successfully used to analyse root tip growth of several species, such as tobacco and maize (Walter *et al.* 2002; Nagel *et al.* 2006) and can easily be adapted to bean roots. The approach is suitable to elucidate how growth and development are affected by environmental factors and to find key genes (e.g. by forward genetics) that are for example responsible for abiotic stress tolerance of bean plants.

Plant root structure and function are tightly linked to abiotic stress tolerance, water and nutrient use efficiency and yield (Walter *et al.* 2009). Therefore, high-throughput phenotyping to screen large numbers of plant varieties and lines for bean genotypes with more beneficial root system architectures will be a valuable tool for breeding programs. Recently, imaging technologies have been developed that allow quantifying important features of root systems automatically (e.g. Armengaud *et al.* 2009; Nagel *et al.* 2009; Le Bot *et al.* 2010). The root system architecture of agar-grown plants can for example be analysed with the novel image-based software, GROWSCREEN-Root (Mühlich *et al.* 2008; Nagel *et al.* 2009). The key element of the software is the extraction of a tree model for root systems. Image sequences of entire root systems are acquired with a high resolution CCD camera and the origin of main roots is detected automatically as a local root element which is defined as a straight line of a few pixels' length. Tracking the main root down to the root tip is automated by concatenating local root elements. Lateral roots branching from main roots axis are searched within a user-defined distance from the main root and tracked downwards to its tip in the same way as for the main root (for details see Mühlich *et al.* 2008; Nagel *et al.* 2009). This image-based software can quantify automatically the root system architecture by measuring root length and density, the distribution of roots within the substrate and branching rates (Fig. 5). In our example, we observed that 10 days after germination bean roots reached a total root length of ~1.5 m, a remarkable root growth potential highlighting the importance of detailed root growth studies even if limited to 2-D imaging methodologies. Such novel approaches when scaled to a desired throughput, will prove valuable in dedicated screening of bean root systems with optimised resource use efficiency.

## Mapping the three-dimensional distribution of roots using MRI

In the sections above root growth was experimentally restricted to two dimensions allowing for straightforward investigation of basic root development. The spatial configuration of the developing root system, the root architecture, however, is of critical importance for plant performance, for example in soil resource acquisition by determining the extent of resource foraging in distinct soil domains (Lynch and Jonathan 2007). Currently, we can pre-select genotypes (based on their root-structure in specific environments) from large populations using high-throughput methods such as rhizotrons but for further and more detailed studies on root development and architecture imaging of roots in natural soil is required.

Root structures often lack symmetries and are dispersed in the opaque soil (Gregory 2006) posing considerable methodological problems. Modern 3-D imaging techniques such as magnetic
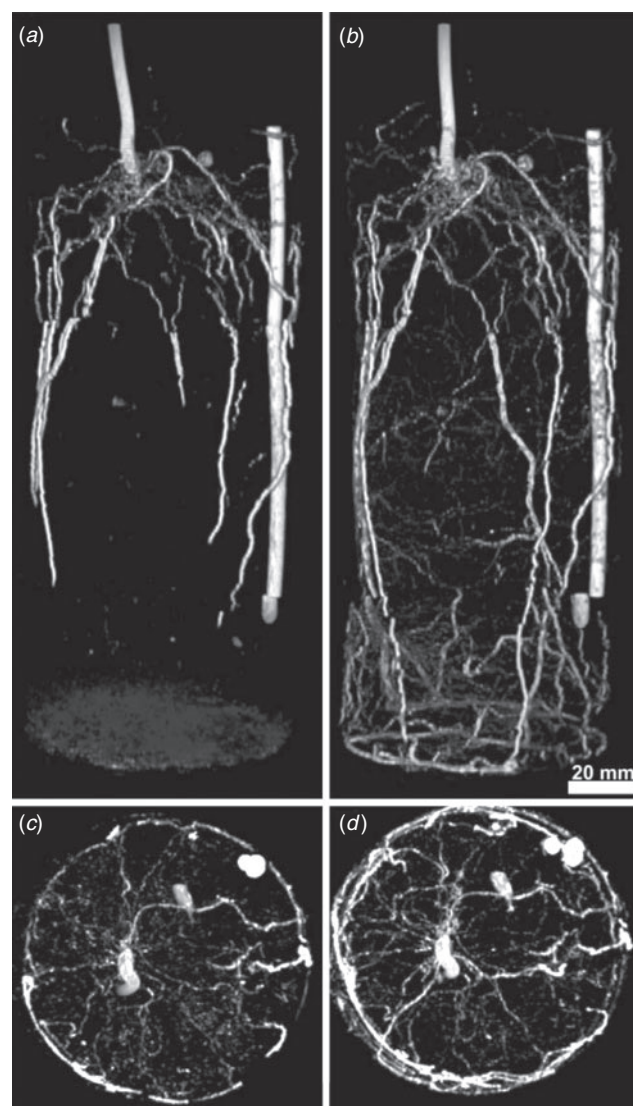


