Root2Res

Root phenotyping and genetic improvement for rotational crops resilient to environmental change

Deliverable 5.1 Development of a statistical evaluation scheme for assessing plasticity data and development of experimental systems for studying root plasticity

Prof. Doris Vetterlein, Helmholtz Centre for Environmental Research – UFZ

Dr. Jessica Clayton, Helmholtz Centre for Environmental Research – UFZ

Dr. Sebastian RGA Blaser, Helmholtz Centre for Environmental Research – UFZ

Funded by the European Union

UK Research and Innovation

Project funded by

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Economic Affairs, Education and Research EAER
State Secretariat for Education, Research and Innovation SERI

Root2Res has received funding from the European Union's Horizon Europe research and innovation programme under Grant Agreement No. 101060124. Its work is supported by Innovate UK through the Horizon Europe Guarantee scheme Grant Agreement No. 101060124 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) grant No. 23.00050.

Deliverable 5.1 includes Root2Res' definition of phenotypic plasticity, describes the derivation and selection of a plasticity index and the statistical procedure for its calculation. Furthermore, a graphical method is described that allows the integration of plasticity indices of different traits into one figure. In the second part the experimental setup is described, which allows for the collection of corresponding data under controlled conditions. The main focus is on the suitability of the setup for X-ray computed tomography (X-ray) analyses and their combination with transcriptome and metabolome analyses. With regard to the need for environmental conditions to be varied, the soil-specific derivation of water availability is described in detail. Practice abstracts will be produced related to part 1 (phenotypic plasticity index – definition, quantification, graphical presentation and interpretation) and part 2 (how to derive substrate specific water availability).

Deliverable Number	**Work Package / Task**
D 5.1	WP5 / T5.1
Lead Beneficiary	**Deliverable Author (S)**
UFZ	Doris Vetterlein Jessica Clayton Sebastian Blaser
Beneficiaries	**Deliverable Co-Author (S)**
BOKU	Eva Oburger
UVIGO	Adela M. Sánchez-Moreiras
IPSP-CNR	Raffaella Balestrini
Planned Delivery Date	**Actual Delivery Date**
31.08.2023	31.08.2023

Type of deliverable	R	Document, report (excluding periodic and final reports)	x
	DEM	Demonstrator, pilot, prototype, plan designs	
	DEC	Websites, patents filing, press & media actions, videos, etc.	
	DATA	Data sets, microdata, etc.	
	OTHER	Software, technical diagram, algorithms, models, etc.	x

Dissemination level	PU	Public, fully open, e.g. web (Deliverables flagged as public will be automatically published in CORDIS project's page))	
	SEN	Sensitive, limited under the conditions of the Grant Agreement	X

Root2Res has received funding from the European Union's Horizon Europe research and innovation programme under Grant Agreement No. 101060124. Its work is supported by Innovate UK through the Horizon Europe Guarantee scheme Grant Agreement No. 101060124 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) grant No. 23.00050.

INDEX

FIGURES

TABLES

1. An index of phenotypic plasticity – RDPI (Relative Distance Plasticity Index)

1.1. Definition of phenotypic plasticity

Phenotypic plasticity is the ability of an organism to alter its phenotype in response to the environment (Sultan 2000) and may involve changes in physiology, morphology, anatomy, development, growth, resource allocation, or mutualistic interactions with other organisms. It is a basic concept in genetics and evolutionary biology and care should be taken in deciphering between the genetic variation within a species resulting in phenotypic plasticity and the phenotypic plasticity of an individual genotype (Valladares et al. 2006 and citations therein). Roots are known to be organs that exhibit very high plasticity so that plants, as sessile organisms, can adapt to the unfavourable site conditions to which they are exposed in the place where their progeny germinate (Hodge 2006; Schneider and Lynch 2020). In an effort to make agricultural systems more resilient, this root characteristic is being considered in breeding programs and agricultural systems management for environmentally resilient crops. However, as agricultural production is (mostly) oriented towards above ground biomass, trade-offs between shoot and root growth should be avoided, *i.e.* root plasticity should increase not only the fitness of plants in general (Dewitt et al. 1998) but should also contribute to maintaining yields under variable conditions (Schneider and Lynch 2020). The concept of phenotypic plasticity should not be confounded with the attempt to define ideotypes which perform well under defined environmental conditions (Schneider and Lynch 2020). In an ideal world, phenotypic root plasticity would result in the particular ideotype for a particular environment when a particular environment is encountered.

To integrate root plasticity in breeding programs, first a measure for the quantification of the plasticity of individual root traits is required. A thorough review of different concepts of quantitative estimation of phenotypic plasticity is provided by Valladares et al. (2006). In the following, we will present some of these findings in more detail. In particular, we will show that the relative distance plasticity index (RDPI) is a useful and easy to use index for the quantification of the plasticity of root traits measured in different experimental setups (laboratory and field conditions), covering different environmental ranges, and not necessarily including a control under optimal conditions. We will provide the background for the choice, the theory and the R-script. In addition, we developed a graphical representation of the results which allows, at one glance, the evaluation of the plasticity of a range of traits.

1.2. Quantification of plasticity

Traditionally reaction norms of different traits can be investigated by directly comparing the response of the trait to an environment by calculating and comparing the slopes or coefficients of variation (Figure 1). However, these can become difficult to compare when reaction norms are non-linear or traits for comparison do not follow similar patterns of reaction norm (Arnold et al. 2019). The use of a plasticity index allows the standardisation of the effect of environment on a particular trait, and can allow for comparison between traits that don't follow the same pattern of reaction norm (within reason).

