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Technical Report

Automatic image recognition to determine morphological development and secondary metabolite accumulation in hairy root networks

This study focuses on the morphological development and secondary metabolite production of the red pigments from the group of betacyanins in hairy roots of *Beta vulgaris*. We demonstrate a working, medium throughput, customized, automatic image recognition solution for hairy roots on agar plates including the evaluation of 12 experimental samples. Image acquisition is conducted under comparable parameters using a tripod with light emitting diode background lighting and a digital single lens reflex camera. The server-based image recognition system developed together with Wimasis GmbH, Munich, Germany helps to obtain not only quantitative values for morphological parameters, such as segment lengths and widths or metabolite concentrations, but also global parameters of root growth, such as total root length or the number of branching points. Using timed diagrams the development of the total root length, the total number of branching points, and the mean pigment concentration during the cultivation period were determined. The generated data present the basis for detailed mathematical modeling in order to achieve a structured growth model for hairy roots. A mathematical model for growth of hairy roots can be used to decrease experimental efforts as well as to optimize cultivation conditions and the bioreactor design.

Supporting information available online

Keywords: *Beta vulgaris* / Hairy roots / Image recognition / Plant cell tissue / Secondary metabolitess

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1 Introduction

Plant cells bare a wide range of nutritional, physiological, and pharmaceutical relevant secondary metabolites [1, 2]. However, conventional industrial production is limited by environmental and geographical influences [1]. The production of secondary metabolites in vegetable cell and tissue cultures (e.g. hairy roots) can be considered alternatively to classical technologies [3]. In this case, a year-round cultivation in the bioreactor under optimal conditions is possible. This approach also does not use harmful substances such as pesticides and therefore allows a sustainable and gentle-on-resource production. Before the industrial scale up of a production process, biomass formation, and the production of the relevant target is investigated experimentally in a laboratory scale and the gained data is starting point for numerical simulations [4, 5].

Hairy roots of *Beta vulgaris* is used as a model system for the expression of betacyanin red pigments and the three growth parameters (tip movement, branching, and secondary thickening [6]) as well as their respective development over time should be investigated experimentally to improve the cultivation conditions and medium composition [7, 8].

In order to get significant data for highly statistical processes an enormous amount of experiments with respective analysis of the gained data is necessary. Therefore, an automatic solution for optical in situ analysis of the root network on petri dishes was developed. To acquire pictures under comparable conditions, a petri dish tripod together with light emitting diode (LED) background lighting was designed.

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Abbreviation: MS, Murashige & Skoog

While it is possible to generate a large number of pictures due to the medium throughput of the image acquisition unit a powerful solution to evaluate the taken pictures is necessary. Automatic image recognition systems have advanced significantly during the last years in terms of quality of object recognition as well as in time consumed [9]. Nowadays, it is possible to analyze images quantitatively in order to evaluate specific characteristics of the image. Objects in each image get selected and their properties such as dimensions or number of occurrences are transferred into a machine-readable format for further computation. Image recognition systems can be considered as a powerful tool for broad investigations of plant tissue growth processes but until now applications in research are mostly limited to medical problems [10, 11].

2 Materials and methods

2.1 Hairy root cultures of *B. vulgaris*

Hairy roots of *B. vulgaris* induced using *Agrobacterium rhizogenes* ATCC 15834 are used as a model system for the investigations of growth morphology and distribution of secondary metabolite concentrations. Subcultures produced as in reference [2] have been used for all investigations. The relevant secondary metabolite contained in *B. vulgaris* hairy roots in this case is the red betacyanin pigments. It is an advantage of the model system that the concentrations of this red pigment can be seen and quantitatively analyzed with imaging techniques.

To induce hairy root cultures, parts of the selected species such as leafs got hurt so that cells get exposed. At those wounded parts phenols are excreted that attract the *A. rhizogenes* via chemotaxis. The used *Agrobacterium* inserted its root-inducing (Ri)-plasmid into the plant genome. Parts of the Ri-plasmid containing the transfer DNA (tDNA) became part of the plant genome [12]. This activity caused the so-called hairy root disease and makes the cells to proliferate and form only small root hair complexes [13]. The genetic alteration of the plant cells is permanent and it is not necessary to stabilize the proliferation with plant hormones [14]. Considering the network structure and its stability, hairy roots can be used for in vitro production of secondary metabolites. For the development process and during the subcultivation cycles, the hairy root tissue on the agar plates can be analyzed optically.