**Fig. 6.** Hypocotyl and major root system of a common bean plant (*Phaseolus vulgaris* L. cv. Fardenlosa shiny) in soil imaged with magnetic resonance imaging (MRI). 3-D MRI images were taken (*a*) 14 and (*b*) 21 days after sowing with a water-filled reference glass capillary on the right-hand side. Corresponding two-dimensional projections in the axial plane of (*a*, *c*) and of (*b*, *d*) are displayed. (*a*) After 14 days the major roots (tap and basal) have reached a depth of 10–15 cm and can be individually followed from their origin down into the soil volume. The same roots have elongated much over the following week. (*b*) Many roots descended along the inner diameter of the container and in a curvilinear path. The disc shaped structure visible towards the bottom of (*a*) originates from high soil water content at the bottom of the container. Plants were grown as described in (Jahnke *et al.* 2009) in a tube-shaped container of 9 cm diameter and 25 cm depth, filled with 1 : 2 v/v mixture of agricultural soil from an experimental site near Kaldenkirchen, Germany and coarse sand. The MRI measurements were performed on a vertical 4.7 T magnet with a 30 cm bore. The MRI spectrometer consisted of a 205 mm gradient coil (gradient strength up to 300 mT m$^{-1}$), a 100 mm birdcage radio frequency coil and a VNMRS console (Varian, Palo Alto, CA, USA). The 3-D images were build from three blocks of 90 slices each 1 mm thick (pixel dimensions $400 \times 400 \times 1000\,\mu$m), generated using a spin echo multiple slice (SEMS) sequence (Haacke *et al.* 1999) with a 9 ms echo time (TE) and a 3 s repetition time (TR) minimising the effects of the relaxation processes $T_2$ and $T_1$ respectively (total measurement time: 51 min).

resonance imaging (MRI) and computerised X-ray tomography (CT) offer solutions for basic research and phenotyping alike. Well known for its biomedical applications, MRI has occasionally also been used in plant sciences for studies ranging from imaging of fruits and shoots for quality and anatomical structure (Kuchenbrod *et al.* 1995; Kockenberger *et al.* 2004; Van As *et al.* 2009) to functional measurements of water status and flow in plant organs (Köckenberger 2001; Windt *et al.* 2006; Van As 2007). Several excellent textbooks describe the general principles of MRI (e.g. Haacke *et al.* 1999). Briefly, MRI is a volumetric imaging technique that enables detailed 3-D imaging of proton containing substances like water, but for which many substrates, including several soil types, are transparent. Roots in water containing soil can also be imaged since, due to

different spatial positions of water relative to the soil particles which modulate the MRI-signal, different bodies of water (i.e. water in the soil and in plant roots) thus can be separated in MRI data.