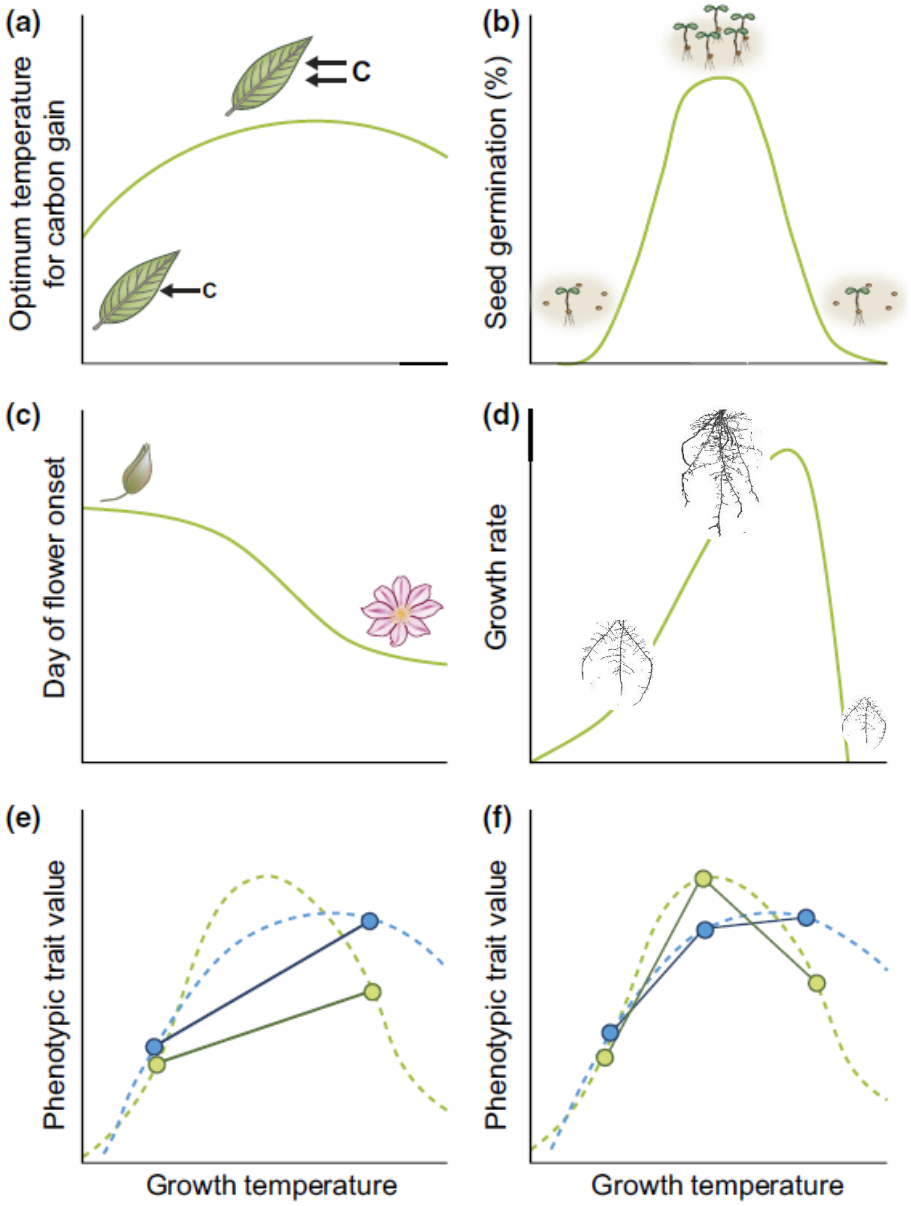


Figure 1: Typical non-linear reaction norm examples demonstrating the variety of shapes of plasticity in response to growth temperature: (a) shallow parabolic reaction norm shape of optimal temperature for carbon gain; (b) peaked response of seed germination percentage; (c) sigmoidal response of day of flowering onset; and (d) threshold response of growth rate of plant roots in response to temperature. (e) Describing reaction norms with only two points (solid lines and points) may miss fundamental and biologically meaningful aspects of the underlying reaction norm shape (dashed line). (f) Adding just one more point (a third environmental level) captures far more of the underlying reaction norm shape. Modified from Arnold et al. (2019)

Here we present a method to estimate plasticity of root and shoot traits using the relative distance plasticity index (RDPI) as described by Valladares et al. (2006).

The index is the average of the absolute pairwise distances between observations where environmental treatment differs. For a numerical trait of a given genotype/species x_{ij}, i denotes the individual observation and j denotes the environment treatment level. Observations from a different individual and different

environmental treatment are thus denoted by i' and j', respectively. The pairwise distance is then calculated as the absolute distance between different observations with differing environmental treatment level: $d_{ij \rightarrow i'j'} = |x_{ij} - x_{i'j'}|$. The distances are then standardised by the sum of the pair, which gives a value between 0 and 1.

Finally, the relative distance plasticity index (RDPI) can be calculated as:

$$RDPI = \frac{\sum \left(\frac{|d_{ij \rightarrow i'j'}|}{x_{ij} + x_{i'j'}} \right)}{n} \quad [1]$$

Where n is the total number of pairwise distances.

With RDPI, information regarding the effect of environment on the trait is conserved in the standardised relative distance between observations with differing levels of environmental treatment. The larger the relative differences, the greater the plasticity of the trait to the environment. As the RDPI takes values from 0-1, 0 indicates no plasticity and 1 indicates total plasticity.

Comparing the standardised pairwise distances between observations at differing factors of environmental treatment negates the need to evaluate and directly compare reaction norms. It is also not necessary to have a control treatment or optimum environmental treatment as reference. This is useful especially for traits where the optimum condition is not known or there is no control treatment. Furthermore, we have chosen this method because it does not require a linear reaction norm of the trait to the environmental treatment compared to commonly used indices such as phenotypic plasticity indices (Balaguer et al. 2001; Cheplick 1995; Navas and Garnier 2002) and measures of coefficient of variation (Schlichting and Levin 1984; Valladares et al. 2002).

With normal or near normal distributed relative distances, taking the mean as estimator of the plasticity index has the advantage that the standard error of the mean can also be evaluated. Valladares et al. (2006) advises that when the reaction norm is non-normal, the median of the relative distances should be taken instead of the mean. However, it is much more complicated and uncommon to estimate the error of the median, because no information of the distribution of the relative distances can be known *a priori*. Therefore, using the median as an estimator for plasticity conveys little or no information on the accuracy of the estimation. For wider use of this method, in cases of non-normal response of the trait to environment, we recommend to follow the advice of Valladares et al. (2006) and transform the raw trait data to achieve normal or near normal distribution before calculating the relative distances.

1.3. Method for using plasticity index to compare effect of environment on different traits

For comparison of plasticity between genotypes and traits, firstly, a statistical analysis of the raw trait data should be performed according to the type of data, testing for the effects of environment and genotype/species on each trait. For example, a two-way ANOVA or MANOVA (when testing across all traits) with interaction could be applicable. This is to test, firstly, that there is an effect of environment on the trait(s), and secondly, that the effect of the environment on the trait between

genotype/species differs (*i.e.* an interaction between genotype/species and environment).

After initial statistical analysis of the raw data, the relative distances can be calculated. This can be performed in R with help of the Plasticity package (Ameztegui 2017). This package was developed for calculating the RDPI as per Valladares et al. (2006).

