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George Themistokleous, Kirsty Binnie, Blair M. McKenzie, Sacha J. Mooney & Tracy A. Valentine

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RootHairAreaFinder: An image processing method for quantifying barley root growth and root hairs simultaneously in a flat rhizotron system.

Authors: George Themistokleous^{1,2,3}, Kirsty Binnie¹, Blair M. McKenzie^{1,4}, Sacha J. Mooney², Tracy A. Valentine^{1*}

1. Ecological Sciences, The James Hutton Institute, Invergowrie, Dundee, Scotland, UK. DD2 5DA
2. Division of Agricultural & Environmental Sciences, University of Nottingham,, Sutton Bonington Campus, Sutton Bonington, Leicestershire, UK. LE12 5RD
3. Eratosthenes Centre of Excellence, Lemesos, Cyprus
4. Geography and Environmental Science, School of Social Sciences, University of Dundee, Nethergate, Dundee, Scotland, UK DD1 4NH

* Corresponding author: Tracy.Valentine@hutton.ac.uk

Abstract

Root system architecture and root hairs highly influence plant resource uptake, yet their simultaneous quantification at the whole-plant scale remains challenging due to the conflicting requirements of high-resolution imaging and non-destructive, repeated measurements. Here, we present a plant growth and imaging system based on A3 sized flatbed rhizotrons, combined with a non-machine-learning image analysis workflow implemented in R, that enables repeated, *in situ* quantification of barley root system architecture and accompanied visible projected root hair area (VP root hair area) over three weeks of growth. The system produces integrated outputs highlighting both whole-root architecture and the spatial distribution of surrounding root hairs from single images. Validation of the image analysis algorithm showed good segmentation performance, with average Matthews Correlation Coefficient values of 0.68 for projected root area and 0.65 for VP root hair area. To demonstrate its experimental applicability, the system was used to assess root growth and root hair responses under controlled environmental conditions, combining three irrigation regimes (2, 4, and 6 irrigation events per day) with three dry bulk density levels (1.4, 1.5, and 1.6 g cm⁻³). In addition to whole-system metrics, the approach enables analysis of root hair expansion at individual root tips. This methodology provides a training-free method for integrated analysis of root traits e.g. projected root size, root growth, root diameter and root hairs under controlled physical conditions similar to soil, facilitating studies of root–soil interactions that require both spatial resolution and temporal continuity.

Keywords

Root hairs, soil physics, image analysis, rhizotron, dry bulk density, drought

Background

Global food security is a major challenge for current and future generations (1). Major issues such as drought stress negatively influence plant growth, survival and reproduction (2) and are responsible for large reductions in crop yield (3). Drought severity is also projected to increase due to increasing global temperatures (4). The projected increase in average global temperature (5) means that roots will experience a higher frequency of mechanical impedance, as reduction in water content of soil is often correlated to an increase in soil strength (6,7). Compaction and climate change can also increase the risk of flooding events due to reduction in pore size

distribution of the soil and the increase in heavy rainfall events. The above climatic effects can be exacerbated when one considers the negative consequences on soil compaction brought upon by heavy agricultural machinery (8). As such, it is important to understand how the soil physical conditions of water and mechanical stresses interact to impact root growth (9,10).

A potential mechanism for overcoming the negative effects associated with drought are changes in root system architecture (RSA), which are of immense importance for the survival and growth of the plant (11,12). Changes in RSA that mitigate drought stress can involve deep rooting, as this enables the uptake of water from deeper soil layers (13). In addition, higher root density, increased lateral root production, and alterations in root diameter can improve water extraction efficiency, enhance soil penetration, and increase the capacity of plants to exploit heterogeneous water resources within the soil profile (14,15).

Root hairs have also been considered to aid plants in overcoming drought effects (16). They are a common anatomical characteristic of most vascular plants which can dramatically increase the surface area of roots (17). They influence the uptake of both nutrients and water (18) with the root hair zone being the most active zone for fluid transfer in the root system due to its high permeability (19). In more precise terms, they are extensions of trichoblasts, specific root epidermal cells which develop in the maturation zone of the root tip (20). Root hair phenotypes can be dynamic, and drought conditions can induce increases in root hair length, thereby expanding the total root surface area and enhancing water absorption capacity (21). Furthermore, root hairs can improve soil penetration and root-soil contact (22), particularly in compact soils (23), where increased soil compaction can reduce root hair length (24).

However, unlike other easily accessible plant organs such as the stem and leaves or small embryo seeds, the imaging of undisturbed root systems is a more complicated process due to the opacity of soil. Recent advances in imaging enable *in situ* quantification of root hairs grown in soil by using synchrotron imaging (25,26), however, the reported dimensions for this plant-soil microcosm are currently small as the germinated seedlings were grown inside syringes with a diameter of only 3 cm. An alternative and less complex method for assessing root growth *in situ* are rhizotrons. Rhizotrons enable non-destructive imaging of root systems grown in closed containers filled with a growth substrate (27–29). In addition to coarse root system structures, i.e., roots, it is also possible to image and quantify finer features of the root system such as root hairs (30).

Root hair analysis methods are available for excised roots, for example Root Hair Sizer (31) and DIRT/ μ (32), however, automated root detection in rhizotron systems is a rather complicated process, because of the complex root background with image quality varying with soil conditions, lighting and root colour and as such, unsupervised machine learning methods have mixed performance results (33). As a result, most of the available software require the user to manually perform the root segmentation from the background (34). In a similar manner, Vincent et al. (30) developed a semi-automated method for analysing root soil images obtained from rhizotron tube cameras using training images. More recently, Lu et al. (35) used a machine learning (ML) based U-net model and transfer learning to segment root hairs with very high accuracy. However, ML methods require prior model calibration for optimal performance. In addition, if tube based rhizotron are used each imaging apparatus ($1.1 \times 1.1 \times 1.2 \text{ cm}^3$) installed only captures spatially localized root information, resulting in numerous small images. As such, it is desirable to develop easy to use, non-computationally demanding, and sufficiently accurate algorithms to measure basic root hair properties that are spatially linked *in situ*, under realistic growth conditions (for example the characterisation of root hairs linked to their spatial position in the root system)

In this paper, a flatbed A3 sized rhizotron system (RS) is introduced along with an image processing algorithm which enables the detection and quantification of the visible root system and its visible projected root hair area (VP root hair area) for barley seedlings grown for up to a period of three weeks. The algorithm is fully automated and can be run from the R console (36) and, is designed for time-lapse studies that investigate root and root hair development. The proposed method is then demonstrated in a phenotyping experiment involving spring barley (*Hordeum vulgare* var. Optic) subjected to different sand water concentrations and dry bulk densities.

Methods

Plant growth system and imaging

Seeds of *Hordeum vulgare* c (Optic) were obtained from the collections at the James Hutton Institute, Dundee, UK and had been previously stored at 4 °C. Plants were grown in a rhizotron system, as described in Supplementary file 1. Briefly, the rhizotron system was constructed of Perspex acrylic sheets approximately A3 size (420 height x 15 depth x 297 width mm³), with side spacer bars, and an automatic irrigation system. Rhizotrons were filled with a dry, black-coloured sand mix from Seachem (Flourite Black). Details about its physical properties,

including penetrometer resistance (37) and volumetric water loss across a range of matric potentials, are provided in Supplementary file 2. A controlled release fertiliser, i.e., “Scotts Osmocote”, manufactured by “Everris” was included by individually grinding to dust 0.3 g of fertilizer with a mortar and pestle for each rhizotron and subsequently mixing the fine powder thoroughly with the required mass of rhizotron substrate prior to rhizotron packing. Images were obtained using an A3 sized flatbed scanner (EPSON Expression 12000XL) at 1,200 dpi, on days 7, 14 and 21 post transfer of seedlings to the rhizotrons.

Water content was adjusted using an automated irrigation system. After 3 days of water equilibration, pre-germinated seeds, germinated as described in Valentine et al. (38), were transferred to the rhizotrons. Each rhizotron was covered in black sheeting (Do4U) to exclude light, placed at an angle of 45° to the horizontal and kept in a glasshouse with the temperature set to 18°C during the day and 14 °C during the night. Artificial lighting was activated at 150 W m⁻² from GAVITA GAN 400AL Ecomax lamps fitted with 400W SON-T sodium bulbs.

To analyse the effect of physical conditions on root hair distribution, a two-way 3 x 3 ANOVA experiment was used with the factors being dry bulk density (DBD) and number of irrigation events (IRE), i.e., water concentration. The experiment had a randomized (blocked) structure with the RS units arranged in two parallel linear rows with 4 treatment replicates. The treatments for DBD were 1.4, 1.5 and 1.6 g cm⁻³ and the treatments of IRE were 2, 4 and 6 irrigation events per day with an event having a duration of 3 minutes. The amount of water added was adjusted to maintain an approximate degree of saturation for each treatment.

Assessment of image quality

Prior to the selection of the 2 mm thick acrylic, the effect of different imaging surfaces on image quality was first assessed. Three surfaces were considered: (1) 2 mm thick acrylic, (2) 4 mm thick, low iron, reinforced glass, and (3) 5 mm thick acrylic. To objectively assess image quality, an image sharpness index was used. The Image Sharpness Index (ISI) is a dimensionless measure of image sharpness, which is computed by estimating the average value of the local greyscale variance at the scale of interest (39). The scale selected was a block radius of 1 pixel, because root hairs are only 1 or 2 pixels thick at the image scale. A higher ISI value indicates a sharper image, as the image intensity variation tends to be smaller when blurriness is stronger (40). Three replicates were prepared per treatment, subjected to identical growth conditions, and imaged on day 7 at 1,600 dpi. The following image processing steps were then applied to each image:

- 1) The skeleton of the visible root system (centre line) was traced manually by using the “Segmented Line” tool of Fiji (41).
- 2) Each of the (n) identified skeleton pixels were assigned a uniform probability ($1 / n$) and then 4 pixels were randomly selected for each image to obtain unbiased estimates.
- 3) A square section of 640 x 640 pixels was formed around each of the 4 randomly selected pixels. The size of this area was empirically determined to sufficiently cover the root hair zone adjacent to the root. If any of the 4 image regions overlapped, then the process was repeated until there was no region overlap.
- 4) The ISI measure described above was then computed for each of the 4 image sections.

Steps 2–4 were implemented in R using a custom-developed script. The 4×3 values associated with each treatment were pooled and analysed using Dunnett’s T3 test for the comparison of mean values.

