doi: 10.17221/121/2015-CJGPB

Two-dimensional Root Phenotyping System Based on Root Growth on Black Filter Paper and Recirculation Micro-irrigation

Wutthida RATTANAPICHAI[1] and Karel KLEM[2]

[1]Department of Agrosystems and Bioclimatology, Mendel University in Brno, Brno, Czech Republic; [2]Global Change Research Institute CAS, Brno, Czech Republic

Abstract

Rattanapichai W., Klem K. (2016): Two-dimensional root phenotyping system based on root growth on black filter paper and recirculation micro-irrigation. Czech J. Genet. Plant Breed., 52: 64–70.

Plant root system architecture (RSA) has an important role in crop production, particularly for water and nutrient uptake under limiting conditions. In the last few years, several root phenotyping methods have been developed. Here we present a new technique which has been developed for non-destructive, inexpensive and high-throughput root growth studies and RSA analyses. To illustrate the potential applications, this method was tested in an experiment with nitrogen and phosphorous deficiencies in a nutrient solution, affecting RSA parameters of two spring barley varieties (Bojos and Barke). This technique is based on root growth on vertically positioned black filter paper (30 × 60 cm) placed between two black plastic (PVC-P) foils and micro-irrigation systems providing the recirculation of nutrient solution. The pre-germinated seeds were placed in the slit between two plastic bars which carry the filter paper and plastic sheets and fix the plant in the vertical position. This system allows easy repeated non-invasive access to roots for their measuring and sampling. Eighteen days after transplanting the root imaging was done using an RGB digital camera. To evaluate the root architecture parameters the "SmartRoot" software was used. The results revealed that the system is able to detect changes in RSA which are caused mainly by P deficiency (particularly changes in lateral root length and total root area). It can be concluded that this technique has a great potential for non-destructive root growth studies, RSA measurement and root sampling.

Keywords: image analysis; nutrient deficiency; root system architecture; spring barley

The root system is an important organ which is responsible for uptake of resources such as nutrients and water from belowground. Improvement of crop production in the last century was directly influenced by modifications in the geometry and function of root system architecture (Hammer *et al*. 2009). The root system architecture (RSA) is controlled by both genetic and environmental factors such as nutrient availability (Fageria 2012). RSA plays an important role in plant productivity, which stems from the fact that about one-third of the earth's land surface is arid and many soil resources are unevenly distributed (Lynch 1995). Therefore, understanding of the functions of roots and RSA in nutrient and water uptake is important for future crop improvement (Hund *et al*. 2009). Numerous methodologies of root measurement have enhanced our ability to visualise, quantify and conceptualise the root architecture and its relationship to plant productivity (Lynch 1995). Non-destructive measurements of root growth under laboratory conditions are carried out in rhizoboxes (Nagel *et al.* 2012) or rhizotrone facilities (Polomski & Kuhn 2002), however, the description of RSA is often done by imaging only the visible roots from the outer surface of the rhizotrone or rhizobox. Furthermore, several non-soil methods for evaluating roots have been developed, including transparent solid medium (Downie *et al*.

doi: 10.17221/121/2015-CJGPB

2012), agar-plate systems (Gregory *et al.* 2009), germination on paper known as "growth pouch" (Hund *et al.* 2009) and hydroponic system (Mathieu *et al.* 2015). However, these methodologies still had numerous limitations such as root size restrictions or laborious access and measurement of root system. One of the most promising non-soil systems for root phenotyping was recently developed on the basis of growing roots on vertically positioned germination paper closed between two acrylic sheets (Le Marié *et al.* 2014).

Nutrient availability has a profound impact on RSA by altering the number, length, angle, and diameter of roots and root hairs (Benjamin *et al.* 2013). When roots grow under phosphorus deficiency, roots exhibit a shallower architecture that results from the inhibition of primary root elongation and increase in lateral root formation (Williamson *et al.* 2001). In contrast, plant growth under nitrogen deficiency stimulates primary root and particularly lateral root elongation but not lateral root initiation (Linkohr *et al.* 2002; López-Bucio *et al.* 2003). Under severe nitrogen deficiency, the formation of lateral roots is almost completely absent (Krouk *et al.* 2010). These examples indicate that the difference in nutrient availability can affect RSA that depends upon the type and concentration of nutrients supplied.

