Chapter 2

High-Throughput Phenotyping of Plant Shoots

Bettina Berger, Bas de Regt, and Mark Tester

Abstract

Advances in automated plant handling and image acquisition now make it possible to use digital imaging for the high-throughput phenotyping of plants. Various traits can be extracted from individual images. However, the potential of this technology lies in the acquisition of time series. Since whole shoot imaging is nondestructive, plants can now be monitored throughout their lifecycle, and dynamic traits such as plant growth and development can be captured and quantified. The technique is applicable to a wide range of plants and research areas and makes high-throughput screens possible, reducing the time and labor needed for the phenotypic characterization of plants.

Key words: Plant imaging, Growth analysis, Leaf area, Shoot morphology

1. Introduction

The remarkable progress in plant genetics over recent years has made increasingly apparent that plant phenotyping is lagging behind and has become the rate-limiting step in plant science and the generation of improved crop varieties. Traditionally, whole shoot phenotyping involves techniques such as visual assessment of plants, manual measurement of height and leaf dimensions, or destructive sampling to determine biomass accumulation, making it a time-consuming and labor-intensive process. High-throughput phenotyping protocols are therefore needed and, as with genetics, this will be a technology-driven process.

The ability to capture and store information in images is not new and has been used for a long time. Automated plant handling and imaging systems have rendered plant shoot phenotyping high-throughput. Using digital imaging as a means of shoot

Jennifer Normanly (ed.), *High-Throughput Phenotyping in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 918, DOI 10.1007/978-1-61779-995-2_2,

phenotyping has several advantages. (1) Whole shoot imaging is nondestructive and noninvasive, making it possible to image the same plant throughout the course of its lifecycle to measure dynamic traits such as growth; (2) it is possible to determine several traits within a single image, thereby increasing the information captured; (3) digital images can be stored and reanalyzed if there are improvements in image processing or different research questions arise; (4) morphological parameters or leaf symptom measurements derived from images are quantitative rather than arbitrary units subject to human assessment; (5) imaging can extend beyond the range of visible light and allows the analysis of traits that are invisible to the human eye.

Nevertheless, the phenotypic traits amenable to high-throughput imaging protocols need to fulfill certain criteria. Capturing the trait reliably in the images and extracting it in an automated manner through image processing are critical. Not all features of a plant shoot obvious to the researcher, such as individual stems of a wheat plant, can easily be identified through image processing. Also, some traits might be subject to circadian rhythms, such as leaf angles or leaf temperature, and the respective protocols to measure those traits need to incorporate a suitable time window for imaging.

The traits measured by imaging will obviously depend on the research question at hand, and it is beyond the scope of this chapter to present an exhaustive list. We will therefore focus on the use of digital color imaging to measure growth dynamics, a trait important for many areas, such as abiotic stress or nutrient use efficiency.

2. Materials

2.1. Seed Treatment

1. Uniformly sized seeds (see Note 1).
2. 70% (v/v) ethanol.
3. 3% (v/v) sodium hypochlorite (see Note 2).
4. Alternatively, Thiram or similar fungicides.

2.2. Growth in Potting Mix

2.2.1. Measurement of Field Capacity of Potting Mix

1. Sintered glass funnel.
2. 1.3-m silicon or clear plastic tubing with diameter to fit the funnel outlet.
3. Retort stand and clamp.
4. Large beaker or bucket as water reservoir.

2.2.2. Pot Preparation and Plant Growth in Potting Mix

1. Plastic pots with a capacity of about 3 L (see Note 3).
2. If the application of nutrients or water to the bottom of the pot is necessary, draining pots should be placed in saucers that enclose the bottom third of the pot.
3. Potting mix (see Note 4).