Image analysis

The proposed image processing algorithm enables the analysis of root systems grown in a rhizotron unit with a black-coloured sand used as a substrate. The algorithm uses functions created by RootHairAreaFinder.cpp. An overview of the process is shown in Figure 1, while further details of individual functions are provided in Supplementary file 3. The main outputs of the algorithm are root length, x and y dimensions of root length, total projected root area (This projected root area refers to the visible area occupied by root in the image, rather than the root’s surface area) and mean root hair width to VP root hair area width per unit segments. The individual steps which compose the algorithm are summarized in Figure 1 (whole A3 rhizotron, Supplementary_file_4.tif) & Figure 2 (individual root analysis, Supplementary_file_5.tif). Briefly, the algorithm uses techniques such as thresholding, mathematical morphology, particle criteria and region growth to separate foreground from background (42). More complex filters based on Gaussian convolution procedures are also used to enhance vascular structures (43). The final segmented image is obtained by applying the (Fiji(44)) IsoData threshold (44) and region growth techniques. The VP root hair area is obtained by applying thresholds on the area adjacent to the detected roots after the process of root detection. VP root hair area is the visible projected area in the image occupied by root hairs as opposed to a measure of the surface area of the root comprised of root hairs. The algorithm is implemented in R via Rcpp (45), with dependencies on the RcppArmadillo (46) and RcppParallel (47) packages. The packages raster (48) and rgdal (49) are also used for image

import, export, and visualization. Images and graphs were produced using cowplot (50) , ggplot2 (51) and ggmap (52), by adding code to save each analysis function output to RDS output files. These were reloaded into R prior to visualisation. For the individual root analysis, the position of the root tip was manually selected. A spline was placed along the centre of the root binary image obtaining the midpoint of the root for each column of pixels. The x, y position of the central spline was plotted using ggplot and a rolling average of positions generated using rollmean (zoo package (53)). The new distance between each column was calculated from the diagonal distance between each column prior to aligning the columns using Pythagoras' distance from the spline point on the x and y scales. Final images were produced by sliding vertical pixel columns to produce a linear spline. Root distance from meristem at each datapoint was calculated as pooled distances between aligned pixel columns. Using the same method each image was aligned and root hair length at each pixel columns calculated from results data

Root growth parameters

Root growth parameters and VP root hair area estimation were measured from the RS images obtained at 7, 14 and 21 days *via* analysis using the proposed algorithm described above. The variables Average Root Growth Rate (ARGR) (mm day^{-1}), Average Vertical Growth Rate (AVGR) (mm day^{-1}), Average Horizontal Growth Rate (AHGR) (mm day^{-1}) and Average Root Hair to Root Ratio (ARHtRR) (mm mm^{-1}), were used to summarise root growth and (relative) root hair abundance. Definitions for those variables are given in Supplementary file 6.

Statistical Analysis

The statistical analysis of the data was performed using the software R (version 4.4.1) and the freely available code provided by Wilcox (54). A non-parametric 2-way ANOVA test was used to statistical compare the ranks of the experimental treatments with the function `bdm2way()`. Post-hoc analysis with pairwise median comparisons was performed with the `mcp2a()` function. Dunnett's T3 test was implemented with the function `lincon()`. The level of significance chosen for this study was 0.05. The graphical outputs were also produced in R with the ggplot2 (51) and the grid (36) packages.

Image analysis validation

The projected root area output from the algorithm was validated as follows: 1) 20 rhizotron images were randomly selected among the pooled set of images for the glasshouse experiment, taken at 14 and 21 days of growth; 2) the outline of the visible root system was traced manually

by using the “Polygon Selections” tool of Fiji creating regions of interest which were binarized;
3) The algorithm predicted projected root area was compared to the manual one.

Root hair detection was validated as follows: 1) 4 rhizotron images were randomly selected among the pooled set of images for the glasshouse experiment, taken at 14 and 21 days of growth.; 2) 5 image sections (300 x 300 pixels) containing root hairs were subjectively chosen by a trained root scientist so that the sections spanned the length of the root system; 3) The root hair boundary was traced manually by using the “Polygon Selections” tool of Fiji; 4) The algorithm predicted root hair boundary area was compared to the manual one. In both cases, the accuracy was quantified with the Matthews Correlation Coefficient (MCC) (55,56).

The MCC values of both traits were compared against a reference value of 0.6 using a one-sample Wilcoxon signed-rank test with a one-sided alternative hypothesis ($MCC > 0.6$), implemented *via* the `wilcox_test()` function from the `rstatix` package (57). This threshold was selected as it is commonly interpreted as the lower bound of substantial agreement in classification and segmentation studies. Effect size (rank-biserial correlation) was computed using the `wilcox_effsize()` function from the same package.

Impact on the resulting VP root hair area and projected root area of varying the `roothair_pixel_radius` was analysed by evaluating the output on multiple images of varying sizes illustrating individual roots up to full rhizotron scale images. The `roothair_pixel_radius` was set at 30 to 250 at 20 pixel intervals. A curve was fitted to the VP root hair area vs. `roothair_pixel radius` using `smooth.spline()`, `spar = 1`), and the first derivative calculated using the `predict()`, `deriv = 1`) function for each image tested.

Algorithm speed

The execution speed of the algorithm was measured for; 1) a set of 8 images of 3-week-old seedlings grown in RS; 2) the images used for Figures 4 & 5; 3) randomly cropped subsets of the image used for Figure 4. The average ($\bar{x} \pm s$) image size of the images was 106.7 ± 63.07 MB. The timings were obtained by using the R base function `system.time()` and extracting the user (user > elapsed, due to parallel processing) time from the outputs. Algorithm timings reported were made by using a PC with the following system properties for 1): Processor: AMD Ryzen 9 7900 12-Core Processor 3.7GHz or for 2) & 3) Intel(R) Xeon(R) Silver 4310 CPU @ 2.10GHz (2.10 GHz) (2 processors); Installed memory (RAM): 32.0 GB (31.2 GB usable); System type: 64-bit operating system, x64 based processor; Windows Version: Windows 11 pro/Enterprise (respectively)

Results

Effect of imaging surface on image quality

The effect of different imaging surfaces on image quality is illustrated in Figure 3. Root hairs can be seen much clearer in the image taken through the 2 mm acrylic surface (Figure 3A), compared with the 4 mm glass and 5 mm acrylic (Figure 3B, C respectively). These visual assessments were evaluated objectively by estimating the ISI value of each image. Figure 3D summarizes the comparison between the different scanning surfaces. The ISI scores support the visual assessments, demonstrating the sharpness of the 2 mm thick acrylic images which had a higher ISI score relative to both the 4 mm glass and the 5 mm acrylic with a 95% confidence interval of (25.033, 55.830) and (34.773, 65.668) respectively. The mean ($\bar{x} \pm s$) ISI values for each treatment were 67.922 ± 15.409 , 27.491 ± 5.17 and 17.702 ± 5.641 respectively.

Assessment of image analysis performance

Root images from the glasshouse experiment (using the 2 mm acrylic imaging front) were analysed using the RootHairAreaFinder algorithm. The algorithm steps are illustrated in Figure 1. Figure 4 shows an example image processed by the algorithm with image outputs at critical stages. An example starting image is shown in Figure 4A, with the image after pre-processing and first Frangi filter (Figure 4B), after root binarization (Figure 4C) after root hair enhancement (Figure 4D) and after root segmentation step for extracting the final results (Figure 4E). The algorithm was estimated to have an average ($\bar{x} \pm s$) execution time of 63.9 ± 41.94 sec.

To assess the potential of examining root hairs along a single root and in relation to the distance from the meristem, an image with a single root was assessed using the algorithm and then post-processed (Figure 5). Image outputs from the analysis are shown in Figure 5A, with the user-marked root tip meristem and the calculated spline fit to the centre of the root based on the output of the RootHairAreaFinder (Figure 5Aii). Figure 5B shows the output images from the algorithm analysis steps aligned so the spline appears linear. Using the spline to measure the distance from the root tip, the diameter of the root, and the average radius of the root hair zone was calculated. Figure 5C shows the root width (C_i , C_{iii}) and root hair length (C_{ii} , C_{iv}) at each pixel distance along the length of the root as raw data ($C_{i,ii}$) or as pooled averages of 5 mm distance from the root tip ($C_{iii, iv}$). This analysis used a `roothair_pixel_radius` (user selected radius of root hair zone outside of the root boundary) value of 100. Root width at distance

along the root was shown to be stable except for the first 5 mm, and the section where there was a gap in the visible root system (Figure 5Ci, iii), where root hair length along the root gradually increased with distance from the root tip (Figure 5Cii, iv)

Algorithm Validation

The validation procedure for the algorithm is summarized in Figure 6. The manually traced VP root hair area enclosed by the traced root hair boundary illustrated in Figure 6A (blue line), was compared with the algorithm predicted VP root hair area (white in Figure 6B). Figure 6C shows the estimated MCC between the manual and automatic assessed VP root hair areas. The average ($\bar{x} \pm s$) VP root hair area MCC value for the analysed sections was 0.65 ± 0.1 . Similarly, Figure 6D illustrates the estimated MCC for the projected root area detection component of the algorithm. The average ($\bar{x} \pm s$) projected root area MCC value for the analysed sections was 0.68 ± 0.09 . The MCC for root area was significantly greater than 0.6 (Wilcoxon signed-rank test, $n = 20$, $p = 0.002$), with a large effect size ($r = 0.62$). Root hair area showed a similar but weaker effect, with MCC values also significantly exceeding 0.6 ($n = 20$, $p = 0.022$) and a moderate effect size ($r = 0.45$).

The `roothair_pixel_radius` sets the area around the root that is searched for root hairs. If the parameter is set too low, over trimming of root hairs occurs producing an output underestimating the root hair region of the image. A higher value increases the potential for errors in the non hair region, and also increases the computational power required for analysis of images. To assess the effect of changing the `roothair_pixel_radius` on algorithm function, we assessed a range of values (from 30 to 250 in steps of 20) on the images used for Figure 4 & 5 and cropped areas (Figure 6 E-H)). The `roothair_pixel_radius` did not, as expected, impact the projected root area (Figure 6E); however, it did impact the VP root hair area depending on image size (Figure 6F). The first derivative (Figure 6G) of the VP roothair area against the `roothair_pixel_radius` shows the extent to which the area increases as the `roothair_pixel_radius` parameter increases. Between `roothair_pixel_radius` values of 100-150, the rate of increase declines for the majority of images suggesting the optimal parameter setting for this set of images is between 100-150. We also assessed the speed of the algorithm on multiple single root image (e.g. Figure 4) image, the image used in Figure 5, and multiple subsets of that image. Figure 6H shows the relationship between the image pixel size and the speed of analysis (when not writing intermediary analysis images to drives).

Root hair responses to environmental constraints

Table 1 shows the estimated metric potential, penetrometer resistance and air-filled porosity of each of the treatments based on the water release curves shown in supplementary file 2). The degree of saturation within the rhizotron was estimated to be 16, 27 and 36% for the 2, 4 and 6 IRE treatments respectively with the values being almost identical across DBD treatments. Based on the established water release curve of the RS substrate (Table 3), the above-mentioned values will respectively correspond to a matric potential of -10.57, -8.76 and -7.66 kPa for the DBD treatment of 1.5 g cm⁻³. The mean penetrometer resistance and air-filled pore space values matching those matric suctions were respectively: 1.78, 1.68 and 1.59 MPa, and 0.41, 0.36 and 0.31%. Across the range of treatments, the lowest DBD (1.4) with the lowest irrigation, had more available water, was at the lower end of the penetration resistance scale and had more air-filled pores. Whereas the highest DBD with the highest irrigations levels, had higher moisture levels, higher penetration resistance but lower levels of air-filled pore space.