Here we present a new technique which has been developed for non-destructive root growth studies and RSA measurement, based on plant growing on vertical black filter paper sheets and recirculation micro-irrigation system. To illustrate the potential applications, this method was tested in an experiment with phosphorus and nitrogen deficiencies affecting different parameters of RSA.

MATERIAL AND METHODS

Root phenotyping system. The phenotyping system (Figure 1) consisted of a plastic tub serving as a reservoir of nutrient solution, 18 growth units, and a micro-irrigation system. The basis of each growth unit was a sheet of black filter paper (type 551, Hahnemühle FineArt GmbH, Dassel, Germany) of dimensions 30 × 60 cm covered from both sides by 30 × 60 cm of black plastic (PVC-P) foil (Aquaplast 805, Fatra a.s., Napajedla, Czech Republic) of 0.5 mm in thickness. The sheet of filter paper and plastic foils were fixed on the top in a holder consisting of two plastic bars. A micro-irrigation channel and slit for the growth and fixation of plants were milled in the middle of the bars. The distance between growth slit and micro-irrigation channel was 3 cm. The recirculation of nutrient solution through the root system growing on filter paper was provided by 20 site drip irrigation system (grow in AG, Berlin, Germany). The pump of this system was placed on the bottom of the reservoir tub and the nutrient solution was fed by small tubes to the micro-irrigation channel of each growth unit. The nutrient solution flowed through the root system on filter paper by gravity and dripped back to the reservoir tub.

Growing conditions. The experiment on the effect of nitrogen and phosphorus deficiency on RSA was carried out in three large growth chambers FS-SI-4600 (Photon Systems Instruments, s.r.o., Brno, Czech Republic). The plants were grown for 18 days from transplantation in the 15 h day and 9 h night regime. The minimum temperature during night was set to 20°C and the maximum temperature during day to 30°C. The maximum relative air humidity during

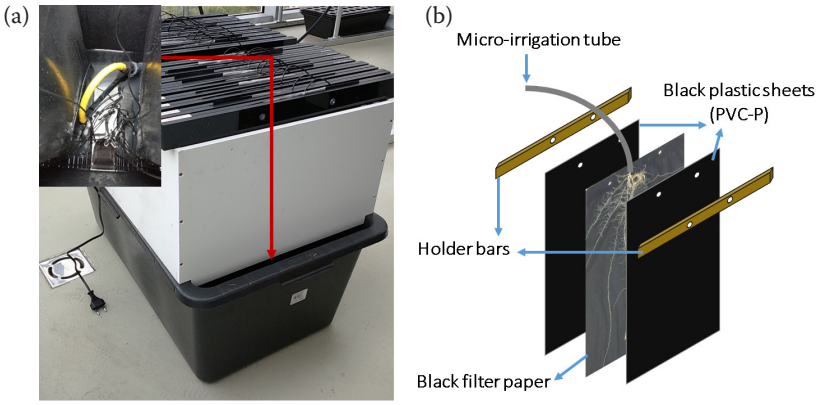

Figure 1. Root phenotyping system consisting of 18 growth units with a micro-irrigation system inside the reservoir tub (a) and detail of the growth unit consisting of two plastic holder bars, two sheets of black plastic foils (PVC-P), micro-irrigation tube fixed between holder bars and black filter paper as a substrate for root growth (b)

doi: 10.17221/121/2015-CJGPB

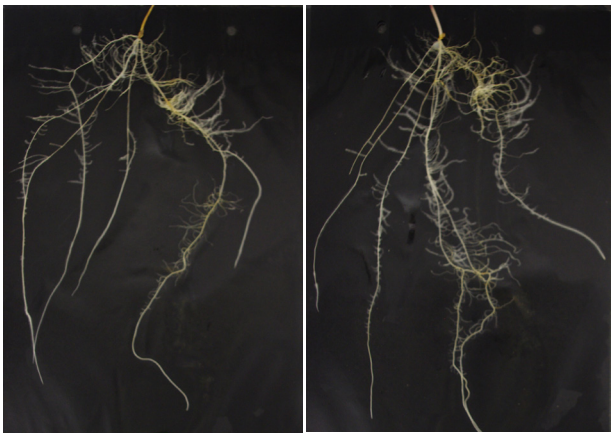

Figure 2. Examples of images of roots growing on black filter paper sheets

night was set to 80% and the minimum during night to 60%. The maximum intensity of photosynthetically active radiation was 680 µmol/m^2/s. The night values were kept constant for 9 h, while the daily maximum was kept for 3 h and the values gradually changed (for 6 h in the morning and 6 h in the afternoon from and to the night values).