2.3. Biological Validation of Shoot Imaging for Biomass Measurements

1. Leaf area meter (e.g., LI-3100C, LI-COR, USA).
2. Drying oven.
3. Analytical balance.

2.4. Image Acquisition

1. Industry grade digital color camera with automated software control (e.g., LemnaTec 3D Scanalyzer system, LemnaTec GmbH, Germany).
2. Automated setup to move plants to the camera or vice versa. If manual systems are used, experiments are usually limited to about 150–200 plants per experiment.
3. Adequate computer hardware for image storage (see Note 5).
4. Adequate illumination equipment.
5. Optional: A color reference card and/or ruler for calibration purposes (e.g., RHS Colour Chart; ColorChecker, X-Rite, USA).

2.5. Image Analysis

1. Adequate computer hardware for high-throughput image processing.
2. Image analysis software package, included with imaging system, e.g., LemnaGrid (LemnaTec GmbH, Germany) and/or standalone software such as MATLAB (Mathworks, USA), Halcon (MVTec Software GmbH, Germany), or Labview (National Instruments, USA). An open source alternative is ImageJ (http://rsbweb.nih.gov/ij).

3. Methods

3.1. Seed Treatment

1. Surface sterilize uniformly sized seeds for 1 min in 70% (v/v) ethanol followed by 5 min in 3% (v/v) sodium hypochlorite.
2. Rinse the seeds several times in deionized water (see Note 6).

   Or

3. Surface coat the seeds with Thiram following the manufacturer's instructions (see Note 6).

3.2. Growth in Potting Mix

3.2.1. Measurement of Field Capacity of Potting Mix

When working in pots, it is important to carefully consider the watering regime to avoid waterlogging and hypoxia (1). Many experiments will adjust watering to "water holding capacity" or "pot capacity," which is the volumetric water content of a free-draining pot. However, this value greatly depends on the height of the pot and might often result in hypoxia, especially with fine potting mixes or field soil. In our experiments, we measure "field capacity," defined as the volumetric water content of the potting mix or soil at 1 m suction.

The setup described here to measure this parameter is comparable to the one shown in Fig. 2 of Passioura (1).

1. Attach the silicon tubing to the funnel outlet.
2. Mount the funnel with tubing on a retort stand about 1 m above the water reservoir (see Note 7).
3. Add about 2 L of water to the water reservoir below the funnel.
4. Fill the funnel and silicon tube with water ensuring that all air bubbles are removed.
5. Add the soil/potting mix to be tested into the funnel and let it settle. About half to two-thirds of the funnel should be filled with soil.
6. Once the water has drained to just above the soil level, cover the funnel with clingfilm to avoid evaporation from the surface.
7. To ensure hydraulic conductivity, there should be no air bubbles present between the filter plate, tubing, and water reservoir.
8. Adjust the position of the filter to obtain a height of 1 m from the sintered filter plate down to the water level in the reservoir.
9. Let the soil/potting mix equilibrate for several days up to one week, ensuring that no air bubbles form.
10. Take out the wet soil from the funnel and record the wet weight (WW).
11. Dry the soil in an oven at 105°C until constant weight is reached.
12. Record the dry weight (DW).
13. The volumetric field capacity is given by the equation (WW – DW)/DW.

3.2.2. Plant Growth in Potting Mix

The following protocol describes growth of plants under well-watered conditions with complete fertilizer present in the potting mix. If experiments for nutrient use efficiency are performed, a fertilizer free potting mix should be used, and nutrients should be supplied through fertilizer solutions with a defined nutrient composition. In the case of drought experiments, the required

watering level for the low watering regimes can be determined through establishing a soil water retention curve, using a pressure plate apparatus (2) or through measuring pre-dawn leaf water potential with a pressure bomb (3).

1. Fill a pot to about 4 cm below the rim after gentle tapping and then weigh it.
2. Use the same weight to fill up all remaining pots.
3. Include several spare pots to monitor water evaporation from the soil during the experiment and at least two pots to determine the oven dry weight of the soil.
4. Once all pots are filled, add enough water for germination.
5. Plant three to four seeds per pot, about 1 cm deep and cover them with soil.
6. Use the soil dry weight to calculate the target weight of a pot at field capacity as determined by Subheading 3.2.1.
7. Adjust the watering level of each pot to field capacity about 2–3 times per week and record the water use.
8. Once the seedlings are about established, thin out to one seedling per pot.
9. Image the plants daily or every second day during the period important for phenotypic measurements.