With this package, relative distances can be calculated and stored for further analysis using the *rdpi_matrix()* function. Using the correct input, this function will output an array of the calculated relative distances for one given trait and one given genotype/species as per equation [1]. With correct structuring of data and the help of other functions and packages in R, the *rdpi_matrix()* function can be applied over the whole dataset to calculate the distances for all traits and genotype/species in just a few lines of code (see R script).

Depending on the distribution of the data, the mean of the distances or median can be calculated to give a value of RDPI for a particular trait and genotype/species of interest. Analysis of variance or means testing can then be performed on the calculated relative distances to test for significant differences between the estimated RDPIs between genotypes/species. Statistical analysis could also be carried out on the relative distances to test for differences between the RDPI of different traits, but caution must be applied as different traits may have very different distributions, which could skew the results. As aforementioned, care must be taken in the pre-processing of the data to assess the distributions of the data. Where possible it is advised to transform trait data to give comparable distributions before calculating relative distances. Where this is not possible, deviations in distributions and extremely different reaction norms need to be taken into consideration when interpreting the plasticity results.

1.4. R-script for calculating RDPIs

Prerequisites for calculating RDPI using the Plasticity package in R (Ameztegui 2017) include installation of R and RStudio (R Core Team 2023), and following packages: agricolae (de Mendiburu 2019), psych (Revelle 2023), dplyr (Wickham et al. 2023a), and ggplot2 (Wickham 2016). The script provides instructions for installing packages on first time use.

For calculation of the relative distances with the rdpi_matrix() function from the Plasticity package, data should be structured as follows: a data frame including one column with data on trait name, one column for environmental factor, one column for species/genotype, and one column on trait value (numerical). Each row must be an independent observation (Figure 2).

ID	genotype	potsize	trait.name	trait.value
C01	BERE	3	HMD	0.18967851
C02	BERE	3	HMD	0.20316664
C03	BERE	3	HMD	0.17921428
C04	BERE	3	HMD	0.26302017
C05	CONCERTO	3	HMD	0.19640800
C06	CONCERTO	3	HMD	0.17490249
C07	CONCERTO	3	HMD	0.26529309
C08	CONCERTO	3	HMD	0.16261942
C09	BERE	5	HMD	0.43320906
C10	BERE	5	HMD	0.33415596
C11	BERE	5	HMD	0.28736033
C12	BERE	5	HMD	0.25244337

Figure 2: Table head from R-Studio showing data structure for using the Plasticity package.

Further functions used in the R script for applying the method over the whole dataset are applied from the following R packages: tidyr (Wickham et al. 2023b), broom (Robinson et al. 2023) and purr (Wickham and Henry 2023). The R-script will be made available *e.g.* via SharePoint.

1.5. Interpretation and graphical representation of results

Once RDPI values have been calculated for the different genotypes and traits, these can be visually presented in the form of a radar/spider chart (in R package ggradar (Bion 2023)) or dot plot. The spider chart allows for quick visual comparison of plasticity index for different traits and genotypes. With a spider diagram, traits can be arranged to show the traits of most interest, for example, root traits *versus* shoot traits, and root traits for soil exploration *versus* traits for soil exploitation. With a dot chart, it is possible to also add the error on the estimation of the index.

Here we show examples of the results that can be obtained with this method using data from a pot size experiment, where barley plants of two genotypes (land race BERE and modern elite CONCERTO) were grown in pots with increasing pot diameter. We investigate the phenotypic plasticity of root and shoot traits due to changes in pot size. Therefore, in this study, pot size is the environmental variable of interest.

The RDPI was calculated as an estimate of phenotypic plasticity related to pot size for leaf area in cm^2 (LA), root surface area in cm^2 (RA), root length in cm (RL), root length density in cm/cm^3 (RL-D), root half mean distance in cm (HMD), root diameter in mm (RD), specific root length in cm/g (Specific-RL), root:shoot surface area ratio (RA:LA) and shoot total nitrogen in mg (STN) and presented in a spider diagram (Figure 3) and a dot plot (Figure 4).

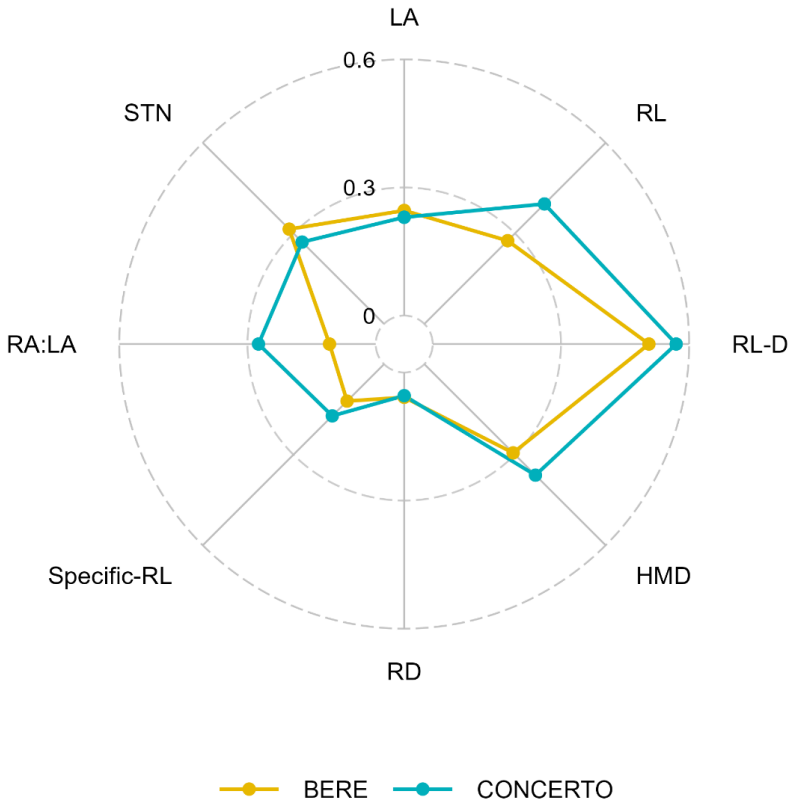


Figure 3: RDPIs for selected root and shoot traits for comparison between two barley genotypes; BERE (yellow) and CONCERTO (blue), illustrated in a spider plot. This enables a quick overview and comparison of the RDPIs although it is limited by the number of traits that can be displayed simultaneously. LA: leaf area in cm^2, RA: root surface area in cm^2, RL: root length in cm, RL-D: root length density in cm/cm^3, HMD: root half mean distance in cm, RD: root diameter in mm, Specific-RL: specific root length in cm/g, RA:LA: root:shoot surface area ratio and STN: shoot total nitrogen in mg.