Hairy root explants used in this study were formed during an earlier study [2]. In order to evaluate the recognition algorithm, a small-scale investigation was conducted containing 12 samples only different in their initial root segment length (IRSL). These 12 samples have been obtained from one single plate.

2.2 Growth pattern of hairy root networks

Generally growth processes in dense root networks can be characterized with at least three different growth parameters [4]. Growth in length is considered tip elongation and can only be found in the ending parts of the roots. It is significant that every root tip zone is pigment free and that zone increases during tip elongation. The betacyanin pigments can be first detected when the cell reaches a certain age and the pigment level increases to a certain maximum [11].

Branching points emerge when two directions of growth are formed out of one single line. During cultivation, there is also a secondary thickening that can be found when older cells increase their mass and volume. Such a defined growth pattern leads to a steady accumulation of biomass in root cultures [15].

2.3 Cultivation techniques

Hairy roots were cultured using standard 92-mm-width single use petri dishes (Sarstedt 821473001) filled with a 7 mm $\pm$ 1 mm high hormone-free cultivation medium layer. The used cultivation medium was standard Murashige and Skoog (MS, Duchefa Biochemistry) solid medium [16], supplemented with 30 g/L sucrose (Duchefa Biochemistry, Haarlem, The Netherlands) and 5.5 g/L plant agar (Duchefa Biochemistry). All samples had an IRSL of 9–19 mm, were single segmented, cultivated at 26°C in darkness and taken from subcultured samples running at 21-day intervals [17].

2.4 Imaging techniques

For image acquisition and with respect to reproducible experimental conditions a tripod was constructed, as shown in Fig. 1 (for constructive details see also Supporting information). A digital single lens reflex (DSLR) camera Canon EOS 1000D with a zoom lens Canon EF-S 18–55 mm was installed on the headboard of the tripod. In the middle of the tripod the petri dish can be stored in a holder with a circular opening.

To eliminate shades and to gain better lighting of the hairy roots during image acquisition, a plate with LEDs (OSRAM QOD expansion) was installed on the bottom of the tripod. This resulted in an optimized lighting of the small hairy root complexes on the petri dish and better picture raw material for image recognition. In order to maximize the raw picture quality specific camera settings have been tested and optimal values have been selected (Table 1).

2.5 Image recognition algorithm

After the acquisition of sufficient picture data, the analysis of the hairy root networks on the agar plates is carried out using a customized image recognition algorithm developed to with Wimasis GmbH, Munich, Germany. The image recognition procedure is structured in several independent processes and starts

Table 1. Camera settings

Description	Setting
White balance	3200 K
Light sensitivity	ISO 200
Shutter speed	0.02 s (1/50)
Distance object-camera	160 mm

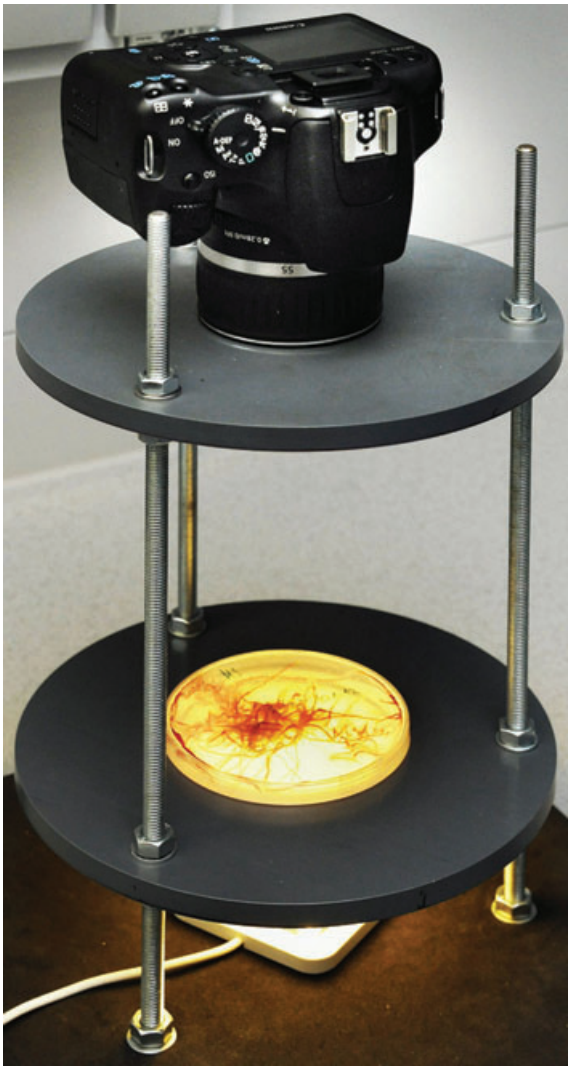

Figure 1. Tripod for image acquisition consisting of digital single lens reflex (DSLR) camera, petri dish holder, and LED background lighting.