The effects of different dry bulk densities (DBD) and differential watering levels (IRE) on root growth parameters are illustrated in Figure 7. Root responses to these conditions, average root growth rate (ARGR), average vertical growth rate (AVGR), and average horizontal growth rate (AHGR) are shown in Figures 7A–C, respectively, while the average root hair to root ratio (ARHtRR) is presented in Figure 7D.

For ARGR, both DBD (ANOVA $F = 8.4935$, $p = 0.003^*$) and IRE (ANOVA $F = 6.2317$, $p = 0.0086^*$) were significant factors, while their interaction was not (ANOVA $F = 1.1427$, $p = 0.3627$). Increased DBD reduced the AVGR across the combined IRE treatments. This was driven mainly by the difference between 1.4 & 1.5/1.6 treatments (Table 2). Increased irrigation (IRE) also decreased the ARGR across the combined DBD treatments driven mainly by the difference in growth between 2/4 IRE treatments and the 6 IRE treatment.

In the case of AVGR, both DBD (ANOVA $F = 7.3805$, $p = 0.0048^*$) and IRE (ANOVA $F = 4.4108$, $p = 0.0269^*$) were significant, with no significant interaction (ANOVA $F = 0.8137$, $p = 0.5163$). Both increased DBD and increased irrigation (IRE) reduced the AVGR across the combined IRE and DBD treatments respectively. The DBD effect was mainly driven by differences in vertical growth between treatment 1.4 and the 1.5/1.6 (Table 2), whereas the IRE effect was driven mainly by differences in 2 IR vs. 4 or 6 IRE (Table 2).

For AHGR, DBD (ANOVA $F = 4.7862$, $p = 0.0257^*$) and IRE (ANOVA $F = 3.9625$, $p = 0.0395^*$) were significant, but their interaction was not (ANOVA $F = 1.6087$, $p = 0.2221$). AHGR reduce with increased DBD, with differences mainly driven by differences between DBD 1.4 & DBD 1.5/1.6 (Table 2). The highest AHGR were found in the intermediate IRE measures (Table 3) with significant differences between 2 & 4, 4 * 6 but not 2 & 6 IRE (Table 2).

Finally, for ARHtRR, neither DBD (ANOVA $F = 1.2022$, $p = 0.3196$), IRE (ANOVA $F = 0.1235$, $p = 0.8639$), nor their interaction (ANOVA $F = 1.8112$, $p = 0.17$) were significant.

Table 3, contains a summary of the rank scores of ARGR, AHGR and AVGR, for each of the experimental treatments. The largest ARGR value corresponded to the 1.4 DBD and 2 IRE treatment while the smallest corresponded to the 1.5 DBD and 6 IRE treatment. The largest AHGR value corresponded to the 1.4 DBD and 2 IRE treatment while the smallest corresponded to the 1.5 DBD and 6 IRE treatment. The largest AVGR value corresponded to the 1.4 DBD and 2 IRE treatment while the smallest corresponded to the 1.6 DBD and 2 IRE treatment. The largest ARHtRR value corresponded to the 1.5 DBD and 2 IRE treatment while the smallest corresponded to the 1.4 DBD and 2 IRE treatment.

Discussion

Root detection

The validation analysis enabled an estimation of the error associated with the proposed algorithm for detecting and quantifying roots grown in the RS. The average ($\bar{x} \pm s$) projected root area MCC value for the analysed sections was 0.68 ± 0.09 ($F1 = 0.67 \pm 0.1$), indicating good overall performance. Scores above >0.5 are considered to suggest strong performance (58,59). Although root segmentation accuracy is good for the proposed RS, it should be stated that there are ML based root detection algorithms that achieved higher root segmentation accuracy ((60): $F1 = 0.7$; Narisetti et al. (61): $F1 = 0.87$), but require prior model training and do not provide an estimate of VP root hair area. In contrast, the algorithm by Lu et al. (35), achieved extremely high accuracy for the species tested (average $F1 = 0.97$), and is also able to segment root hairs with similarly high precision. However, the algorithm is again ML-based, and runs on small images (< 400 KB – based on Supplementary file) capturing only local root patches, not whole root architecture. It is also not clear, what the runtime of the algorithm is, especially when provided with much larger images. For the proposed algorithm, the average ($\bar{x}$

$\pm$ s) user time required to execute the algorithm was just 63.9 ± 41.94 sec for seedlings at 3 weeks of growth, highlighting its use for analysing images in near real-time.

The proposed method is therefore a fast alternative to manual annotation, but achieves agreement with expert segmentation within the expected range for human-derived labels in complex root imaging tasks. The observed MCC values (~ 0.68) indicate performance comparable to expert-level interpretation while retaining full automation. MCC values were consistently larger than 0.6, a commonly used threshold for substantial agreement in segmentation tasks, supporting robustness across samples. Thus the approach combines expert-comparable rapid extraction of roots and root hairs via a fully automated processing pipeline, eliminating user variability, and scalability to whole-root-system images, rather than exceeding manual annotation accuracy.

Although the algorithm was validated using spring barley, it is expected to work for other plant species with bright white roots. Because the RS is standardized and the algorithm requires no prior training, unlike other ML-based methods, it is expected to generalize naturally. This could be readily verified in future experimental work.

Root hair detection

Intuitively, the ability to detect fine root structures such as hairs, will be positively correlated with image quality. As such, the first step was in assessing in a more objective way the effect of different imaging surfaces on image quality. The method used to quantify image sharpness although simple, tends to be more robust to noise relative to most other candidates (62) and led to the selection of a 2 mm thick acrylic object (Figure 3) for scanning. The next challenge was to reduce the effect of the growth substrate itself on image quality. Figure 8, illustrates why sand was selected as a substrate as opposed to the more mechanically complex soil since its shrink-swell cycles effectively lead to particles covering most of the root material.

As for the validation, the segmentation accuracy for VP root hair area was similar to that of roots, achieving an MCC value of 0.65 ± 0.1 ($F1 = 0.73 \pm 0.1$). The observed variability in MCC values across images reflects differences in image complexity, including variation in root density, root hair visibility, and background texture inherent to minirhizotron imaging. Despite this spread, all MCC values remained positive, with a minimum of 0.46, indicating consistent agreement between predicted and ground truth segmentation across heterogeneous conditions. Across the three cultivars used to validate their algorithm, Vicent et al. (30) obtained F1 scores ranging from 0.32–0.72, with an average value of 0.48 (estimated from Table 3). The accuracy

of their method was generally lower than that of the proposed algorithm, but was comparable for papaya grown in peat and perlite, likely due to the dark root tones. However, the method is semi-automated, requiring user input, and focuses on relatively small regions of the root system. Conversely, similar for root segmentation, the algorithm of Lu et al. (35) achieved a higher F1 score 0.852 for VP root hair area. However, the same limitations mentioned above for root segmentation also apply for the root hair component of that algorithm. Neither of the previously mentioned algorithms can extract overall root architecture and root hairs from a single image while also maintaining locational information. However it should be noted that the analysis is restricted to the focal plane of the image which means that there will always be root hairs that are not visible, either because they are out focus or are covered by other root material or sand particles. This is however, an intrinsic limitation of the RS, where the analysis is restricted to the thin interface of the scanning surface the substrate/root mixture. Furthermore, although a number of authors tend to consider only clearly visible root hairs (26,63) in their analysis, this was avoided here because it was found to give a misleading estimate of the total VP root hair area, especially at areas of high root hair density. Another limitation is that the algorithm identifies VP root hair area but not actual root hair count or more complex parameters. This is unfortunately not possible with the RS as the images are restricted to a 2D image plane. Furthermore, because the root hairs were 1 pixel in width, the process of correctly counting them will be especially challenging and likely prone to errors (30,35).

Root growth responses to experimental treatments

This ranges of penetrometer resistance, matric potential and air-filled pore space imposed in this study fall within those commonly associated with the least limiting water range, where root growth is jointly constrained by water availability, aeration and mechanical impedance (64). The review of Bengough et al. (7) suggested that in maize root elongation rate is halved at a matric potential of -500 kPa and a penetrometer resistance value of 2 MPa. Similarly in soils spring barley roots have been shown to decrease root elongation rates by more than 50% in response to penetrometer resistance measures of 1 MPa at a matric potential of -20kPa (38). The matric potential achieved in this study ranged from only a fraction of that magnitude was achieved in this study ranged from 10.57 to -7.66 kPa. The reason for this narrow range in matric suction and (relatively) larger range in air-filled pore space was clearly due to the dominance of the large pores in the RS substrate and the level of irrigation. Nevertheless, the gradual decrease offered a spectrum of increasing water availability and enabled the

manipulation of water stress, although it was restricted to the macro-pore scale, with an average absolute treatment difference of 20.14% in ARGR between the 2 and 6 IRE treatments for each DBD treatment. The estimated penetrometer resistance range was also from 1.08 to 1.95 MPa. This range of penetrometer resistance has been shown to halve barley root growth in soil (38) and similarly the root growth in the RS system was more than halved by the increased DBD treatments (Figure 7). The reduction in root growth indicates that an increase in the DBD translates to an increase in mechanical impedance by reducing root elongation rates. Our findings were thus, in agreement with the general trend of an inverse relationship between DBD and root elongation rates (65).

Bengough et al. (66) concluded that root hairs enhanced root penetration ability in low density ($1.0\text{--}1.2\text{ g cm}^{-3}$) soils but not to higher density (1.5 g cm^{-3}). In this study, no significant differences could be detected across the $1.4\text{--}1.6\text{ g cm}^{-3}$ density range ($p = 0.3196$). Although, the difference in penetrometer resistance was large enough to induce a statistically significant difference (1.4 DBD treatment) in terms of ARGR this effect was perhaps not enough to induce a change in VP root hair area. Root hairs are also critical for solute absorption (67) and as such, intuitively at least, one might expect a reduced ARHtRR for the higher IRE treatments. However, there was no evidence of a statistically significant difference between the IRE treatments ($p = 0.8639$). Results from Haling et al. (68) also suggest a differential response in root hair length among different strength and water treatments. Although, root hair length is not reported here a differential response between treatments will have affected the estimates of VP root hair area which will in turn affect ARHtRR but, no such variation was detected here. However, it must be considered that the minimum and maximum penetrometer resistance values of the treatments used by the authors were 0.03 and 4.45 MPa respectively so the range used was wider than the range used here, i.e., 1.08 to 1.95 MPa. In a similar manner, the low and high-water concentration treatments had a matric potential of around -7.5 and -40 kPa respectively with a significantly lower matric potential limit in comparison to the one used in this study, i.e., -11.36 kPa. This could help explain why the corresponding ARHtRR responses were not detected in the results reported here.