The spider plot shows at a glance the estimated RDPI for each trait as per equation [1] and traits are arranged to highlight the plasticity of the shoot (LA) compared to traits related to root size (RL), those related to soil exploration (RD, HMD), to root morphology/anatomy (RD, Specific RL), root versus shoot size (RA:LA) and a root function (STN representing shoot nitrogen content). Note, that for traits showing a very high correlation with each other (root length, root volume, root surface area, root dry weight), only one was selected for the spider web plot. Figure 4 shows RDPI estimates for each trait in a dot plot including standard error of the mean.

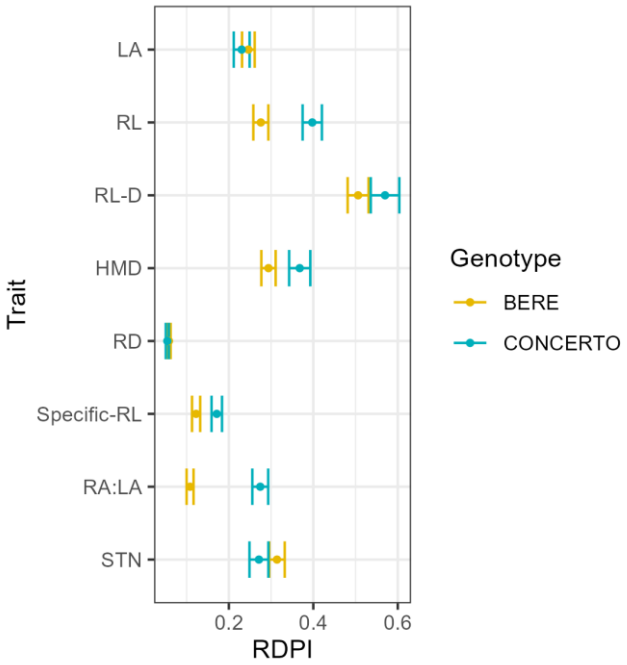


Figure 4: Dot chart showing the RDPIs of a higher number of selected traits. The respective error (standard error of the mean) on the estimation of the index is also depicted. LA: leaf area in cm^2, RA: root surface area in cm^2, RL: root length in cm, RL-D: root length density in cm/cm^3, HMD: root half mean distance in cm, RD: root diameter in mm, Specific-RL: specific root length in cm/g, RA:LA: root:shoot surface area ratio and STN: shoot total nitrogen in mg.

1.6. Concluding remarks - RDPI

The calculation of plasticity index RDPI can be conducted for a large range of experimental setups including laboratory and field experiments and allows for integrating studies conducted for the same species and environmental driver in analogy to a meta data analysis at a later time point. Note that in such an integration, care has to be taken to account for the fact that the environmental range covered with the experimental conditions might differ between experiments. Likewise, the phenotypic plasticity of an individual trait may be afflicted by an ontogenic effect, and hence differences in plant development cannot be neglected (Correa et al. 2019).

The spider web diagrams allow quick comparison of the plasticity of different root properties at a glance. In addition, shoot traits, representing above ground performance can be integrated as well as the root:shoot ratio which is a measure for the relative investment in root versus shoot growth. The number of nodes in the spider web diagrams can be limited by testing correlation between traits prior to selection of nodes. Grouping of nodes by potential function (*i.e.* exploitation related traits versus exploration related traits) further increases the amount of information available at a glance.

2. Experimental systems

2.1. Boundary conditions for the choice of an experimental system

To study the plasticity or root traits related to exploration and exploitation of soil environments, a range of methods need to be applied simultaneously. Importantly, all methods have their specific prerequisites which need to be considered in the experimental design. Here, we specifically address X-ray computed tomography (CT) for the visualisation and characterisation of root system architecture (RSA) in 3D during growth; WinRHIZO-analysis for quantification of root system size and diameters; root gene expression analysis (Transcriptomics) for adaption of the plants to actual growth conditions, and root metabolome analysis (Metabolomics) for information on the history of growth conditions. In addition, the system should enable the analysis of root exudation and related changes in root microbiome composition.

Key factors to be considered are:

- the trade-off between sample size and resolution for CT analysis;
- the need to sample material for metabolome and transcriptome analyses, which must be kept small enough not to compromise WinRHIZO analyses;
- the short time window available to collect and clean the samples for metabolome and transcriptome analysis.

For the experiments under controlled conditions in Root2Res WP5 'In-depth traits assessment and understanding of plasticity', environments are reflected by different:

- soils (differing in terms of texture and chemical properties);
- water availability (ranging from waterlogging to drought); and,
- nutrient supply (covering a potential range from deficiency to excess regarding nitrogen and phosphorous).

The work load associated with the experimental system should allow for investigation of at least two core genotypes of the main crops barley (*Hordeum vulgare*) and faba bean (*Vicia faba*) at different water availabilities and satellite experiments investigating the impact of soil type and of nutrient supply. The selection of two genotypes from the four core genotypes per species will be done in consultation with Roo2Res WP2 'The phenotyping toolbox' and will be based on the initial results of WP2, Task 2 'Phenotyping toolbox for root and related rhizosphere traits'.

2.2. Description of the experimental system

2.2.1. Plant growth columns

The experimental system is characterised by the use of acrylic glass tubes with a height of 25 cm and different diameters. These columns are sealed at the bottom with a water-permeable nylon mesh (30 µm mesh size) and can be filled with the soil material of choice. To minimise the influence of soil structure (*e.g.* grain size sorting of particles), the soil is homogenised, sieved and carefully filled into the columns to ensure uniform conditions for root growth. The soil is usually filled up to a height of 23 cm. The soil surface is covered with coarse gravel to minimise evaporation. Each column is placed in a tray, which allows irrigation via capillary rise in addition to watering from the top. Soil filled columns are wrapped with aluminium foil to

Funded by the European Union
UK Research and Innovation
Project funded by
Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra
Swiss Confederation
Federal Department of Economic Affairs, Education and Research EAER
State Secretariat for Education, Research and Innovation SERI

minimise algal growth and light exposure of the roots. The trays are covered to prevent evaporation. The columns are available with different diameters. The size to be used depends on the type of plant and the associated root diameter. Columns with a larger diameter can be used for plants with larger root diameter, *e.g.* faba bean. Columns with a smaller diameter are used for plants with finer roots like barley. This limitation is related to the resolution of the CT images, which is necessary to allow for detection of the roots with sufficient accuracy. More on this is found in section 2.2.4 X-computed topography.