with image preprocessing and moves on to the plate detection to define the relevant region for recognition. During the next step of hairy root detection, a skeleton of the network is created. Finally, the measurements of the skeleton are written into a comma-separated value-file (*.CSV) and a new image file for visual confirmation is created.sThe steps of the image recognition process are described in detail in the next paragraph.

2.5.1 Image preprocessing

First a saturation copy is obtained from the original red-green-blue (RGB) image. This new layer of the image allows separating the hairy root network in the foreground from the agar gel in the background. Furthermore, an adaptive noise-removal system and a contrast-enhancing filter are used in order to remove background artifacts and to get a clearer image of the root skeleton.

2.5.2 Plate detection

As only a small part of the picture is relevant for the image recognition process, the edge of the used plate has to be detected and, therefore, a circular region of interest (ROI) that is part of the image gets defined. The recognition algorithm also obtains the diameter of the circular ROI in pixels and converts it into millimeter scale using the particular diameter of the actual petri dish with is encoded in the image filename. This step is necessary as the diameter of the used petri dishes varies by ±1.5 mm and an output in pixel is not sufficient. Using the following equation, it is possible to determine an individual conversion factor (CF) from pixels to millimeter for every single petri dish used during the cultivation campaign.

$$\mathrm{CF}\left[\frac{\mathrm{mm}}{px}\right] = \frac{\mathrm{plate}_{diameter_{\mathrm{mm}}}[\mathrm{mm}]}{\mathrm{ROI}_{diameter_{\mathrm{px}}}[px]} \quad (1)$$

2.5.3 Hairy root network detection

The core hairy root detection process is structured into three substeps. At first all pixels belonging to the root network have to be identified, after that these pixels are consolidated into a coherent skeleton on that and finally the measurements are being conducted.

With the help of a binary mask, the parts of the roots that are in the ROI are identified using a combination of standard deviation and morphological filters. Also, those parts with high red pigmentation are detected. This is done by comparing the red fraction of every image pixel that is part of the root network with the green and blue fraction at the same position.

After obtaining the root binary mask, the image pixels not belonging to the root network are singled out and cropped in order to achieve a skeleton is coherent and does not break apart. In this step, all segments are numbered. This is followed by an analysis of the obtained skeleton as parts in which two or more segments divide or merge, respectively, are either considered as:

- *Branching points*, if a new root sprouts from a previous one in that region, or
- *Crossing points,* if a root overlaps and hides another one in that region.

Occurring crossing points are separated from branching points as those have a characteristic merging before the division from a single point starts. Therefore, the segments containing crossing points are transferred into a new layer. After differing crossing from branching points, the skeleton is divided into independent, numbered segments, which are classified into two layers:

- *Layer 1:* segments that are not overlapped by others.
- *Layer 2:* segments that are partially hidden.

Finally, all recognition data collected from one image is written into a CSV-file that includes segment metrics and branching point metrics and an image visualizing the recognition success is produced. The obtained metrics recovered from every image are the following:

Single segment length (*SSL*): The single segment length is defined in millimeter of a part of the hairy root network that starts

at a branching point or the initial starting point of the root explant and ends at another branching point or a root tip. The values of each length of a single segment are estimated in pixels first taking into account that the distance between two adjacent pixels ranges between a value of 1 px to SQRT(2) px due to the following assumptions: if the segment forms a horizontal or vertical line, the distance between their centers is 1 px, as expected. If it forms a diagonal line, the distance between the centers is the same as the hypotenuse of a right-angled triangle whose cathetus length are 1 px, so it results in a distance of SQRT(2). Summing up to each pixel distances following to both rules mentioned above the SSL for each segment is formed. This means that the SSL in pixel is always greater or equal to the number of pixel forming the examined segment and an exact value of the SSL is calculated. After obtaining the SSL in pixel, it is converted in a millimeter value using the specific CF.