The growth material used here was a mixture of sieved gravel and sand combinations, and the combination used produced an open matrix. However, further evaluation of size fractions along with root and root hair evaluations using the algorithm presented here, including smaller particle ranges, and a wider range of cultivars will allow further understanding of the impact of soil physical conditions on root and root hair responses to soil physical conditions.

Conclusions

This study demonstrates that the proposed RS, combined with a non-ML image analysis algorithm, provides a robust, fast, and potentially generalizable approach for simultaneous quantification of whole-root architecture and VP root hair area from single images. Validation analysis showed good segmentation performance for both roots and root hairs ($MCC \approx 0.65$ – 0.68 ; $F1 \approx 0.67$ – 0.73), comparable to or exceeding existing semi-automated approaches, while avoiding the need for prior model training. Although higher accuracies have been reported for ML-based methods, these typically operate on small image patches, require training data, and do not readily scale to whole-root systems. In contrast, the proposed method enables near real-time analysis of large images, making it well suited for high-throughput or time-sensitive experimental workflows.

Application of the method to controlled water and bulk density treatments confirmed that the RS can impose realistic gradients of matric potential, aeration, and mechanical impedance within the least limiting water range. Root growth responses were primarily driven by changes in dry bulk density, with increased mechanical impedance significantly reducing axial root growth rates, consistent with established relationships reported in the literature. In contrast, no significant treatment effects were detected for relative VP root hair area, likely reflecting the comparatively narrow ranges of matric potential and penetrometer resistance achievable in the RS compared to studies spanning more extreme conditions.

Overall, the proposed RS and analysis framework offers a practical compromise between accuracy, speed, and scalability, enabling integrated assessment of root system architecture and root hair development under controlled physical soil conditions. Future work should focus on extending validation to additional plant species, exploring wider ranges of mechanical and drought stresses, and integrating complementary imaging steps to further enhance accuracy.

List of abbreviations

Root system architecture (RSA)

Dry bulk density (DBD)

Number of irrigation events (IRE)

Image Sharpness Index (ISI)

Average Root Growth Rate (ARGR)

Average Vertical Growth Rate (AVGR)

Average Horizontal Growth Rate (AHGR)

Average Root Hair to Root Ratio (ARHtRR)

Matthews Correlation Coefficient (MCC)

Declarations

Ethics approval and consent to participate – Not applicable to this manuscript

Consent for publication – Not applicable to this manuscript

Availability of data and materials

The RootHairAreaFinder.cpp file, R script and example data files for rhizotron analysis will be made available through github <https://github.com/TracyValentine/RootHairFinder> and <https://zenodo.org/uploads/19288922>

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

TV, GB, SM designed the initial project and obtained the funding from the STARS programme and performed project management. GT designed the rhizotron system, designed and ran the experiments, wrote the code for the algorithm and ran validation tests. KB contributed to the design and running of the rhizotrons and rhizotron experiments. TV contributed to testing the algorithm and writing code for single root analysis. GT compiled the original manuscript with additions from TV. Supervision – TV, BM & SM. All authors contributed to writing, commenting, editing and approving the manuscript.

Acknowledgements

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Tables

Table 1: Approximate matric potential (ψ), penetrometer resistance (PR) and air-filled porosity (f_a) based on rhizotron system (RS) water release and strength curves, across dry bulk density (DBD) and irrigation event (IRE) treatments.

DBD (g cm ⁻³)	IRE	ψ (kPa)	PR (MPa)	f_a (%)
1.4	2	-11.36	1.19	0.44
	4	-8.99	1.12	0.38
	6	-7.97	1.08	0.33
1.5	2	-10.57	1.78	0.41
	4	-8.76	1.68	0.36
	6	-7.66	1.59	0.31
1.6	2	-9.92	1.95	0.38
	4	-8.45	1.85	0.33
	6	-7.25	1.77	0.29

Table 2: Post Hoc comparisons for the impact on dry bulk density and irrigation treatments on root growth parameters obtained using RootHairAreaFinder.

Root parameter	Comparison		psihat	P	
	DBD	IR			
ARGR	1.4 : 1.5		7.2097	<0.0001	***
	1.4 : 1.6		7.876	0.0017	***
	1.5 : 1.6		0.6664	0.4508	ns
		2 : 4	-2.2182	0.1319	ns
		2 : 6	3.4424	0.0317	*
		4 : 6	5.6606	<0.0001	***
AVGR	1.4 : 1.5		9.9035	<0.0001	***
	1.4 : 1.6		9.9211	0.003	**
	1.5 : 1.6		-0.7806	0.2654	ns
		2 : 4	-0.7806	0.2654	ns
		2 : 6	5.7689	0.0100	*
		4 : 6	6.5496	<0.0001	***
AHGR	1.4 : 1.5		2.851	0.0250	*
	1.4 : 1.6		4.571	0.0217	*
	1.5 : 1.6		1.72	0.2604	ns
		2 : 4	-3.9048	0.0284	*
		2 : 6	1.20231	0.2988	ns
		4 : 6	4.9278	0.0117	*

Table 3: Root system rankings for average root (ARGR), horizontal (AHGR) and vertical (AVGR) growth rates, and root hair to root ratio (ARHtRR) medians across dry bulk density (DBD) and irrigation event (IRE) treatments. Highest and lowest ranks are highlighted in bold.

DBD (g cm ⁻³)	IRE	ARGR (mm day ⁻¹)	AHGR (mm day ⁻¹)	AVGR (mm day ⁻¹)	ARHtRR (mm mm ⁻¹)
1.4	2	9.19 (1)	5.9 (2)	11.22 (1)	2.99 (9)
	4	8.66 (2)	6.13 (1)	9.98 (2)	3.35 (8)
	6	5.62 (5)	4.07 (6)	6.63 (4)	4.06 (2)
1.5	2	5.33 (6)	3.47 (7)	6.46 (5)	4.1 (1)
	4	5.89 (4)	5.16 (4)	6.37 (6)	3.55 (6)
	6	5.04 (7)	4.62 (5)	5.09 (9)	3.71 (4)
1.6	2	4.33 (9)	3.3 (8)	5.2 (8)	3.5 (7)
	4	6.51 (3)	5.28 (3)	7.32 (3)	3.83 (3)
	6	4.75 (8)	2.95 (9)	5.39 (7)	3.61 (5)

Figures

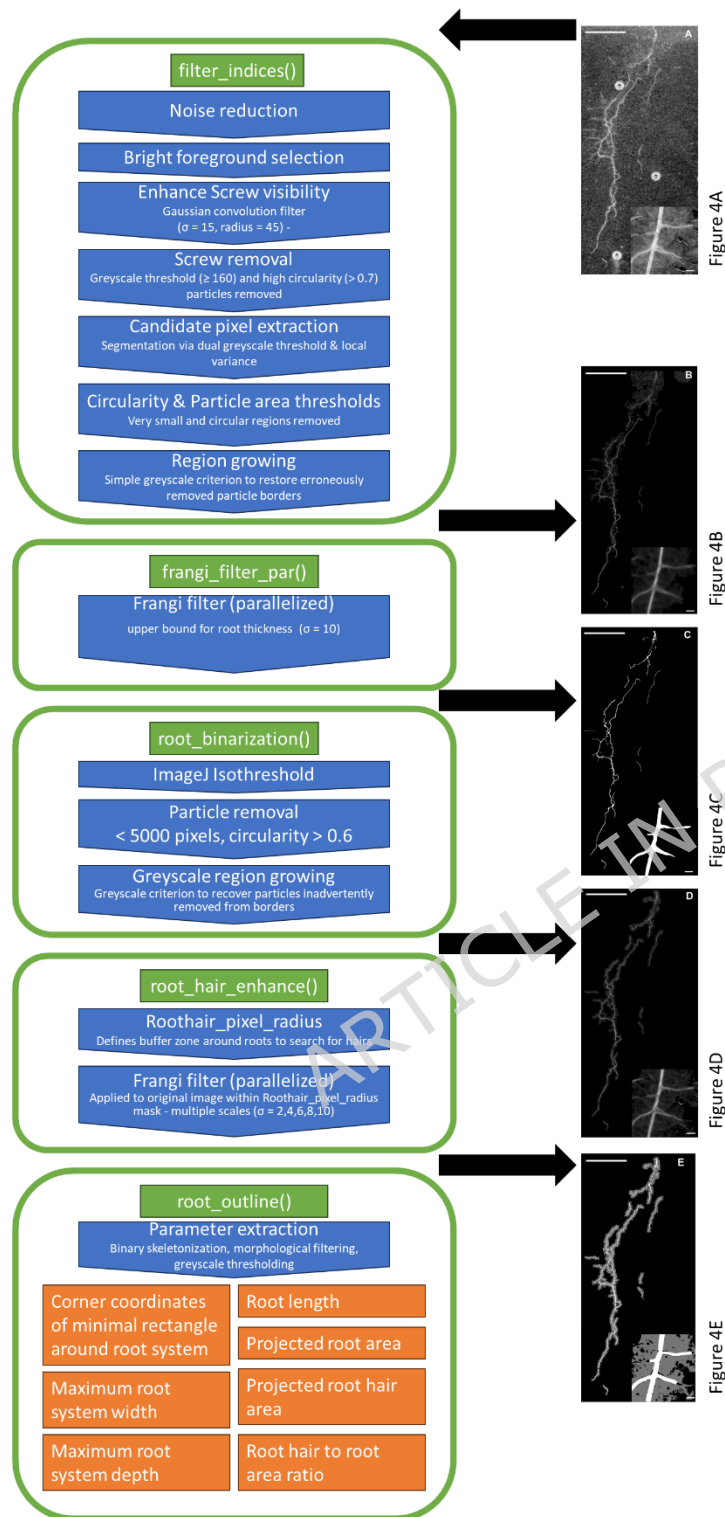


Figure 1: Schematic diagrams of the analysis process. Each Green ringed area represents a function created by RootHairFinder.cpp. Images generated after each function step used (see “RootHairAreaFinder.R”). Blue areas indicate processes included within each function. Orange areas indicate numerical parameters calculated. Images on the right illustrate example

outputs from the analysis, showing the image matrices generated by each RootHairAreaFinder function.