Plant material and experimental design. The experiment was carried out on two barley varieties (Barke and Bojos). Seeds of both varieties were provided by the barley gene bank of the Agricultural Research Institute Kroměříž Ltd., Czech Republic. Seeds were germinated on moistened germination paper for 48 h at 26°C in the dark. Germinating seeds were then transplanted to the growth unit and these were subsequently placed in growth chambers for 18 days. The plants were subjected to three nutrition treatments provided by Knop's hydroponic solutions: complete nutrient solution (control), nitrogen deficiency (N–) and phosphorus deficiency (P–). The composition of nutrient solutions is shown in Table 1. The pH of all nutrient solutions was between 5 and 6 and pH was controlled every 2 days. Nutrient solution was replaced every 7 days of the experiment. Each combination of treatments (variety × nutrients) was replicated three times and completely randomized within each phenotyping system (variety) and randomized in time (every 7 days) among growth chambers.

Table 1. Composition of Knop's nutrient solutions for complete nutrient treatment (control), nitrogen deficiency treatment and phosphorus deficiency treatment

Solution		Control	N deficiency	P deficiency
Composition	(g)			
$Ca(NO_3)_2$	1	√	–	√
$MgSO_4 \cdot 7H_2O$	0.25	√	√	√
$FeCl_3 \cdot 6H_2O$	0.01	√	√	√
KH_2PO_4	0.25	√	√	–
KCl	0.125	√	√	√
$CaCl_2$	0.03	–	√	–
Total volume (ml)		1000	1000	1000

Image acquisition and analysis. The root images (Figure 2) were taken with a Pentax K-x digital SLR camera (Ricoh Imaging Company, Ltd., Tokyo, Japan) fixed on a tripod from a distance of approximately 60 cm. The sensitivity ISO-800, aperture size F4 and shutter speed 1/10 second were used. The resolution of the images was 6500 pixels per cm^2. Subsequently, the root architecture parameters were evaluated by the SmartRoot freeware (Université Catholique de Louvain, Belgium, Lobet *et al*. 2011) based on ImageJ software. The data we obtained from software were seminal root (SR) length, lateral root (LR) length, lateral root density, total root area and mean root diameter.

The effects of barley genotype and nutrition treatment were analysed by two-way analysis of variance (ANOVA) using the SPSS 11.5 statistical software (SPSS Inc., Champain, USA).

RESULTS AND DISCUSSION

The main objective of this study was to verify the functionality of a newly developed root phenotyping system for evaluation of differences in RSA within the experiment with two spring barley genotypes and nitrogen and phosphorus deficiency. This root phenotyping system enables repeated, non-invasive imaging of RSA during plant growth. Moreover, this system provides recirculation of nutrient solution, enabling its regular monitoring and rapid exchange. The system represents a combination of hydroponic cultivation with growth on 2D medium (filter paper), which allows easy scanning and evaluation of RSA simultaneously with the evaluation of impacts of environmental factors on the rhizosphere. In particular, it is possible to study the effects of nutrient deficit or excess, pH, osmotic potential, salinity, toxic substances and their mutual interactions. The system is thus especially suitable for evaluating dynamic changes in RSA of different genotypes in response to environmental conditions.

A similar system of so called "rhizoslides" was developed by Le Marié *et al.* (2014). Rhizoslides utilize

doi: 10.17221/121/2015-CJGPB

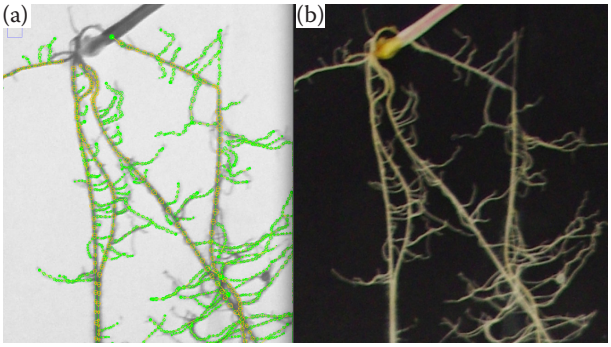

Figure 3. The root image after tracing by the SmartRoot software (a) compared with the original root image (b)