Starting, middle, and end point segment width (*SSW, MSW, ESW*): An important property of each hairy root segment is the width in millimeter at the beginning, in the middle, and at the end of each segment. This information can be used to calculate the volume and, therefore, the biomass of each segment piece. For the middle point and the two end points, the mean root radius is calculated by summing up the pixels orthogonally located on the main skeleton vector. The conversion is done analogically to SSL.

Total number of branching points: Branching points are root regions where a skeleton vector divides and two new roots sprout. These points appear as isolated white areas on the segments skeleton control images. By identifying the number of branching points overlapping root segments have to be isolated and should not be considered as branching point. This is a major difficulty in the image recognition process.

Mean segment length: The mean segment length (MSL) is the mean value of all identified single segment lengths and calculated using the following equation:

$$MSL_{[mm]} = \frac{\Sigma SSL_n}{n} \quad (2)$$

Red pigment concentration: The red pigment concentration (RPC) is defined as the rate of red-pigmented area of the root segment. It is calculated by dividing the number of pixels that belong to these areas by the total number of pixels of the detected root. This value is calculated for each segment, by associating each pixel of the root with the nearest segment.

2.5.4 *Control images*

An important point during the image recognition process is the formation of control images to visualize the results and to identify weaknesses. It is possible to put the root skeleton as a new layer on the image and therefore verify the recognition success.

In Fig. 2, an example detail shows the numbered segments defined from one white dot to another. The trajectory corresponding to the relevant root segment is marked as a red line. In order to visualize the volumetric dimensions of each segment the relevant areas is colored in blue.

In the middle of Fig. 2, an overlaying segment is shown that has been stored on a new layer and been numbered subsequently. In order to distinct correctly between branching points and overlaying segments it was necessary to create a second layer for the skeleton.

A main aim of the research is to identify the areas in the hairy root network where and to what extent secondary metabolites are accumulated. Therefore, a threshold for the RPC value was implemented and the results visualized in a control image. On the far right of Fig. 2, yellow areas of the root network that have an RPC value less than the threshold and red colored areas bare a higher RPC value compared to the threshold are shown consequently. These control images give a clear hint were secondary metabolites and in this case the concentration of betacyanin in hairy roots of *B. vulgaris* are accumulated [18].

3 Results and discussion

The next step in developing a working solution to identify the growth parameters of hairy root networks was a small campaign of experiments to validate the recognition process. A small-scale experiment of 12 parallel petri dishes was conducted with approximately 1 to 2-cm-long explants of hairy roots from *B. vulgaris* producing the red betacyanin pigments. The growth process was monitored over a cultivation time of 11 days and

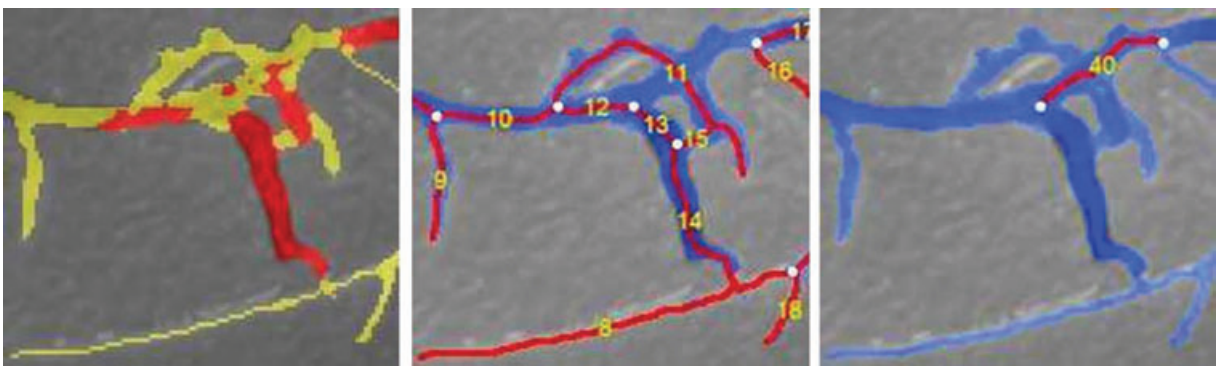

Figure 2. Control image section showing root skeleton with numbered segments, overlaying segments, and metabolite concentration threshold visualization.

Figure 3. Control images with numbered segments, skeleton, and pigment concentration for line 2, day 5, 1st.

Table 2. Individual segment metrics.