2.2.2. Soils

The experiments will be carried out with three different soils. Based on the chemical analysis (WP2) and the texture analysis (WP5), two soils have been selected representing two of the core sites of Root2Res. They differ in texture and chemical properties (Table 1). These are the soils from ARVALIS (France) and PTUJ (Slovenia). The third soil will be selected at a later date based on the results of WP2, Task 2.

Table 1: Texture and chemical properties of selected soils representing core sites of Root2Res.

Soil	Clay %	Silt %	Sand %	TOC g/kg	C/N	N g/kg	TIC g/kg	EC µS/cm	pH 0.01 M CaCl2	CAL-K mg/kg	CAL-P mg/kg
Arvalis	33	62	5	12.8	12.5	1.0	0.1	179.4	6.9	87.6	92.6
PTUJ	8	46	46	13.0	11.6	1.1	24.9	299.7	7.4	92.7	81.0
Red Vertisol	63	26	11	10.4	15.8	0.7	0.1	139.7	6.5	19.1	14.7
New field	28	37	35	23.7	10.3	2.3	0.7	183.6	6.9	82.4	118.1

Due to strong swelling and shrinking behaviour, the Red Vertisol will not be used.

2.2.3. Climate chamber and plant growth period

The plant growth takes place in a walk-in climate chamber, offering sufficient space (12 m²) for the placement of numerous columns at the same time. In the chamber, light exposure time and intensity, day/night duration, temperature and relative humidity can be set as desired. Typical settings are 12/12h day/night cycles at 19-22 °C and 16-18 °C respectively, 350 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) and 65% relative air humidity (*e.g.* Blaser et al. 2020; Lippold et al. 2021), but can be adapted in consultation with other WPs to ensure similar conditions. The change in weight of the soil columns is determined using scales to derive plant transpiration rates. Considering the growth conditions and the column sizes used, the experiment duration is usually limited to 3 weeks.

2.2.4. X-ray computed tomography

A major advantage at the UFZ location is the availability of an industrial X-ray micro-CT (X-TEK XTH 225, Nikon Metrology) in the immediate vicinity of the climate chamber. This enables repeated imaging of the same individual plants with advancing root

development to record dynamic expression of the RSA (relevant for model development in Root2Res WP6 'The modelling toolbox'). To minimise negative impacts, CT imaging is performed during the night phase in the climate chamber.

UFZ has several years of experience with the use of X-ray CT as an imaging method for root growth in soil (Blaser et al. 2020; Carminati et al. 2012; Lippold et al. 2021) and are aware of the limitations of the method. The spatial resolution of the CT images must be sufficiently high to achieve an adequate root detection rate. There is a fixed geometric relationship between sample size and image resolution. The larger the sample, the coarser the spatial resolution of the acquired CT images. Figure 5 shows 2D slices of raw grey value images from X-ray CT data of a sandy substrate with barley roots inside. The gradual decrease in sample size (*i.e.* column diameter) and the related change in resolution illustrates the increasing degree of details that can be observed in the CT images.

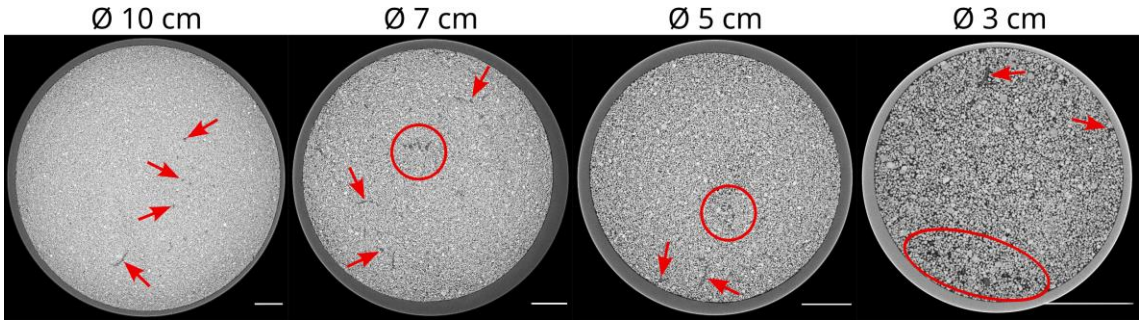


Figure 5: 2D sections of raw grey-scale images from X-ray CT scans of columns filled with the same sandy substrate and barley roots growing inside (partly indicated by arrows and circles). Scale bar represents 1 cm in length. The gradual reduction of sample diameter from 10 cm to 3 cm illustrates the relationship between sample size and spatial resolution of the acquired images. The higher the resolution, the better the features are recognisable. Roots as well as soil particles are resolved in higher detail with increasing image resolution. The two middle column sizes with 7 cm and 5 cm in diameter represent the best compromise and are most suitable for use in the experimental system.

In the 10 cm column, the roots are barely visible which results in a limited root detection rate when analysing the images. In contrast, the 3 cm column enables a high spatial resolution, but the available soil volume is so small that the growth period of the plants would have to be shortened, which severely limits the possible investigations. In addition, many more CT scans would be needed to cover a relevant share of the columns. This would increase both the radiation dose and the workload to an unreasonable extent. For these reasons, the two extreme variants (3 cm and 10 cm) are not suitable for continuous use in the experimental system. Therefore, only the column sizes 5 cm and 7 cm are being considered.

The 7 cm columns are particularly suitable for plants with larger root diameters, *i.e.* faba bean. For barley having thinner root diameters, the 5 cm columns are more suitable, although a certain loss of root coverage cannot be fully excluded depending on the growth conditions. The radiation dose needs to be considered especially for faba bean (Blaser et al. 2018). Potential impacts of X-ray CT on microbiome composition and the transcriptome are not expected in case sampling takes place more than 24 hours after last CT scanning (Ganther et al. 2020). The image analysis is carried out with an adapted version of the in-house technique "Rootine v2" (Phalempin et al. 2021). From the CT data, RSA development can be visualised (Figure

6) and parameters including root length, diameter distribution and seminal root angle will be analysed.