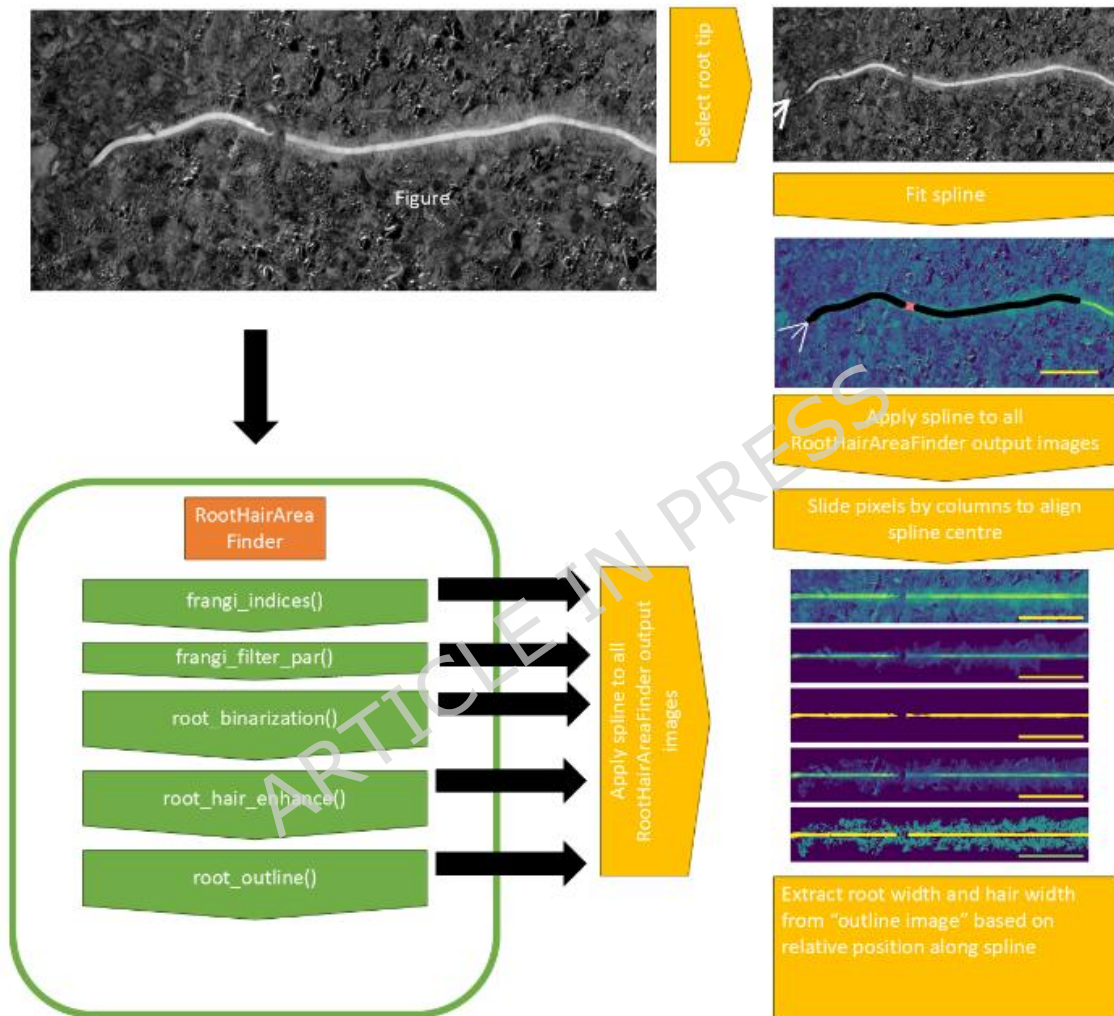


Figure 2: Schematic diagrams of the analysis process of a single root. Each Green ringed and green area represents a function created by RootHairFinder.cpp. Images generated after each function step used (see “RootHairAreaFinder.R”). Light orange areas indicate processes included during the single root analysis.

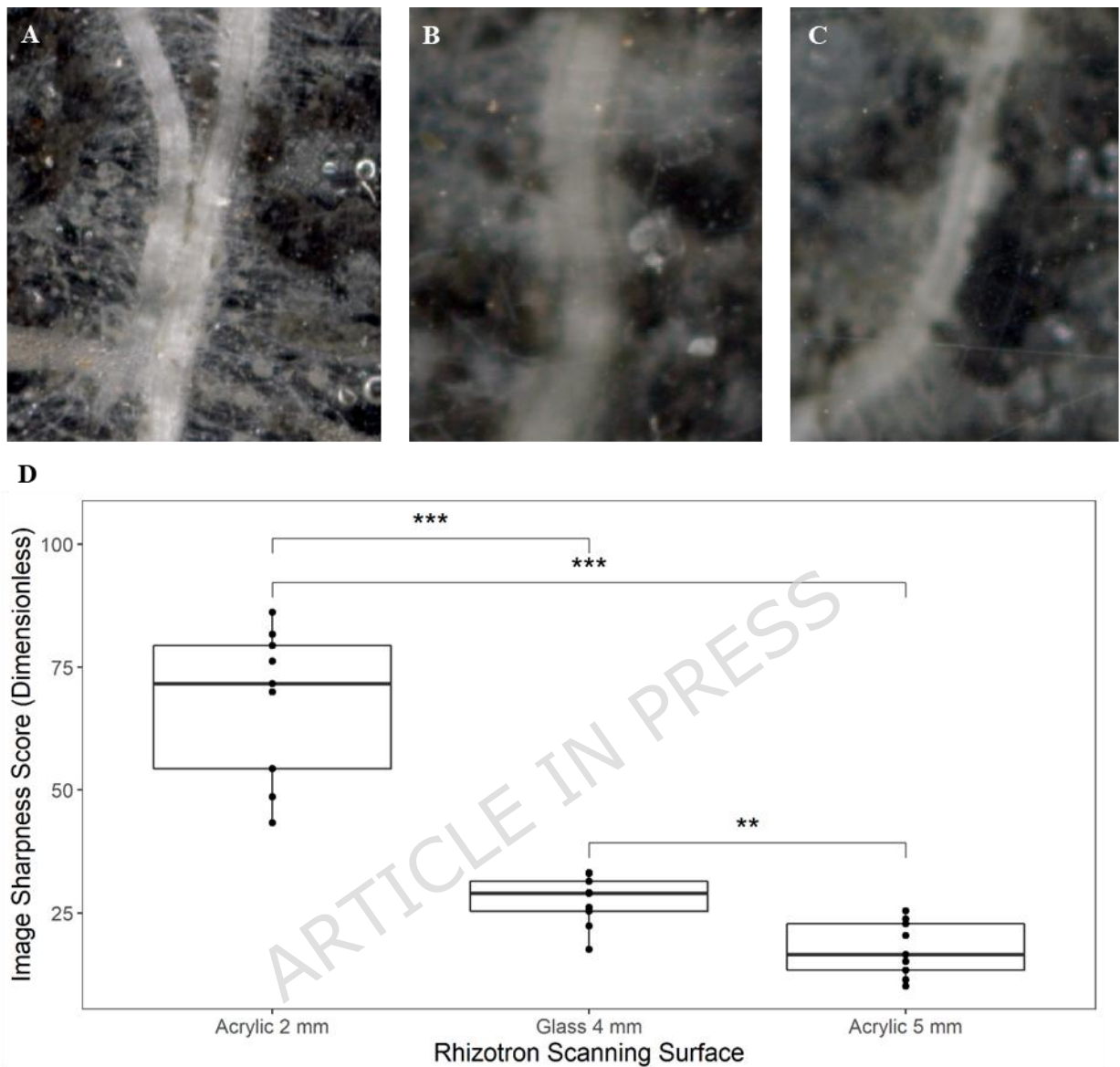


Figure 3: Rhizotron scanning surface experiment comparing imaging quality for **A)** acrylic - 2 mm thick, scanned at 1,600 dpi, **B)** 4 mm thick, low iron, reinforced glass, **C)** acrylic - 5 mm thick and. **D)** ISI boxplot comparison for the three different minirhizotron scanning surfaces; A higher Image Sharpness (ISI) score indicates a higher quality image.

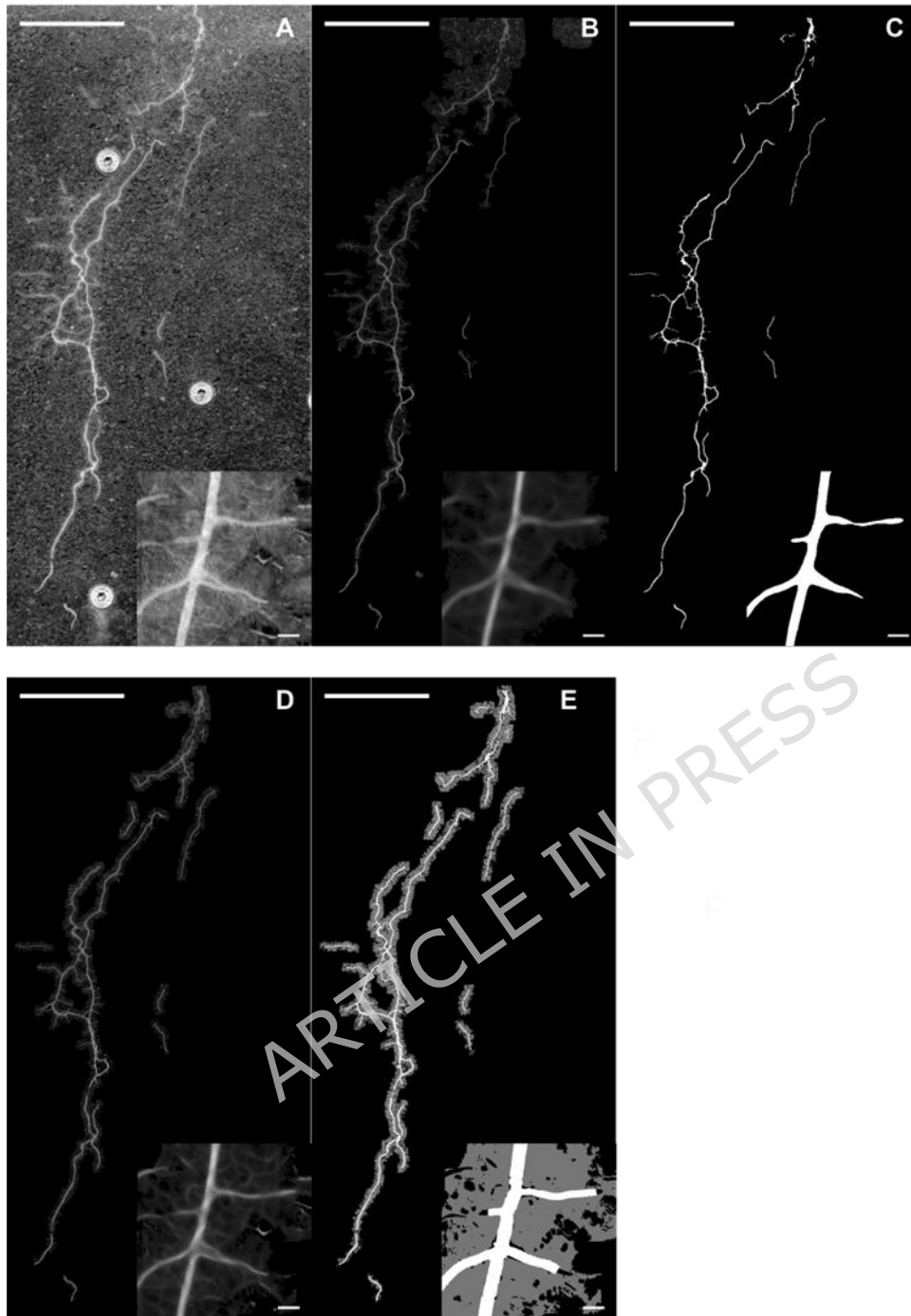


Figure 4: A) Image of A3 rhizotron with 14 day old spring barley seedling obtained with using a flatbed scanner at 1,200 dpi, B) output from frangi_filter_par C) output from root_binarization steps, D) output from root_hair_enhance E) results matrix showing roots and hair area, Main images bar = 5 cm and magnified images bar = 0.1 cm.