Segment ID	RPC [%]	SSL [mm]	SSW [mm]	MSW [mm]	ESW [mm]	Volume [mm^3]
1	75.6	12.00	0.26	0.38	0.43	1.19
2	34.9	0.38	0.17	0.30	0.47	0.03
3	82.8	2.60	0.43	0.47	0.60	0.50
4	14.9	4.64	0.21	0.34	0.64	0.57
5	22.6	2.09	0.21	0.34	0.60	0.24
6	74.8	1.28	0.64	0.47	0.64	0.34
7	39.8	0.77	0.68	0.38	0.21	0.11
8	88.6	0.98	0.64	0.51	0.81	0.33
9	49.5	3.32	0.26	0.51	0.81	0.72
10	66.5	0.64	0.89	0.60	0.64	0.25
11	19.1	1.66	0.04	0.26	0.51	0.09
12	81.7	3.19	0.60	0.38	0.47	0.58
13	0.5	1.74	0.21	0.43	0.47	0.19
14	97.2	1.53	0.55	0.30	0.30	0.18

ESW, end segment width; ID, identification number; MSW, mean segment width; RPC, red pigment concentration; SSL, single segment length; SSW, single segment width.

pictures of the petri dish have been taken outside the thermostat chamber every 12, respectively, 24 h. Using the image recognition system developed together with Wimasis GmbH in Munich, the following parameters characterizing the hairy root network have been obtained from all taken pictures:

- length of every single segment,
- segment volume,
- segment width at either ends and in the middle,
- number of branching points,
- total length of all segments, and
- red pigment concentration in percentage for each single segment.

Exemplary for all taken pictures, one image (Fig. 3) has been selected. Every obtained segment of the hairy root network is marked with a unique identification number (ID) and its properties are listed under the respective ID (Table 2).

After each segment's properties have been analyzed it is possible to derive segment metrics for the whole skeleton. Relevant parameters include the total number of segments (14 for line 2, day 5, 1st), the total root length (TRL = 36.81 mm), the mean segment length (MSL = 2.63), and the total volume that equals 5.32 mm^3. Using these values corresponding to the total network at a specific time during the cultivation period, the change or steadiness during the development of the hairy root network can be monitored.

For the analysis of the branching point development, total metrics can be generated to display the total number of branching points (TBP = 6) and their mean distance from each other equals 1.68 mm.

A major part of the image recognition process is the evaluation of the red pigment concentration in every single segment (Table 2) and a mean value indicating the total concentration of the red betacyanin pigments in the hairy root network is generated for the timed overview (mean pigment concentration [MPC] = 60.5%).

Analogically to the values presented above, the data from all taken images have been processed to form mean values. As all investigated growth processes are highly statistical, these mean values have been used to perform an analytical analysis.

Results obtained from this small campaign are presented as timed diagrams indicating the change or the steadiness of the chosen parameters characterizing the root network.

The TRL is an indicator for biomass formation. For the analytical analysis, the campaign has been divided into two scenarios by the IRSL. The displayed results in Fig. 4 show two graphs, one for biomass accumulation of initial segments with 9- to 12-mm length and the other graph indicating values for an IRSL of 17–19 mm. Both values strongly follow an exponential curve. In the end of the cultivation period, the total root length TRL is about 22 times higher as the IRSL. As the total biomass can be derived from the length and the matching width parameters belonging to each segment the observed growth factor can also be assumed for the total biomass accumulation.

In order to characterize the growth pattern and the change in the morphology of the hairy root network the total number of branching points TBP has been monitored. The data displayed in

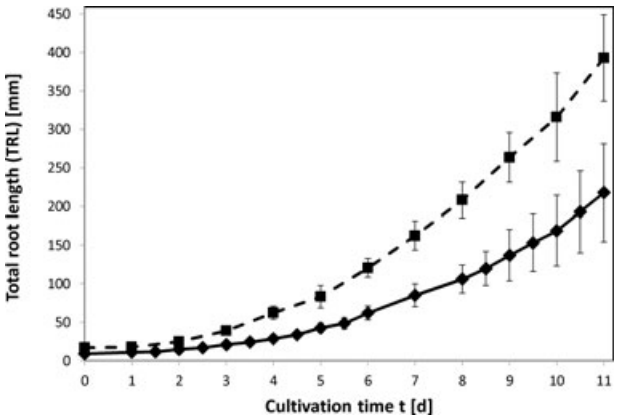

Figure 4. TRL over a cultivation time *t* of 11 days on MS medium with IRSL of 17–19 mm and IRSL of 9–12 mm including standard deviations.