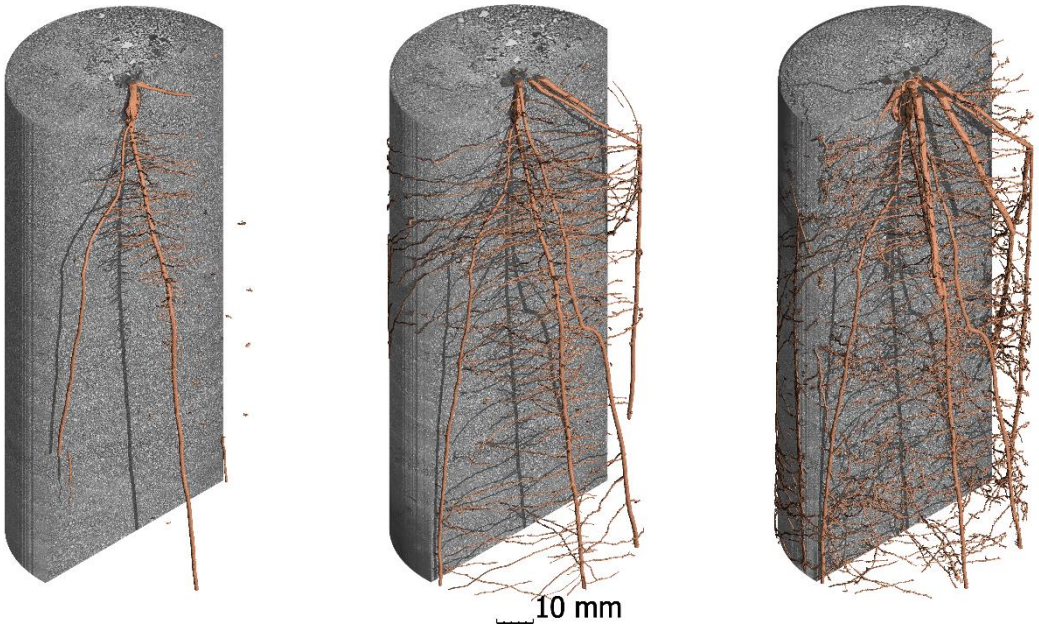


Figure 6: 3D time series of the growth of a maize root system in sandy soil acquired with X-ray CT. The root system is shown after 7, 14 and 21 days of growth in columns with a diameter of 7 cm. The spatial resolution is 45 µm. The tomograms were segmented with RootineV2 (Phalempin et al. 2021) and visualised with VGStudio Max. Figure created by Sebastian Blaser.

2.2.5. Destructive sampling

At the end of each experiment, destructive sampling is carried out. This takes place in cooperation with the project partners within the WP. Subsamples of both, leaves and roots, are required by project partners for their analyses of gene expression and metabolomics. For barley, one seminal root including all laterals developed from this seminal can be cut off quickly after pushing out the soil column. This subsample is quickly rinsed in deionised water, plotted dry and after recording the fresh weight dipped in liquid nitrogen prior to transfer into the -80 °C freezer. The subsampling procedure has already been tested and was evaluated as very successful based on the good collaboration of comparative analyses in both partner laboratories in Italy (IPSP/CNR) and Spain (UVIGO), meaningful separation of trait expression according to treatments as well as agreement between biological replicates. Time from cutting the shoot to harvesting the roots (free of soil) and shoot tissue subsamples in liquid nitrogen was less than 15 minutes. Subsamples can be stored at -80 °C and further shipped on dry ice to the relevant partner institutions for transcriptomic and metabolomic analyses. The remaining samples (3-5 seminal roots including their laterals) can be used at the UFZ for further WinRHIZO analysis. The root fresh weight of each sample is used to extrapolate any feature for the entire root system. The standard traits that are recorded for all experiments are listed in Table 2.

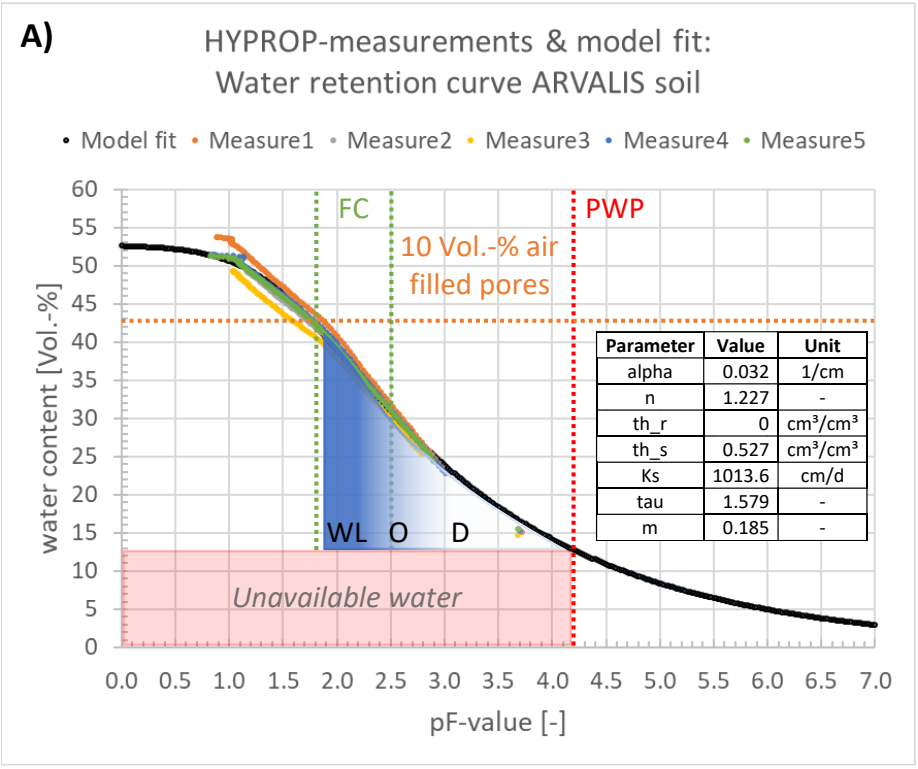
.

Table 2 Shoot and root traits that are recorded for all experiments.

Shoot traits	Root traits
Fresh & dry weight	Fresh weight
Leaf area	Root length, surface & volume
C & N analysis → plant N uptake	Diameter distribution
(further nutrient analysis, depending on the amount of available biomass)	Seminal root angle for barley; 1st order lateral root angle for faba bean
Transpiration	(Branching pattern if possible. More feasible for faba bean)

2.3. Derivation of water stress treatments

The water stress treatments are supposed to cover a wide range from waterlogging to drought stress. Water availability for soil grown plants can be described by the water retention curve, *i.e.* a characteristic relationship relating the volumetric water content to the soil water potential. The latter is a measure of the force required by the root to extract soil water. Water potential is given as pF values defined as the negative decadic logarithm of the soil water tension in hectopascals. Water retention curves allow to derive the volumetric water content for typical key characteristics like field capacity FC (pF 1.8-2.5) or permanent wilting point PWP (pF 4.2). In addition, water holding capacity or air-filled pore volume can be derived. Water retention curves reflect the pore size distribution in the soil and thus depend on particle size distribution (texture, sieving) and soil bulk density (packing of soil into the columns). For these reasons, water retention curves have to be established for each soil and for the respective feasible bulk density. For the two soils F-ARVALIS and Slo-PTUJ, the water retention curve was already determined by an evaporation experiment with HYPROP®. Figure 7 shows both the individual measurements and the model fit (constrained van Genuchten & Mualem model) for the two soils at a bulk density of 1.22 g/cm³ for F-ARVALIS and 1.32 g/cm³ for Slo-PTUJ.