Few studies have investigated plant roots in (large) soil volumes, most likely because of the problem of local distortions of the magnetic field due to the presence of ferromagnetic particles. Nevertheless, early in MRI-history, Bottomley *et al.* (1986) demonstrated the possibility of imaging roots of seedlings and young plants of *Vicia faba* L. potted in various substrates. With modern instruments and data analysis tools, we have now set up a dedicated laboratory for MRI on plants with a focus on root research and phenotyping. The customised Varian MRI-system (Palo Alto, CA, USA) used in our
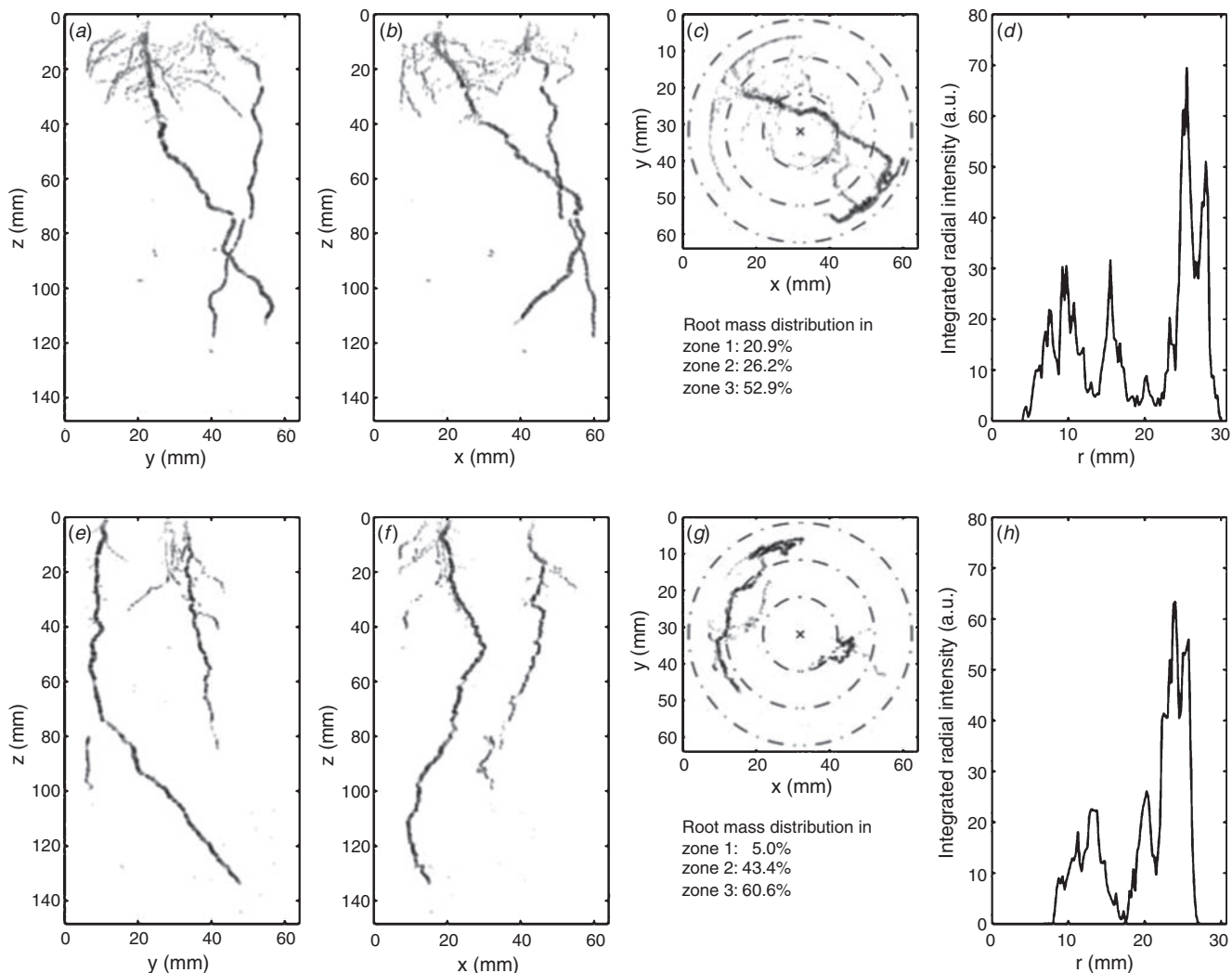


**Fig. 7.** Root distribution of plants grown with a neighbour of the same species (conspecific) or a heterospecific neighbour. Roots systems (14 days after transplanting seedlings into pots) showing either two conspecific individuals (*a–c*): maize with maize (*Zea mays*) or two heterospecific individuals (*d–f*) maize with *Onobrychis viciifolia* agg., a non-nodulated legume species (the individual with the shorter root system). (*a*, *b*, *d*, *e*) Two different 3-D side views, (*c*, *f*) a 2-D top view (projection in the axial plane) with specification of three concentric rooting zones of the root systems. The 3-D images clearly show the different types of root systems of these two species and in this particular case, how the root systems of the individuals can either overlap or not. Plants were pre-germinated and seedlings transplanted into containers (7 cm diameter, 20 cm depth). magnetic resonance imaging (MRI) measurements were made daily over a period of 2 weeks with the same magnet and radio frequency coil in the spectrometer as in the study described in Fig. 6.

example on the root system of common bean plants (*Phaseolus vulgaris* L. cv. Fardenlosa shiny; Fig. 6) features a vertical bore 4.7 Tesla (T) superconducting magnet that can accommodate plants/pots up to a maximum of 17 cm diameter and 2 m length. Figure 3 shows 3-D images of a whole-root system and the lowest part of the hypocotyl of the same individual at an age of 14 (Fig. 6*a*) and 21 days (Fig. 6*b*). Image quality allows for a detailed characterisation of the root architecture, such as separating tap roots and basal roots and their individual distribution (Fig. 6). We estimate that currently roots down to a diameter of 300 µm could be imaged. After 21 days (Fig. 6*b*) roots tend to pack in the lower part of the cylinder, indicating that the container size may already be limiting for a relatively young plant. Projection of root distribution in the axial plane (Figs 6*c, d*) provides a simpler visualisation of the angular distribution of roots and their development.