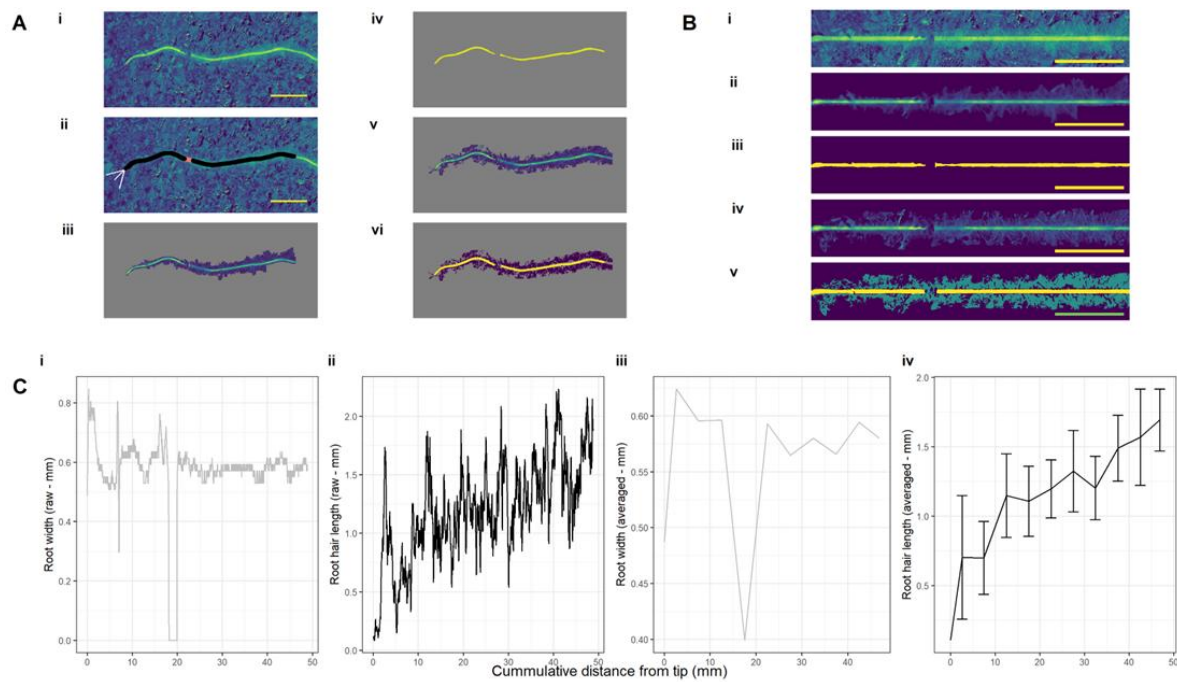


Figure 5: Analysis of single root:

A) Original image analysis – viridis false coloured: (i) Original image; (ii) with spline marked in black and spline fill marked in red; output from (iii) frangi_filter_par; (iv) root binarization; (v) root_hair_enhance; (vi) combined final results. **B)** Extracted root output aligned along the spline (post analysis): (i) original image; (ii) frangi_filter_par; (iii) root binarization for extracting root; (iv) root_hair_enhance; (v) combined results. **C)** Raw (i,ii) and averaged (iii,iv) root width (i,iii) and root hair length (ii, iv) relating to distance from the root tip. Error bars show the variance in root hair length along the 5 mm section of root ((Root hair area width - the root area width)/2). Scale bar = 1 cm

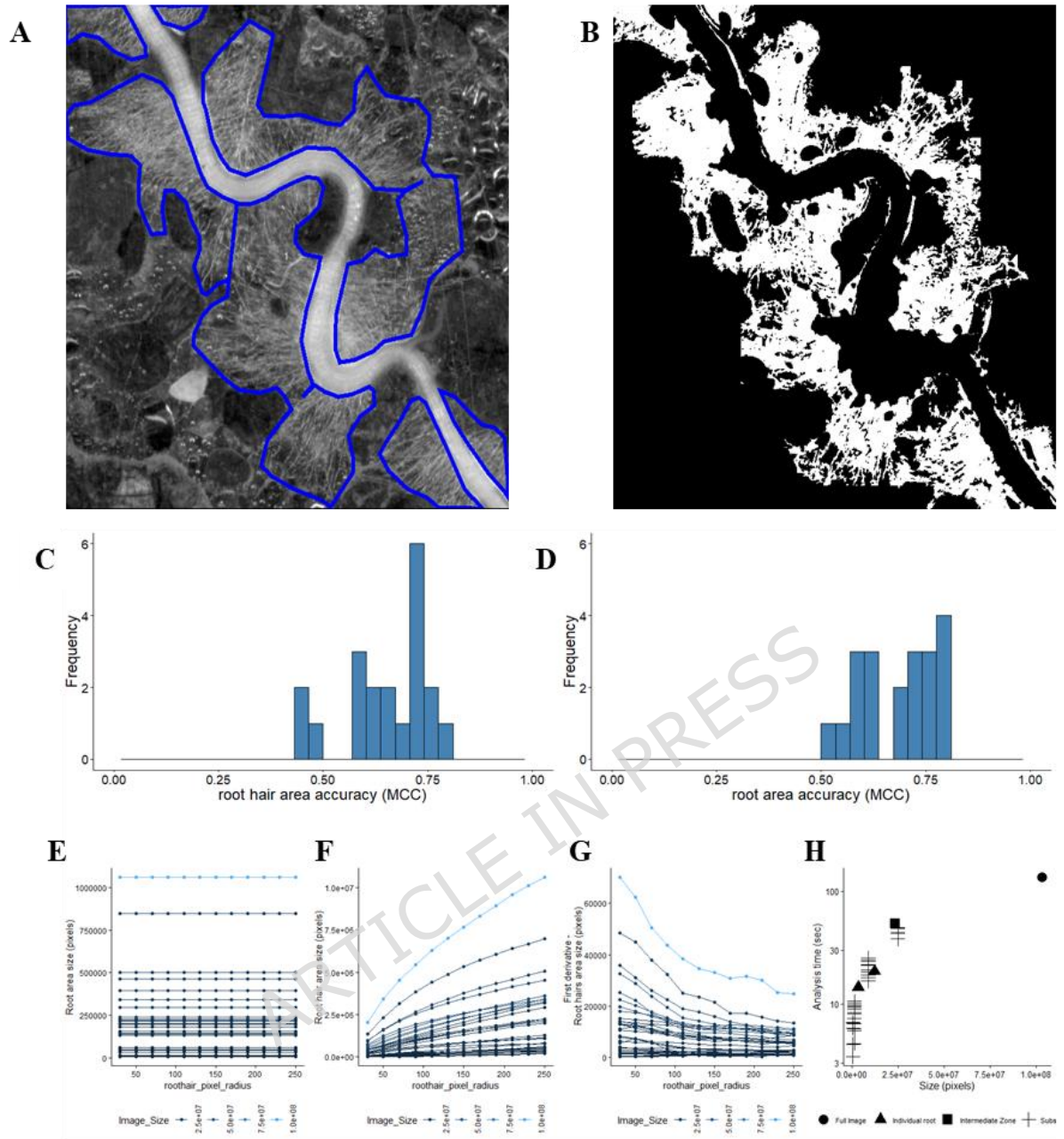


Figure 6: Assessment of the functioning of the root hair algorithm.

Example test image showing manually traced VP root hair area (blue line) (A). VP root hair area (white) predicted by the algorithm after thresholding the area adjacent to the root (B). Histograms of Matthews correlation coefficient (MCC) for predicted VP root hair area (C) and projected root area (D), computed from pixel-wise comparisons of binarized predictions against manually traced root hair masks (300×300 pixels) and projected root area masks, respectively. Evaluation of the effect of increasing the `roothair_pixel_radius` parameter on projected root area (E), VP root hair area (F), and the first derivative of the VP root hair area (G). Evaluation of analysis time of images of different size ranges (H).