3.3. Biological Validation of Imaging for Shoot Biomass Measurements

We found a good correlation of the plant area measured from three images (two images from the side at 90° rotation and one image from the top) and shoot biomass for a variety of plants including wheat, barley, sorghum, and tomato. However, this might not be the case for all plant types and certainly not for the whole lifecycle of the plant. It is therefore necessary to establish a calibration for the specific plant type analyzed and the developmental stages of the plant critical for phenotyping.

1. Grow several replicates of plants to the desired growth stage under the same conditions used for the phenotyping experiments (see Note 8).
2. Image the plants immediately prior to destructive harvest (see Note 9).
3. Harvest the shoot and measure the shoot fresh weight. If individual organs, such as leaf and stem, can be differentiated in the images, measure them separately.
4. Measure the leaf area with a leaf area meter (see Note 10).
5. Dry the shoot or separated shoot organs in a drying oven until constant weight is reached.
6. Measure the shoot dry weight or the dry weight of the individual organs.

7. Establish a calibration curve for the projected shoot area extracted from the images (see below) and shoot area or shoot biomass. If the images of the shoot can be differentiated into individual organs, take the different biomass for those organs into account when establishing the calibration curve; e.g., the same pixel area of stem may account for more shoot biomass than the same pixel area of leaf.

3.4. Image Acquisition to Monitor Plant Growth

How images are acquired will greatly depend on the hard- and software available to the researcher and the trait to be measured. There are complete systems available from LemnaTec (LemnaTec GmbH, Germany) that combine plant handling, imaging hardware, and the control software. Other institutes might have the capability to build their own automated in-house solutions (4, 5) or use a fairly simple camera setup and manual handling of plants. We will therefore only present aspects of image acquisition that are generally applicable and important for any type of setup.

1. The aim of any imaging setup should always be to obtain the best possible image of the plants for measuring the trait of interest. Image acquisition should be done as consistently as possible. This will greatly facilitate the image analysis and ideally allow the generation of automated image analysis algorithms that require minimum user input.
2. In general, there are two methods for image acquisition.
   (a) The plants are stationary and the camera is moved to the plant. This is most commonly used for plants with a simple architecture, such as, Arabidopsis, where a single image from the top often provides sufficient data.
   (b) The plants are moved to a stationary camera setup. This is an advantage for plants with a complex morphology, such as wheat and barley, where images from several angles will greatly increase the quality of data obtained through imaging. In addition, the imaging environment, such as background and illumination, is easier to control.
3. Illumination conditions should be as uniform as possible, both over time and throughout the field of view. It is important to preheat the lamps until constant illumination is reached before the first images are taken. Hunter et al. (6) give detailed information on how to achieve optimal lighting and avoid shadows and reflections.
4. Use of a color card and ruler allows calibration of the imaging setup. If both are present in an image, it is possible to normalize the recorded colors and calibrate for the zoom factor used. This allows comparisons between different imaging setups that differ in lighting conditions and the cameras used.

5. The imaging background should be chosen carefully to facilitate the identification of the plant in subsequent analysis. Backgrounds, such as white or blue, are preferable, since the green of the plant will be easy to differentiate.
6. Green and gray should be avoided as pot colors. White, blue, and black are suitable for most plant types and white has the advantage of keeping the soil cooler than darker colors. Materials with a flat finish reduce undesired reflections.
7. The soil surface can become challenging in the image analysis, since sandy or drying soils can have very similar colors to senescent leaves. Colored plastic mulch or white gravel on the surface can reduce this problem and have the further advantage of reducing water loss from the soil surface.
8. Many plants, especially wheat and barley, will need some sort of support when grown in pots, such as carnation frames. Again, they should not be green and if metal they need to be tested to determine if they can be easily eliminated in the image analysis. In some cases, it might be easier to get color-coated frames to avoid problems during the automated image analysis.
9. When choosing the exposure for the images, it is generally better to have a lower exposure. Overexposure will lead to white spots and thus a loss of color information that cannot be compensated for by image analysis.
10. The file format for storing the images should not lead to loss of image information (e.g., JPG or BMP). PNG and TIFF are the commonly used formats and do not lead to loss of information through compression.