colour germination paper, and to provide a sufficient contrast between germination paper and roots the red spectral band is used. The main advantage of our system is the use of a recirculation micro-irrigation system which ensures a uniform flow of nutrient solution through the root zone, and thus the quality of nutrient solution in the proximity of roots is not affected by withdrawal of nutrients or root exudates. Recirculation of nutrient solution and a loose placement between two sheets of black plastic also ensures adequate air supply to the roots and does not restrict the root growth space, as may be in the case of rhizoslides. Additionally, the black filter paper presents a marked contrast to the roots, which simplifies an image analysis, whether the images were taken with digital SLR camera or flatbed scanner. The main advantage of rhizoslides is the ability to separate and evaluate the crown and seminal roots in maize (Le Marié *et al.* 2014). It is thus evident that each system has its advantages for certain specific applications.

Previously, the cultivation of roots on germination paper was used in the form of paper-roll setup (Watt *et al.* 2013) or growth pouches (Hund *et al.* 2009). These studies showed a good correlation with the length of the roots under field conditions in the early stages of growth, but not in the reproductive stage (Watt *et al.* 2013). The length of filter paper in our study was 60 cm, but in principle, an almost unlimited extension is possible as both black filter paper and PVC-P foil are available in rolls. The possibility to extend the length of the system together with uniform flow of nutrient solution down through the root zone potentially enables the monitoring of RSA development up to the generative phase.

Every root image was analysed using the root image analysis software SmartRoot (Figure 3). SmartRoot is the semi-automated image analysis software where an individual root-tracing algorithm is triggered by

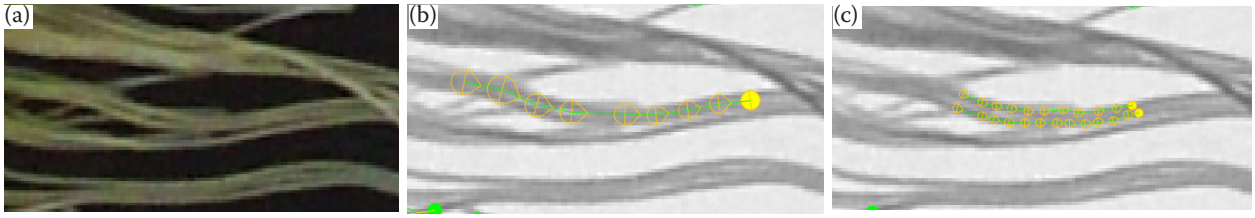

Figure 4. The original image of adjacent lateral roots (a), result of automatic tracing function (b) and result of manual tracing within the SmartRoot software (c)

Table 2. The mean square error and *F*-value from ANOVA test

Traits	Mean square error	*F*-value		Significance	
		varieties	nutrient deficiency	varieties	nutrient deficiency
Total SR length	277.285	0.866	0.387	0.370^{ns}	0.687^{ns}
Total LR length	0.464	2.319	33.250	0.154^{ns}	0.000^{**}
Total root area	43.783	3.931	61.927	0.071^{ns}	0.000^{**}
Lateral density	1.745	0.093	3.188	0.767^{ns}	0.081^{ns}
Mean diameter	0.000	1.600	42.100	0.230^{ns}	0.000^{**}
Root dry weight	0.001	1.250	2.120	0.286^{ns}	0.163^{ns}
Shoot dry weight	0.001	0.028	8.212	0.869^{ns}	0.006^{**}

$^{**}P < 0.01$; ns – not significant; SR – seminal root; LR – lateral root

doi: 10.17221/121/2015-CJGPB

a mouse click. It determines the centre (midline) of the root near the picked position and proceeds with the stepwise construction of a segmented line approximating the root midline, progressing forward and backward to the tip and base of the root. The algorithm estimates the root diameter at each node of the segmented line and uses this information to set the orientation of the segmented line (from the root base to the root tip) (Lobet *et al.* 2013). Root-tracing of seminal roots is very quick and precise, however the software needs a decision from the user for lateral root or thin root tracing. Therefore, the time consumption to analyse root images depends on the number of lateral roots and complexity of the root system. If the roots are adjacent, the software is not able to distinguish individual roots resulting in the overestimation of the node size. In this case it was necessary to manipulate these nodes manually (Figure 4). Therefore, the throughput of image processing using SmartRoot depends on the type of root system, the quality of the image, and the required information (Lobet *et al.* 2011).