Imaging the whole-root system allows extraction of different parameters from the same measurement session: number and length of different root types, branching angles, root mass and their spatial distribution. The current spatial resolution would be sufficient for studying development and distribution of root-nodules in legumes hosting $N_2$-fixing root symbionts (the plant shown here was not nodulated). The non-invasive nature of MRI allows the study of individual root (system) development and thereby also dynamic responses to stress and environmental conditions (including the vicinity of other plants, soil fauna and abiotic stress). This will enable us, for the first time, to follow the complex 3-D development of below-ground structures *in vivo* for gaining a mechanistic understanding of interactions between the root system and its abiotic and biotic environment.

In addition to root–soil interactions, pronounced plant-plant interactions between species can occur (e.g. de Kroon 2007; Bezemer *et al*. 2010). Results from grassland biodiversity experiments have shown positive effects of plant diversity on overall productivity and nutrient cycling (Roscher *et al*. 2011), mainly related to complementary use of overall resources between species with different resource foraging traits and to beneficial effects of $N_2$-fixing legume presence. We used MRI to map the development of root systems over time of two different plant individuals (either of the same species or of different species) compared with one individual (control). We addressed the hypothesis that growth with an interspecific neighbour will result in increased root growth away from that neighbour's roots (avoidance), vs a lesser effect with a conspecific neighbour. 3-D images allowed individual-specific observation of tap and lateral root development over time (Fig. 7). For a quantifiable inter-treatment comparison, the 3-D data cubes were translated into 2-D images and developed an algorithm for separating the diameter of the tube into three zones (Fig. 7*c, f*) for calculating the percentage of total root mass found in each zone. This now allows a comparison of the effects of interacting species identity on overall root distribution.

Thus, we believe that root MRI, especially when used with automated image analysis tools, will enhance the mechanistic understanding of development and architecture of the root system and its interaction with the soil and neighbouring plants. These methods provide us with tools for non-invasive, high-precision imaging of dynamics of the 'hidden half'. Insights from MRI studies will be of high ecological and economical value because

resources such as water and mineral nutrients are becoming increasingly limited in many soils and expensive in agriculture (Lynch and Jonathan 2007) and an understanding of the response of root development to biotic and abiotic factors can indicate how and where fossil fuel-based fertilisers could be complemented by positive plant–plant interactions and by 'green fertilisers' ($N_2$-fixing legumes) to supplement and sustain crop species development.

### The challenge of measuring root distribution in the field

Mapping root structure in the field remains an unsolved challenge. Root growth and root function in natural soils are known to be fundamentally different to root growth in pots, which stresses the importance of developing approaches to quantify root distribution and function in nature. To our knowledge, there is no method available at the moment that allows a non-invasive mapping of root systems in natural soils. Researchers still have to dig out soil cores and manually score root traits (Trachsel *et al*. 2011), which does not allow monitoring of root growth and development and does not address the 3-D structure. Additionally, fine roots that constitute the functionally most important component of the root system are destroyed by destructive measurements and there is no method available to directly assess the fine root system (Hendricks *et al*. 1993). One potentially useful approach may emerge from NMR technologies. For example, the oil industry spent substantial effort in the past decades to adapt so called inside-out NMR techniques for the exploration of oil wells (Brown *et al*. 2001). This technology was recently transferred to map water distribution and pore size in natural soils (Haber *et al*. 2010). Even though still far from a routine application, this approach may permit mapping the 3-D structure of roots in the field.

### Conclusions

Maximising yield per unit of resource use will remain a main focus of future plant improvement programs. Phenotyping methodologies that can feasibly be scaled up to achieve higher throughput (such as several of those presented here) will form an essential component of such programs, by integrating spatial and temporal patterns in plant development and function that are subject to dynamic resource limitations. Despite significant challenges to the development of such *in situ* techniques, interdisciplinary approaches provide great promise for the development of practical, usable tools to monitor above-ground, below-ground and transport processes.

Several non-invasive phenotyping sensors and protocols can be adapted to bean populations and breeding material. Quantifying root growth in relation to resource availability may help to understand how beans exploit below-ground nutrients and interact with their neighbours. Imaging spectroscopy will further our understanding of how leaves and shoots are affected by environmental constraints and of spatio-temporal stress responses of plants and canopies. Non-invasive and spatially resolved measurements of transport processes and resource allocation may be used to better understand and potentially guide transport between plant organs and pods. Finally, we stress that single high-throughput phenotyping pipelines (e.g. high-throughput estimation of shoot biomass)

are useful, but that only the combination of several phenotyping protocol 'chains' and at different scales (from semi-controlled environments to field) will significantly contribute to a deeper understanding of the dynamic processes in both individual plants and canopies.

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