3.5. Image Analysis to Measure Projected Shoot Area

Since plant imaging allows daily recordings, simple image analyses such as plant size measurement yield valuable information about plant growth and performance. Nevertheless, basic image analysis also requires the use of specialized software, computing infrastructure and database management if it is to be performed at high-throughput.

Depending on the software solution used, different levels of prior knowledge in image analysis and programming are necessary to develop image analysis algorithms, and collaboration with scientists experienced in that area is advisable.

MATLAB (MathWorks, Massachusetts, USA) is possibly the most commonly used and powerful software to develop image analysis algorithms and offers solutions for automated image acquisition. Halcon (MVTec Software GmbH, Germany) is a fairly comprehensive application for image analysis, and it is compatible with common programming languages such as C, C#, and .NET. ImageJ

(http://rsbweb.nih.gov/ij) presents a Java-based solution for image analysis that is open source, so it is easily accessible. However, all three software programs require a certain amount of programming skills to write and implement analysis algorithms. The built-in image analysis solution of LemnaTec setups, LemnaGrid, is designed to allow researchers without prior programming knowledge to create algorithms for image analysis through drag-and-drop software where individual operators can be connected to create a processing pipeline. Unfortunately, algorithms can only be shared among LemnaTec users and the functionalities are not as comprehensive as those of specialized image analysis software.

Since the specific algorithms will depend on the software used and the imaging setup, we will only discuss general steps common to digital image processing (7) that are necessary to measure the size of the plant and to perform subsequent growth analysis.

1. *Image retrieval*. Recorded images need to be loaded into the software from a database or storage folder. Images may need to be cropped or a region of interest (ROI) may need to be set to shorten the computing time and/or to remove unnecessary parts of the image that can become a source of noise.
2. *Image preprocessing*. The application of filters to minimize noise or increase sharpness can improve the outcome of the subsequent analysis steps. However, there is a possibility of losing information that cannot be retrieved in later steps. If thresholding is used to make a binary image in the next step, the color image needs to be converted into a grayscale image by transforming the 3D RGB color information into a single channel.
3. *Image segmentation*. The next step is the segmentation of the image into objects of interest and objects that will later be discarded, such as the background, pot, support frames, or soil. Depending on the composition of the image, there are several options to produce a binary image. Classification by color with a supervised nearest neighbor algorithm or thresholding of a grayscale image is commonly used. In both instances, the result is a binary image, where pixels that belong to the object of interest are set to a value of 1, all others to 0.
4. *Noise reduction*. Morphological operations such as erosion-dilation steps or filling holes can be used to correct for unavoidable imperfections in the binary image that result from noise from image acquisition or difficulties in distinguishing between parts of the object and background that have similar colors.
5. *Image composition*. Leaves can often become fragmented in earlier steps due to curling of the leaves, and the individual fragments need to be merged to create one single object, the plant.

6. *Image description*. Features of the identified object, such as area, height, width, convex hull, or compactness, are quantified. The features mostly consist of mathematical characteristics calculated from the object.
7. *Color classification*. The identified object, the plant, can now be extracted from the original RGB image. Based on the color information of the original image, the leaves can be subdivided according to their color and the respective areas quantified using supervised nearest neighbor color classification. This can be used to quantify necrotic or senescent leaf area. A similar approach that uses the color information of the plant to determine the chlorophyll content is presented in chapter 6, this volume.