As a proof of the concept, we analysed the effect of nutrient deficiency on root architecture (Table 2 and Figure 5). The result showed that there was no significant effect of spring barley variety on root traits in general. Moreover, there was no significant effect of either nutrient deficit treatment or variety on total SR length, LR density and root dry weight. The effect of nutrient deficiency was significantly

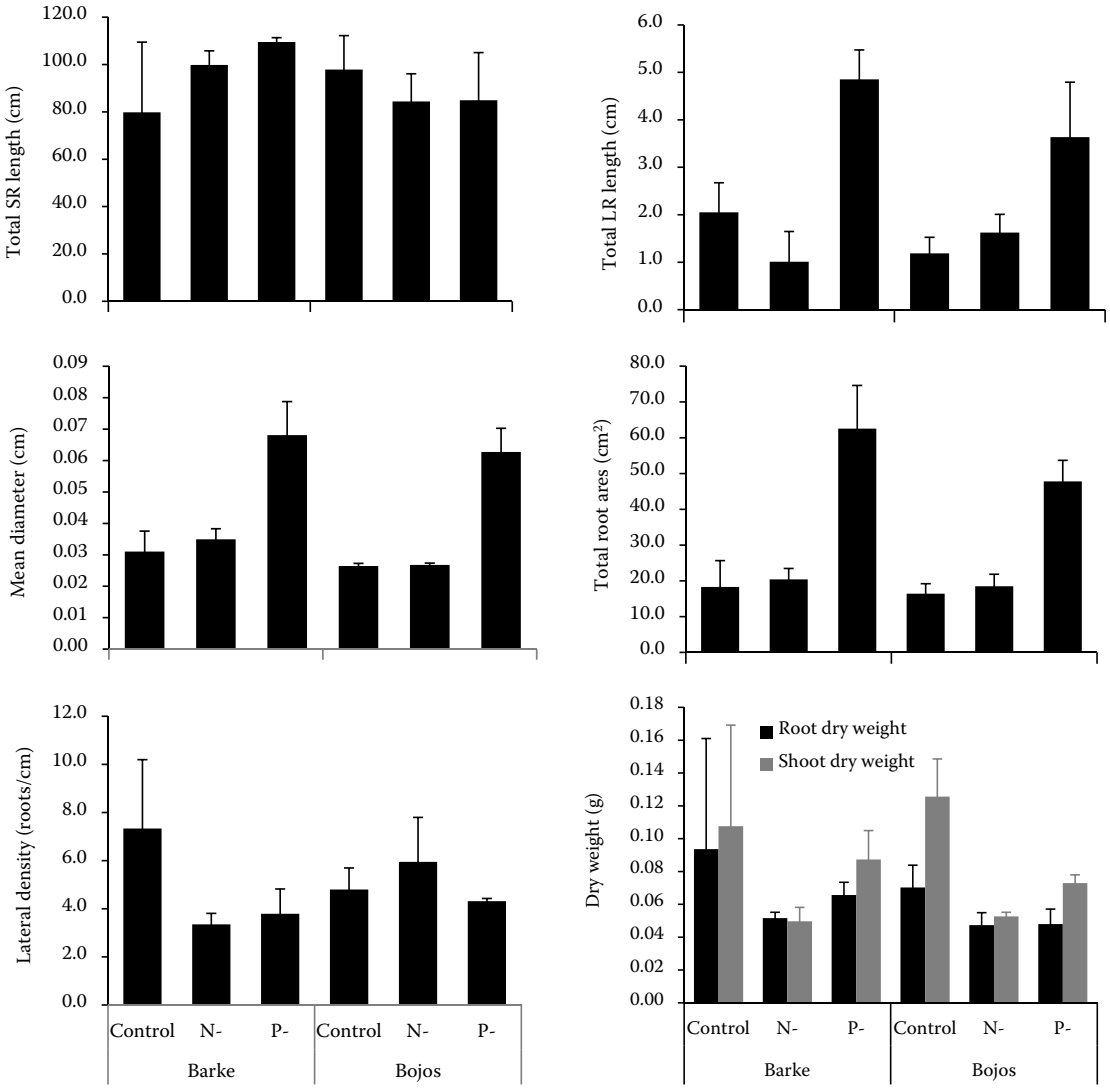

Figure 5. Effect of nutrient deficiencies on total seminal root (SR) length, total lateral root (LR) length, mean diameter, total root areas, lateral density and dry weight of spring barley (mean ± SD, n = 3)