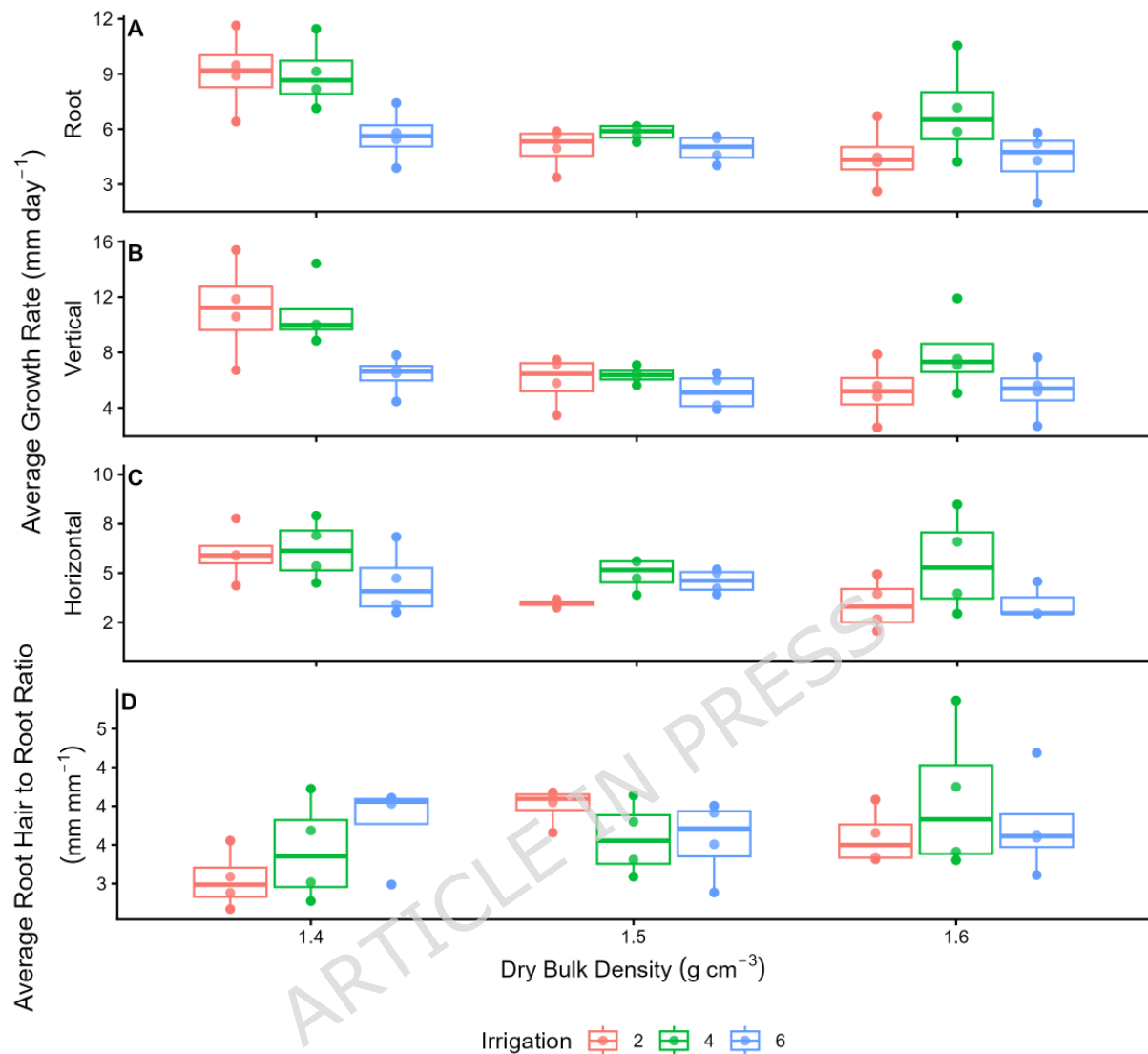


Figure 7: Spring barley root variables at the end of a 21-day growth period in the sand-based minirhizotron system as a function of dry bulk density (DBD) and grouped by irrigation event (IRE). Average growth rates of **A)** Root (ARGR) vs. DBD, **B)** Vertical (AVGR) vs. DBD and **C)** Horizontal (AHGR) vs. DBD, and **D)** Root Hair to Root Ratio (ARHtRR) vs. DBD.

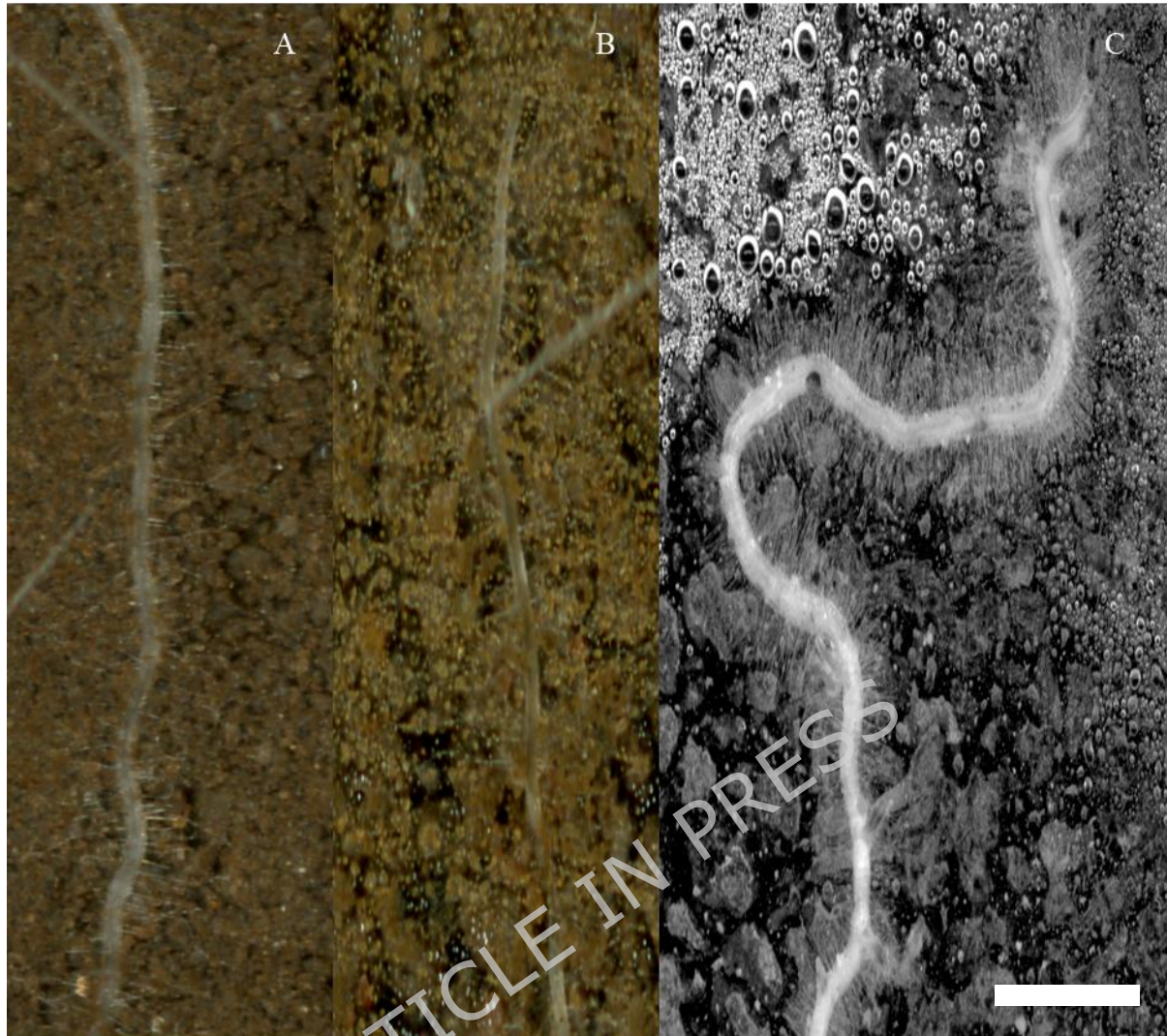


Figure 8: Spring barley imaged at 1,600 dpi after 14 days of growth inside the rhizotron system. The swelling behaviour of both clay (A) and sandy (B) soils results in a significant fraction of the root material being covered by soil particles, especially fine structures such as root hairs. In contrast, such effects could be significantly reduced by using non-swelling inorganic sand (C) as a growth substrate. Bar = 4 mm

Supplementary file 1

Custom made acrylic-based rhizotron units (Figure S1) were filled with a black coloured sand substrate (Flourite Black) which was sieved to have a uniform 16.67 % of its mass for each size fraction (mm): 4–2.8, 2.8–2.0, 2.0–1.4, 1.4–1, 1–0.5, 0.5–0. Leaky pipes were placed at the 2 opposite sides of the rhizotrons and irrigation cables were fitted to them to slowly saturate the rhizotrons over a series of irrigation events rather than forcing a large amount of water in turn connected to an irrigation pump (Boyu FP-1500 Adjustable Pump) in a black light-absorbing water container. The irrigation pump could then be controlled through a custom-made fitted timer to operate when required. Once the substrate had reached field capacity two pre-germinated spring barley (*var. Optic*) seedlings with a root length between 1–2 cm were randomly chosen and placed on top of the sand and were subsequently covered with moistened sand. The rhizotrons were then covered with a black light absorbing cloth until the termination of the experiment in three weeks. The second barley seedling was removed after 48 hours so that only one actively growing seedling remained after the initial establishment phase.

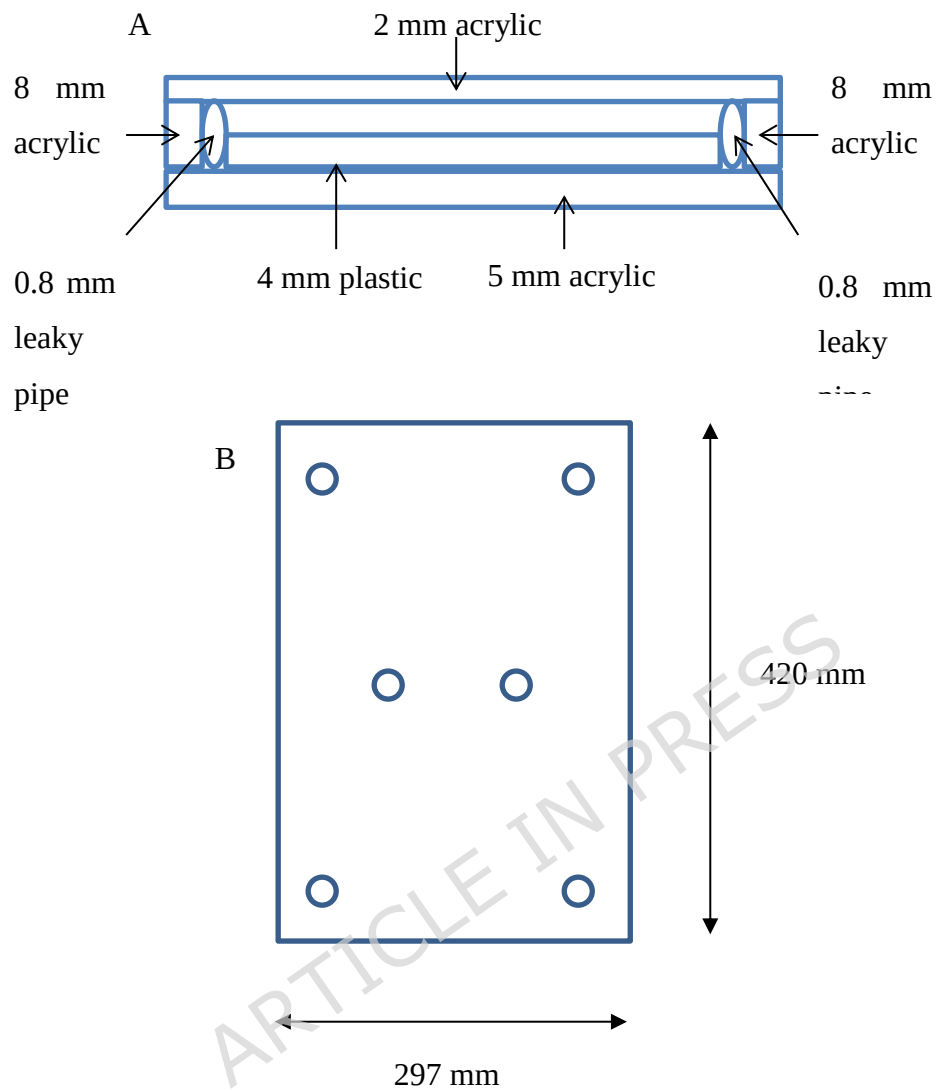


Figure S1: Schematic diagram of new minirhizotron design B **A)** Top view and **B)** Front view (circles indicate screws).