3.6. Basic Plant Growth Analysis

The following protocol describes basic measurements of several growth parameters. For more detailed plant growth analyses, refer to the excellent publications by Hunt (8, 9). All steps presented here assume a linear correlation between plant biomass and the projected shoot area measured from the images. If this is not the case, the calibration established in Subheading 3.3 should be used to convert the measured projected shoot area to estimated biomass or leaf area.

1. Increase in shoot area (A) over time (t). For a first evaluation of the data, plot the shoot area for individual plants or treatment groups over time. This will allow a visual assessment of treatment or genotype effects and the identification of biological outliers (entire growth curve is affected) or technical outliers from the imaging process (generally only individual points of the growth series are affected). Most plant species have a sigmoid growth curve when imaged from seedling stage to early reproductive stage, consistent with other measuring techniques. Once leaves start to senesce during seed ripening, this will obviously result in a decrease in projected leaf area, which is then no longer a good indicator of plant biomass. It is possible to overcome this technical challenge by using the color information of the leaves to differentiate between green and senescent leaf areas if experiments need to extend over the whole growth cycle; however, this needs to be tested for each plant species.
2. Use the data of shoot area over time to generate a growth model through curve fitting. Growth models, such as higher order polynomials or cubic splines that make no prior assumption about the data, are preferable. Higher order polynomials can be generated with basic spread sheet software, such as Microsoft Excel (Microsoft Cooperation, USA). Spline curves generally need statistical software packages.

3. Use the growth model to compute the absolute growth rate of the plants, which is the first derivative (dA/dt) of the growth model. The absolute growth rate will reveal how much area the plant gained per day at any time during the experiment. If plants were imaged over most of the lifecycle, the absolute growth rate will show an increase during early growth, reaching a maximum when plants shift from vegetative to reproductive growth and a subsequent decline as plants mature. The time interval for plants to reach maximum absolute growth can be regarded as a trait. Certain stress treatments, such as drought or salinity can alter the length of the interval, indicating altered plant development.
4. Relative growth rate ($dA/dt \cdot 1/A$). In addition to the absolute growth rate, a growth model can be used to calculate the relative growth rate (RGR) at any given time. The RGR is generally highest for young seedlings and then declines gradually. Since RGR is independent of plant size, it allows comparison of plants and varieties with fairly different growth habits. Analysis of RGR over time can reveal when genotype or treatment effects become apparent.
5. Leaf area duration (LAD). The expression of leaf area duration was used by Watson in 1947 (10) for the integral of the leaf area over the entire lifecycle and was described as the "whole opportunity for assimilation" of the plant. Using the previously developed growth model, it is possible to calculate LAD for the entire experiment or certain intervals relevant to the treatment. LAD will give a measure of the leaf area and its persistence over the chosen period.
6. Morphological measurements. As for total size of the plant, morphological measurements are most powerful when considered over the whole growth period, rather than just at a single time point. There are numerous morphological parameters that can be extracted and quantified from images. Most obvious is probably height and width of the plant. Another frequently used measurement is compactness. This is defined as the ratio of the plant area to the convex hull, the area that entirely encloses the plant. Compactness can be a very useful measure to describe the morphology of fairly rigid plants such as Arabidopsis. However, it is prone to noise for grassy plants such as wheat or barley, where leaves are highly flexible and do not stay in the same position. A simple alternative in this case is to calculate the ratio of leaf area to plant height. This value will increase with increasing tiller number and can show clear differences between control and stressed plants. Another option would be to divide the side view images into several segments, e.g., horizontal sections in 10 cm intervals extending both above and below pot level. Quantifying the percentage of leaf area in those segments will allow a description of the leaf denseness at various heights.