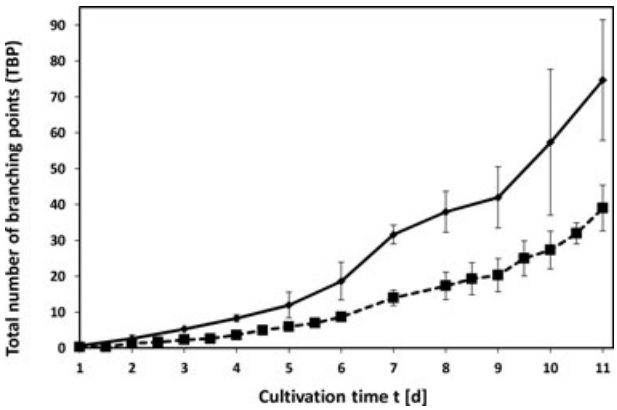

Figure 5. TBP over a cultivation time *t* of 11 days on MS medium with IRSL of 17–19 mm and IRSL of 9–12 mm including standard deviations.

Fig. 5 clearly show an exponential development of the TBP value for both IRSL values. During the cultivation time of 11 days, an individual starting segment from the 9–12 mm category develops more than 200 branching points on average while an individual starting segment from the 17–19 mm category develops nearly 400 branching points. The observed branching takes place at the tip of each growing root segment.

The main objective of the research with hairy root organ complexes is the production of valuable secondary metabolites with them. Exemplary for hairy roots of *B. vulgaris*, the red betacyanin pigments are produced and visible due to its red color. Evaluated data from the experiments with 12 samples of *B. vulgaris* hairy roots on agar plates show an average increase of 15% in the total betacyanin concentration in the root network during a cultivation period of 11 days as indicated by the trend line (Fig. 6). Due to the small database of analyzed images, a quite high scatter for the red pigment analytics has been observed. To get more significant data, a larger number of experiments is obviously necessary.

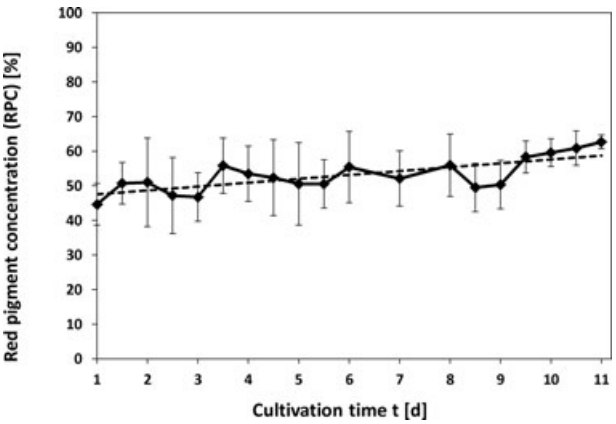

Figure 6. MDC of betacyanin in the whole hairy root organ complex including standard deviations and trend line over a cultivation time *t* of 11 days on MS medium.

4 Concluding remarks

The presented image recognition system for hairy roots on agar plates filled with solid MS medium has been tested in a small campaign with 12 different samples during a cultivation period of 11 days. Valuable quantitative data about the evolution of the morphology and the production of secondary metabolites in hairy roots of *B. vulgaris* has been generated and is the basis for detailed mathematical modeling in order to achieve a structured growth model for hairy roots.

First data analysis shows clear significance for an exponential growth of the root network and the development of branching points. It has been quantitatively observed that the overall betacyanin concentration increases during the cultivation period.

Ongoing development on the image recognition algorithm concentrates on segment recognition over time that will enable observation of single segments during the cultivation period. Larger experiments with more samples using nominal cultivation procedures but also experiments with elicitors are currently on investigation and will be used to calibrate the growth model and eliminate statistical uncertainties.

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The authors have declared no conflict of interest.

Nomenclature

CF	[mm/px]	conversion factor
ESW	[mm]	end segment width
IRSL	[mm]	initial root segment length
MSL	[mm]	mean segment length
MSW	[mm]	mean segment width
RPC	[%]	red pigment concentration
ROI	[-]	region of interest
SSL	[mm]	single segment length
SSW	[mm]	single segment width
TBP	[-]	total number of branching points
TRL	[mm]	total root length

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