doi: 10.17221/121/2015-CJGPB

reflected mainly in total LR length, total root area and mean diameter. P deficiency promoted particularly the total LR length and this effect was significant in both varieties (Figure 5). Williamson *et al.* (2001) presented evidence that lower concentrations of P favoured lateral root growth over primary root growth. López-Bucio *et al.* (2002) reported that the number of lateral roots was up to five times higher in plants grown at a limiting P concentration compared to an optimal concentration. Under low P concentrations the lateral roots arise in close proximity to each other and are densely covered by root hairs (reviewed by López-Bucio *et al.* 2003). On the other hand, no significant changes in parameters of RSA were observed under N deficiency. Zhang and Forde (1998) revealed that high nitrogen (nitrate) concentration reduces the lateral root elongation. This effect was observed in our study only in Bojos variety and this effect was also statistically non-significant. Conversely, Hansson and Andren (1987) reported improved production of lateral roots under higher nitrogen concentration. It is well known that roots tend to proliferate in nitrogen-enriched soil zones and to be thinner (Durieux *et al.* 1994). In our study the root length, density and diameter were less affected by N than the root dry weight. Baligar *et al.* (1998) explained differences in the root response to N by different stage of development.

There were no significant differences in root and shoot dry weight between varieties. Nutrient deficiency affected significantly shoot dry weight but not root dry weight. This effect was more pronounced in nitrogen deficiency. This effect led to an increase in the root to shoot ratio but only in Bojos variety. According to optimal partitioning theory the plants that encounter limited nutrient or water supply are expected to partition more biomass to their roots and less to their stems and leaves, and thus increase their root to shoot ratio (Ågren & Franklin 2003).

CONCLUSIONS

In this study, we presented the newly developed technique for root phenotyping, which is based on the root cultivation in a black filter paper and micro-irrigation recirculation system. This system allows the non-destructive analysis of the root system and thus provides an opportunity for evaluating the dynamics of changes during development. The advantage of the system is a possibility of monitoring and controlling the quality of nutrient solution in terms of nutrient content, pH, and osmotic potential. The system was tested on two spring barley genotypes and three nutrition treatments (control, N and P deficiency). RSA parameters were analysed using the SmartRoot software, wherein it was shown that the system is able to detect the changes in RSA which are caused mainly by P deficiency (particularly lateral root length and total root area).

Acknowledgements. This article was set up at Mendel University in Brno as a part of the project of Internal Grant Agency of Agronomy Faculty, Mendel University (IGA AF Mendelu) No. TP 7/2015 with the support of the Specific University Research Grant, provided by Ministry of Education, Youth and Sports of the Czech Republic in 2015. K. Klem was supported by Ministry of Education, Youth and Sports of the Czech Republic within NPUI, Project No. LO1415.

References

Ågren G.I., Franklin O. (2003): Root: shoot ratios, optimization and nitrogen productivity. Annals of Botany, 92: 795–800.

Baligar V.C., Fageria N.K., Elrashidi M.A. (1998): Toxicity and nutrient constraints on root growth. HortScience, 33: 960–965.

Benjamin D.G., Ricardo F.H.G., Swetlana F., von Nicolaus W. (2013): Plasticity of the *Arabidopsis* root system under nutrient deficiencies. Plant Physiology, 163: 161–179.

Downie H., Holden N., Otten W., Spiers A.J., Valentine T.A., Dupuy L.X. (2012): Transparent soil for imaging the rhizosphere. PLoS ONE, 7: e44276.

Durieux R.P., Kamprath E.J., Jackson W.A., Moll R.H. (1994): Root distribution of corn: the effect of nitrogen fertilization. Agronomy Journal, 86: 958–962.

Fageria N.K. (2012): The Role of Plant Roots in Crop Production. Boca Raton, CRC Press.

Gregory P.J., Bengough A.G., Grinev D., Schmidt S., Thomas W.T.B., Wojciechowski T., Young M. (2009): Root phenomics of crops: opportunities and challenges. Functional Plant Biology, 36: 922–929.