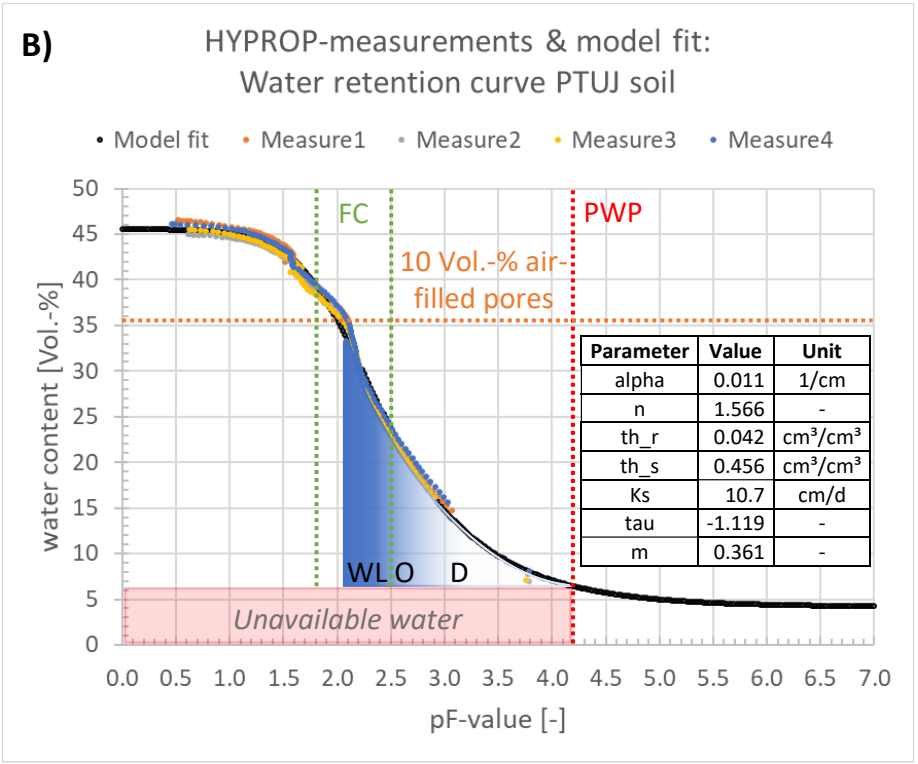


Figure 7: Water retention curves for (A) ARVALIS and (B) PTUJ soils measured via evaporation experiment (HYPROP®). Measurements are depicted in colour code, model fit (constrained van Genuchten & Mualem) in black. Green dashed lines indicate field capacity (FC) which is defined in the pF range of 1.8-2.5, and the red dashed line marks the permanent wilting point (PWP) at pF 4.2. The pF value is defined as the negative decadic logarithm of the soil water tension in hectopascals (hPa), e.g. pF 2.0 translates to -100 hPa. The orange horizontal line represents the volumetric water content when 10% of the sample volume is air-filled. The blue

area between the curve and the unavailable water represents the intended water regimes from waterlogging (WL) to optimum conditions (O) to drought (D). Respective van Genuchten parameters are given in the inset tables.

The PWP indicates that remaining water is so strongly bound within the soil that it cannot be absorbed by the plants (here 12.8 Vol.-% in ARVALIS, 6.4 Vol.-% in PTUJ). It is also important to have at least 10 - 15% (rule of thumb) of the pores filled with air to avoid lack of oxygen, which would be critical for the roots. These boundary conditions must be considered when designing the experiments on water availability. Optimum water supply will be defined at pF 2.5, thus in both substrates the content of plant available water (FC minus PWP) is about 18 Vol.-% (Table 3,). The treatment with optimum water supply will have a fairly constant water content throughout the plant growth period by frequent re-watering of the soil columns. For the waterlogging treatment, it is planned to increase the water content from about 7 days after germination to optimum water content plus 10 Vol.-%, *i.e.* 40.8 Vol.-% in ARVALIS and 33.8 Vol.-% in PTUJ. For both substrates, this results in water contents in the range of the threshold of 10 - 15% air-filled pore space (11.9 Vol.-% in ARVALIS and 11.8 Vol.-% in PTUJ, respectively). If water content is increased too early, the risk of mouldy seeds is too high.

For drought stress treatments, plants will not be re-watered after germination. Based on transpiration rates measured in previous experiments with faba bean (Koebernick et al. 2015) and barley (Kemanian et al. 2005), it is expected that plants in the drought stress treatments will reach permanent wilting point by the end of week 3. The stress intensity and speed of stress development will be monitored by measuring total transpiration. It should be noted that due to feedbacks between plant size, water requirement and soil water extraction identical stress conditions can never be maintained for longer time periods in soil-based systems.

Table 3: Soil specific characteristics for water stress treatments.

Soil characteristics for water stress treatments	F- ARVALIS	Slo-PTUJ
Total pore volume in Vol.-%	52.7	45.6
Total pore volume minus 10% air-filled pore volume in Vol.-%	42.7	35.6
Volumetric water content at FC (pF 2.5) in Vol.-%	30.8	23.8
Volumetric water content at PWP (pF 4.2) in Vol.-%	12.8	6.4
Plant available volumetric water content (FC minus PWP) in Vol.-%	18.0	17.3
Volumetric water content at waterlogging (FC plus 10 Vol.-%) in Vol.-%	40.8	33.8

An often-used approach for establishing different low volumetric soil water content levels (at certain percentage of water holding capacity) by frequent re-watering fails to account for the heterogeneous water distribution in the soil due to decreasing soil hydraulic conductivity with decreasing water content. Root architecture under such conditions mirrors artificial patterns of water distribution within the soil profile rather than plasticity of root growth as a response to decreasing water availability.

Furthermore, vertical water distribution within the column is not homogeneous due to gravity. For ARVALIS and PTUJ soils, this vertical gradient is estimated to be in the range of only 3 Vol.-% for a mean pF value of 2.5 and is thus neglected for the definition of treatments. In soils with a very sandy texture (> 90%) vertical gradients may result in waterlogging at the bottom of the columns while water availability is limiting in the top of the columns.