4. Notes

1. We have used the described methods for numerous species including wheat, barley, maize, sorghum, tomato, and chickpea. Since the assay presented here is based on growth analysis, it is extremely important that the seeds and seedlings used are as uniform as possible. If sufficient seed is available, one should always plant excess amounts to be able to select for evenly sized seedlings. If it is known that the lines being used germinate at different rates, the sowing should be staggered to have evenly sized seedlings at the start of the experiment.
2. The sodium hypochlorite solution can be prepared using a household product such as Domestos®, when taking into account the lower active concentration of Cl^- compared to a lab grade solution.
3. The color of the pot should allow an easy distinction from the plants in the image-processing step, preferably white or blue. Black is possible, but it leads to an increased soil temperature. Standard green nursery pots should not be used.
4. The choice of potting mix will obviously depend on the experiment. Some might require controlled nutrient application and should therefore be free of fertilizer. If the pots are placed on an automated conveyor system, the substrate should not be too loose (such as pure sand) since it might shift through the movement on the belt and damage the root system. The clay content should not be too high since there is the potential for compaction on the conveyor belt and consequently root anoxia.
5. We generally take three images per plant (two from the side at 90° rotation and one from the top) at about 15–20 time points throughout an experiment. With a file size of about 4 MB, this amounts to 4 MB × 3 images × 20 time points = 240 MB per plant. Even a smaller scale experiment with 200 plants will therefore need 47 GB of storage.
6. Seed treatment might not be necessary, depending on the source of the seed. However, fungal infections of young seedlings can influence the growth rate and their sensitivity to certain stress treatments.
7. If no large retort stand is available, a smaller one can be placed on a table with the water reservoir on the ground.
8. The growth conditions can influence parameters such as leaf thickness and will consequently also influence the correlation between leaf area and biomass.
9. The number of images taken per plant will depend on the shoot morphology and the desired throughput. We found that

three images (two from the side and one from the top) are sufficient for most plants. Plants such as Arabidopsis generally require only a single image from the top.

10. If no leaf area meter is available, a simple flat-bed scanner can be used. However, this requires mounting the leaves on paper and extracting the leaf size from the acquired images. It is generally more labor intensive and not suitable for a large sample number.

References

1. Passioura JB (2006) The perils of pot experiments. Funct Plant Biol 33:1075–1079
2. Klute A (1986) Water retention: laboratory methods. In: Klute A (ed) Methods of soil analysis, Part I, 2nd edn. Agronomy Monograph 9, American Society of Agronomy and Soil Science Society of America, Madison
3. Scholander PF, Hemmingsen EA, Hammel HT et al (1964) Hydrostatic pressure and osmotic potential in leaves of mangroves and some other plants. Proc Natl Acad Sci USA. doi:10.1073/pnas.52.1.119-125
4. Granier C, Aguirrezabal L, Chenu K et al (2006) Phenopsis, an automated platform for reproducible phenotyping of plant responses to soil water deficit in *Arabidopsis thaliana* permitted the identification of an accession with low sensitivity to soil water deficit. New Phytol 169:623–635
5. Jansen M, Gilmer F, Biskup B et al (2009) Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via growscreen fluoro allows detection of stress tolerance in *Arabidopsis thaliana* and other rosette plants. Funct Plant Biol 36:902–914
6. Hunter F, Biver S, Fuqua P (2007) Light-science & magic: an introduction to photographic lighting. Focal Press, Elsevier, Oxford, UK
7. Gonzalez RC, Woods RE (2006) Digital image processing, 3rd edn. Prentice-Hall, Upper Saddle River
8. Hunt R (1978) Plant growth analysis. Edward Arnold, London
9. Hunt R, Causton DR, Shipley B, Askew AP (2002) A modern tool for classical plant growth analysis. Ann Bot 90:485–488
10. Watson DJ (1947) Comparative physiological studies on the growth of field crops. Ann Bot 11:41–76

http://www.springer.com/978-1-61779-958-7

Liver Proteomics
Methods and Protocols
Josic, D.; Hixson, D.C. (Eds.)
2012, XIII, 346 p., Hardcover
ISBN: 978-1-61779-958-7
A product of Humana Press