Hammer G.L., Dong Z., McLean G., Doherty A., Messina C., Schussler J., Zinselmeier C., Paszkiewicz S., Cooper M. (2009): Can changes in canopy and/or root system architecture explain historical maize yield trends in the US corn belt. Crop Science, 49: 299–312.

Hansson A.C., Andrén O. (1987): Root dynamics in barley, lucerne and meadow fescue investigated with a mini-rhizotron technique. Plant and Soil, 103: 33–38.

Hund A., Trachsel S., Stamp P. (2009): Growth of axile and lateral roots of maize: I development of a phenotying platform. Plant and Soil, 325: 335–349.

doi: 10.17221/121/2015-CJGPB

Krouk G., Lacombe B., Bielach A., Perrine-Walker F., Malinska K., Mounier E., Hoyerova K., Tillard P., Leon S., Ljung K., Zazimalova E., Benkova E., Nacry P., Gojon A. (2010): Nitrate- regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Development Cell, 18: 927–937.

Le Marié C., Kirchgessner N., Marschall D., Walter A., Hund A. (2014): Rhizoslides: paper-based growth system for non-destructive, high throughput phenotyping of root development by means of image analysis. Plant Methods, 10: 13.

Linkohr B.I., Williamson L.C., Fitter A.H., Leyser H.M.O. (2002): Nitrate and phosphate availability and distribution have different effects on root system architecture of *Arabidopsis*. The Plant Journal, 29: 751–760.

Lobet G., Pages L., Draye X. (2011): A novel image analysis toolbox enabling quantitative analysis of root system architecture. Plant Physiology, 157: 29–39.

Lobet G., Pages L., Draye X. (2013): Novel scanning procedure enabling the vectorization of entire rhizotron-grown root systems. Plant Methods, 9: 38.

López-Bucio J., Hernandez-Abreu E., Sanchez-Calderon L., Nieto-Jacobo M. F., Simpson J., Herrera-Estrella L. (2002): Phosphate availability alters architecture and causes changes in hormone sensitivity in the *Arabidopsis* root system. Plant Physiology, 129: 244–256.

López-Bucio J., Cruz-Ramírez A., Herrera-Estrella L. (2003): The role of nutrient availability in regulating root architecture. Current Opinion in Plant Biology, 6: 280–287.

Lynch J. (1995): Root architecture and plant productivity. Plant Physiology, 109: 7–13.

Mathieu L., Lobet G., Tocquin P., Perilleux C. (2015): "Rhizoponics" A novel hydroponic rhiztron for root system analyses on mature *Arabidopsis thaliana* plants. Plant Methods, 11: 3.

Nagel K.A., Putz A., Gilmer F., Heinz K., Fischbach A., Pfeifer J., Faget M., Blossfeld S., Ernst M., Dimaki C., Kastenholz B., Kleinert A.K., Galinski A., Sharr H., Fiorani F., Schurr U. (2012): GROWSCREEN-Rhizo is a novel phenotyping robot enabling simultaneous measurements of root and shoot growth for plants grown in soil-filled rhizotrons. Functional Plant Biology, 39: 891–904.

Polomski J., Kuhn N. (2002): Root research methods. In: Waisel Y., Eshel A., Beeckman T., Kafkafi U. (eds): Plant Roots: The Hidden Half. 3rd Ed. New York, Marcel Dekker, Inc.: 313–314.

Watt M., Moosavi S., Cunningham S.C., Kirkegaard J.A., Rebetzke G.J., Richards R.A. (2013): A rapid, controlled-environment seedling root screen for wheat correlates well with rooting depths at vegetative, but not reproductive, stages at two field sites. Annals of Botany, 112: 447–455.

Williamson L.C., Ribrioux S.P.C.P., Fitter A.H., Leyser H.M.O. (2001): Phosphate availability regulates root system architecture in *Arabidopsis*. Plant Physiology, 126: 875–882.

Zhang H., Forde B.G. (1998): An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. Science, 279: 407–409.

Received for publication August 31, 2015
Accepted after corrections June 14, 2016

Corresponding author:

Ing. Karel Klem, Ph.D., Ústav výzkumu globální změny AV ČR, v.v.i., Bělidla 986/4a, 603 00 Brno, Czech Republic; e-mail: klem.k@czechglobe.cz