A concluding overview of the main growth conditions for the two core plant species barley and faba bean used in the experiments in WP5 is depicted in Table 4.

Table 4: Overview of the main growth conditions for the core plant species barley and faba bean used in the experimental systems in WP5.

Growth condition	Barley	Faba bean
Column diameter	5 cm	7 cm
Available soil volume (height =23 cm)	452 cm^3	885 cm^3
Day / night duration	12h / 12h	
Temperature ranges (day / night)	19-22 °C / 16-18 °C	
Photosynthetic active radiation	350 µmol m^{-2} s^{-1}	
Relative air humidity	65%	
Growth period	3 weeks	
Optimum water treatment	pF 2.5 with frequent re-watering	
Water logging treatment	pF 2.5 + 10 Vol.-% water content	
Drought treatment	pF 2.5 initially; without re-watering	

References

Ameztegui A (2017) Plasticity: An R package to determine several plasticity indices. GitHub repository.

Arnold PA, Kruuk LEB, Nicotra AB (2019) How to analyse plant phenotypic plasticity in response to a changing climate. New Phytol 222: 1235-1241. doi: 10.1111/nph.15656.

Balaguer L, Martínez-Ferri E, Valladares F, Pérez-Corona ME, Baquedano FJ, Castillo FJ, Manrique E (2001) Population divergence in the plasticity of the response of Quercus coccifera to the light environment. Functional Ecology 15: 124-135. doi: 10.1046/j.1365-2435.2001.00505.x.

Bion R (2023) ggradar: Create radar charts using ggplot2.

Blaser S, Koebernick N, Spott O, Thiel E, Vetterlein D (2020) Dynamics of localised nitrogen supply and relevance for root growth of Vicia faba ('Fuego') and Hordeum vulgare ('Marthe') in soil. Sci Rep-Uk 10: 15776. doi: 10.1038/s41598-020-72140-1.

Blaser S, Schluter S, Vetterlein D (2018) How much is too much?-Influence of X-ray dose on root growth of faba bean (Vicia faba) and barley (Hordeum vulgare). PloS one 13: e0193669. doi: 10.1371/journal.pone.0193669.

Carminati A, Vetterlein D, Koebernick N, Blaser S, Weller U, Vogel HJ (2012) Do roots mind the gap? Plant Soil 367: 651-661. doi: 10.1007/s11104-012-1496-9.

Cheplick GP (1995) Genotypic Variation and Plasticity of Clonal Growth in Relation to Nutrient Availability in Amphibromus Scabrivalvis. The Journal of Ecology 83. doi: 10.2307/2261599.

Correa J, Postma JA, Watt M, Wojciechowski T (2019) Soil compaction and the architectural plasticity of root systems. J Exp Bot 70: 6019-6034. doi: 10.1093/jxb/erz383.

de Mendiburu F (2019) Agricolae: statistical procedures for agricultural research.

Dewitt TJ, Sih A, Wilson DS (1998) Costs and limits of phenotypic plasticity. Trends Ecol Evol 13: 77-81. doi: 10.1016/s0169-5347(97)01274-3.

Ganther M, Yim B, Ibrahim Z, Bienert MD, Lippold E, Maccario L, Sorensen SJ, Bienert GP, Vetterlein D, Heintz-Buschart A, Blagodatskaya E, Smalla K, Tarkka MT (2020) Compatibility of X-ray computed tomography with plant gene expression, rhizosphere bacterial communities and enzyme activities. J Exp Bot 71: 5603-5614. doi: 10.1093/jxb/eraa262.

Hodge A (2006) Plastic plants and patchy soils. J Exp Bot 57: 401-411. doi: 10.1093/jxb/eri280.

Kemanian AR, Stockle CO, Huggins DR (2005) Transpiration-use efficiency of barley. Agricultural and Forest Meteorology 130: 1-11. doi: 10.1016/j.agrformet.2005.01.003.

Koebernick N, Huber K, Kerkhofs E, Vanderborght J, Javaux M, Vereecken H, Vetterlein D (2015) Unraveling the hydrodynamics of split root water uptake experiments using CT scanned root architectures and three dimensional flow simulations. Front Plant Sci 6: 370. doi: 10.3389/fpls.2015.00370.

Lippold E, Phalempin M, Schlüter S, Vetterlein D (2021) Does the lack of root hairs alter root system architecture of Zea mays? Plant Soil 467: 267-286. doi: 10.1007/s11104-021-05084-8.

Navas M-L, Garnier E (2002) Plasticity of whole plant and leaf traits in Rubia peregrina in response to light, nutrient and water availability. Acta Oecologica 23: 375-383. doi: 10.1016/s1146-609x(02)01168-2.

Phalempin M, Lippold E, Vetterlein D, Schluter S (2021) An improved method for the segmentation of roots from X-ray computed tomography 3D images: Rootine v.2. Plant methods 17: 39. doi: 10.1186/s13007-021-00735-4.

R Core Team D (2023) R: A language and environment for statistical computing.

Revelle W (2023) psych: procedures for psychological, psychometric, and personality research. R package version 2.3.6.

Robinson D, Hayes A, Couch S (2023) broom: Convert Statistical Objects into Tidy Tibbles.

Schlichting CD, Levin DA (1984) Phenotypic Plasticity of Annual Phlox - Tests of Some Hypotheses. American Journal of Botany 71: 252-260. doi: Doi 10.2307/2443753.

Schneider HM, Lynch JP (2020) Should Root Plasticity Be a Crop Breeding Target? Front Plant Sci 11: 546. doi: 10.3389/fpls.2020.00546.

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. Trends in Plant Science 5: 537-542. doi: 10.1016/s1360-1385(00)01797-0.

Valladares F, Balaguer L, Martinez-Ferri E, Perez-Corona E, Manrique E (2002) Plasticity, instability and canalization: is the phenotypic variation in seedlings of sclerophyll oaks consistent with the environmental unpredictability of Mediterranean ecosystems? New Phytol 156: 457-467. doi: 10.1046/j.1469-8137.2002.00525.x.

Valladares F, Sanchez-Gomez D, Zavala MA (2006) Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. Journal of Ecology 94: 1103-1116. doi: 10.1111/j.1365-2745.2006.01176.x.

Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Wickham H, François R, Henry L, Müller K, Vaughan D (2023a) dplyr: A Grammar of Data Manipulation. R package version 1.1.2.

Wickham H, Henry L (2023) purrr: Functional Programming Tools. R package version 1.0.1.

Wickham H, Vaughan D, Girlich M, Ushey K, Posit, PBC (2023b) tidyr: Tidy Messy Data (1.3.0).