Supplementary file 2

The “Flourite Black Sand” came in two different size fractions both gravel (8–4 mm) and sand (4–0 mm). The gravel and the sand were first mixed in a 50:50 ratio (gravimetric) and the entire mixture was subsequently sieved at 4.0, 2.8, 2.0 and 1.4 mm to create mixtures of 4.0–2.8, 2.8–2.0, 2.0–1.4 and 1.4–0 mm respectively. Each mixture was then washed with tap water for three times and subsequently oven dried at 105 °C.

A series of cylindrically shaped (4.5 mm diameter x 5.0 mm height) repacked sand cores were packed at different dry bulk densities (1.4, 1.5 and 1.6 g cm⁻³) (n = 4). The gravimetric moisture content of each sand core was first adjusted to 20 % to give it consistency. Cores were saturated with degassed water, then subjected to a sequence of different matric suctions (5, 10, 20 and 50 kPa) *via* the use of sand and tension tables. A needle penetrometer (1 mm diameter, 30° cone angle, 4 mm min⁻¹ penetration rate, readings; averaged at 1–mm intervals from 5–15 mm depth range) fitted to a mechanical test frame (Instron model 5544; Instron, MA, USA), with a 50 N load cell accurate to 2 mN at maximum load, was used to measure penetration force at each matric suction and calculate penetrometer resistance (force divided by cone cross-sectional area) (37,38) (69,70). The measurements of mass and penetrometer resistance enabled the estimation of the sand water release and strength curves respectively (Figure S2). This enabled an assessment of the differences between samples with contrasting physical properties and how that may affect their strength and water holding capacity.

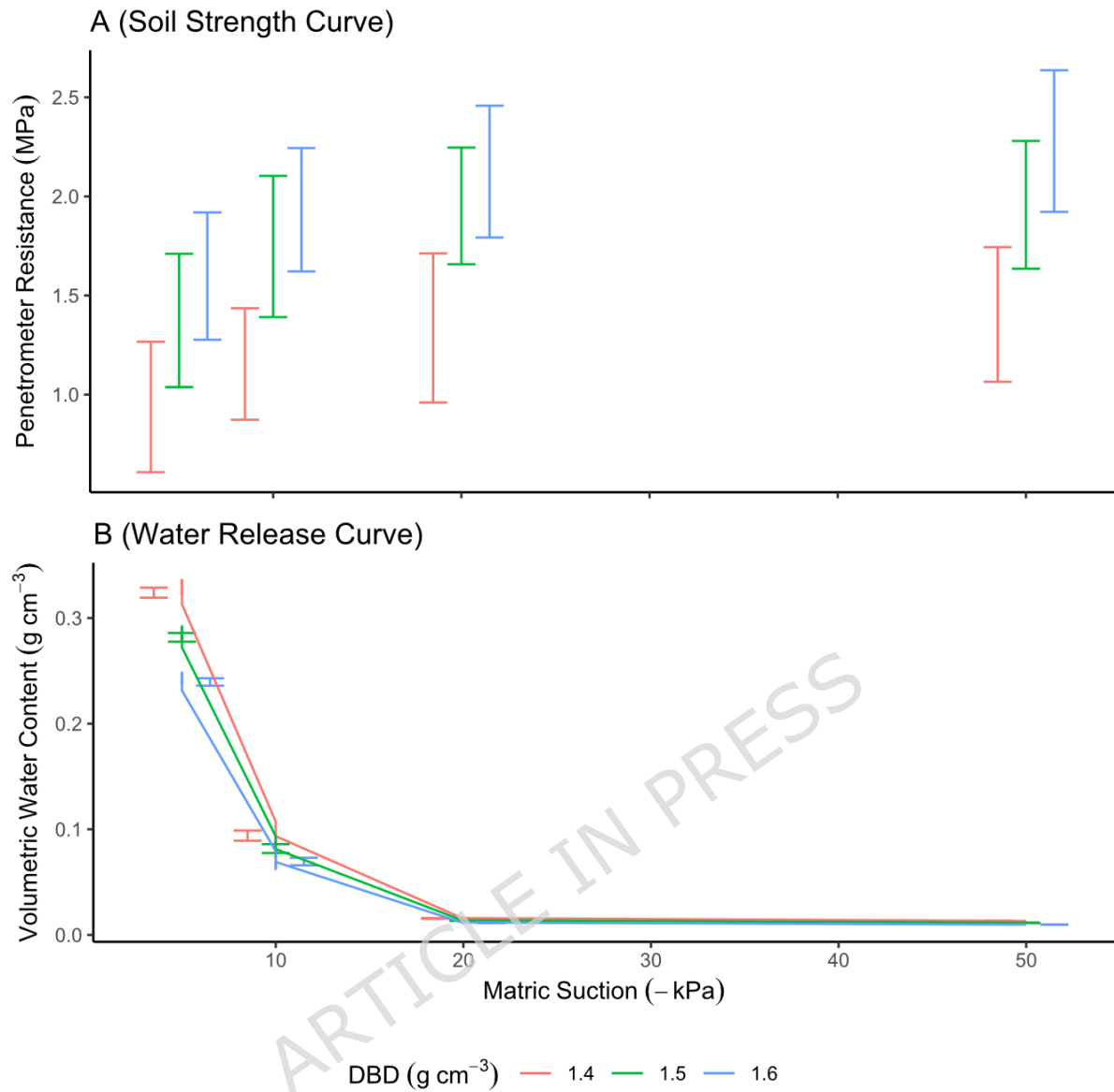


Figure S2: Sample mean $\pm$ standard deviation of rhizotron system substrate strength curves (**A**) and water release curves (**B**) for dry bulk density values of 1.4, 1.5 and 1.6 g cm^{-3} with connecting lines for the mean values.

Supplementary file 3

`filter_indices(grey_matrix)`

The first step pre-processes the image to reduce noise and select a subset with a high probability of being foreground. A Gaussian convolution filter ($\sigma = 15$, radius = 45) is applied to enhance screws visible in the rhizotron images. A grayscale threshold (≥ 160) is then applied, and particles with high circularity (> 0.7) are removed.

The remaining candidate pixels are further segmented using a dual threshold based on grayscale intensity and local variance (2×2 kernel). Circularity and particle area thresholds are applied to the resulting mask to isolate potential foreground pixels. A region-growing algorithm based on a simple grayscale criterion is then used to restore pixels that may have been erroneously removed along particle borders. These pixels are passed to the next step.

`frangi_filter_par(grey_matrix, foreground_id, sigma)`

This step enhances vascular structures by applying a parallelized version of the Frangi filter to the candidate pixels from the previous step. A hyperparameter σ value of 10 is used as the upper bound for root thickness.

`root_binarization(vessels_root)`

The enhanced vascular image is thresholded to extract the root foreground. The ImageJ IsoData threshold is first applied, after which particles are removed based on size (< 5000) and circularity (> 0.6). A region-growing algorithm, based on a grayscale criterion, is then used to recover pixels that may have been mistakenly removed along the borders of remaining particles.

`root_hair_enhance(grey_matrix, root_binary, roothair_pixel_radius)`

The resulting binary root image is analyzed to enhance root hairs extending beyond the detected roots. The hyperparameter `roothair_pixel_radius` defines a buffer zone around the roots within which root hairs may be present. The Frangi filter is applied again to the original grayscale image at multiple scales ($\sigma = 2, 4, 6, 8, 10$), and the maximum response across scales is retained.

`root_outline`:

The final step uses information from the previous stages to quantify root traits such as root length and projected areas of roots and root hairs. These traits are extracted using a combination of binary skeletonization, morphological filtering, and grayscale thresholding.

Supplementary file 4

This is a separately uploaded file “Supplementary_file_4.tif”

Supplementary file 5

This is a separately uploaded file “Supplementary_file_5.tif”

Supplementary file 6

The following definitions are used for the variables that characterise root growth in images of the rhizotron system (RS):

$$F1 = \frac{2 * TP}{2 * TP + FP + FN}$$

$$MCC = \frac{(TP * TN) - (FP * FN)}{\sqrt{(TP + FP) * (TP + FN) * (TN + FP) * (TN + FN)}}$$

where TP, TN, FP, and FN correspond to true positive, true negative, false positive, and false negative pixels obtained by comparing automated segmentation outputs against manually annotated binary masks.

- Vertical Root Length (VRL): The difference between the minimum and maximum vertical coordinates of the visible root system grown in the RS. The measured value is expressed in units of mm.
- Horizontal Root Length (HRL): The difference between the minimum and maximum horizontal coordinates of the visible root system grown in the RS. The measured value is expressed in units of mm.
- Vertical Growth Rate (VGR): The difference between the VRL values at 2 different time points (t) divided by the time interval (δt), i.e.,
 - $VGR_{ti} = [(VRL_{ti} - VRL_{t(i-1)}) / \delta t]$.
 - The calculated value is expressed in units of mm day^{-1} .
- Horizontal Growth Rate (HGR): The difference between the HRL values at 2 different time points (t) divided by the time interval (δt), i.e.,
 - $HGR_{ti} = [(HRL_{ti} - HRL_{t(i-1)}) / \delta t]$.
 - The calculated value is expressed in units of mm day^{-1} .
- Root Growth Rate (RGR): The weighted sum of the VGR and HGR values. The weights are defined by the corresponding RS dimensions of height (400 mm) and length (261 mm), i.e.
 - $RGR_{ti} = (VGR_{ti} * 0.605144) + (HGR_{ti} * 0.394856)$
 - The calculated value is expressed in units of mm day^{-1} .
- Average Root Growth Rate (ARGR): The average value of the RGR values from each time point (t), i.e.,
 - $ARGR = (\sum_{ti=1}^{ti=n} RGR_{ti}) / n$
 - The calculated value is expressed in units of mm day^{-1} .
- Average Vertical Growth Rate (AVGR): The average value of the VGR values from each time point (t), i.e.,
 - $AVGR = (\sum_{ti=1}^{ti=n} VGR_{ti}) / n$
 - The calculated value is expressed in units of mm day^{-1} .
- Average Horizontal Growth Rate (AHGR): The average value of the HGR values from each time point (t), i.e.,
 - $AHGR = (\sum_{ti=1}^{ti=n} HGR_{ti}) / n$

- The calculated value is expressed in units of mm day^{-1} .
- Root Hair to Root Ratio (RHtRR): The ratio of the VP root hair area (VPRHA) to the projected root area (PRA).
 - $RhtRR = VPRHA/PRA$
 - The measured value is dimensionless (mm mm^{-1}).
- Average Root Hair to Root Ratio (ARHtRR): The average value of the RHtRR values from each time point (t), i.e.,
 - $ARHtRR = (\sum_{ti=1}^{ti=n} RHtRR_{ti})/n$
 - The calculated value is dimensionless.