Chapter 7

High-Throughput Scoring of Seed Germination

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Abstract

High-throughput analysis of seed germination for phenotyping large genetic populations or mutant collections is very labor intensive and would highly benefit from an automated setup. Although very often used, the total germination percentage after a nominated period of time is not very informative as it lacks information about start, rate, and uniformity of germination, which are highly indicative of such traits as dormancy, stress tolerance, and seed longevity. The calculation of cumulative germination curves requires information about germination percentage at various time points. We developed the GERMINATOR package: a simple, highly cost-efficient, and flexible procedure for high-throughput automatic scoring and evaluation of germination that can be implemented without the use of complex robotics. The GERMINATOR package contains three modules: (I) design of experimental setup with various options to replicate and randomize samples; (II) automatic scoring of germination based on the color contrast between the protruding radicle and seed coat on a single image; and (III) curve fitting of cumulative germination data and the extraction, recap, and visualization of the various germination parameters. GERMINATOR is a freely available package that allows the monitoring and analysis of several thousands of germination tests, several times a day by a single person.

Key words *Arabidopsis thaliana*, Automatic scoring, Curve-fitting, Germination, High-throughput analysis, Image analysis

1 Introduction

Fundamental and applied seed biology research relies heavily on accurate quantification of seed germination. Nowadays, large-scale experiments using large genetic populations or mutant collections are popular tools to unravel molecular aspects of seed development, germination, dormancy, and seed performance [1, 2]. Detailed analysis of these traits requires the complete cumulative germination curve and extraction of parameters such as start, rate, and uniformity of germination. *Arabidopsis thaliana* is one of the most used model species for plant science and it has also become a very useful model plant to study seed biology. However germination of the very small *A. thaliana* seeds (200–400 μm) is often evaluated by using a binocular microscope, which makes this a very
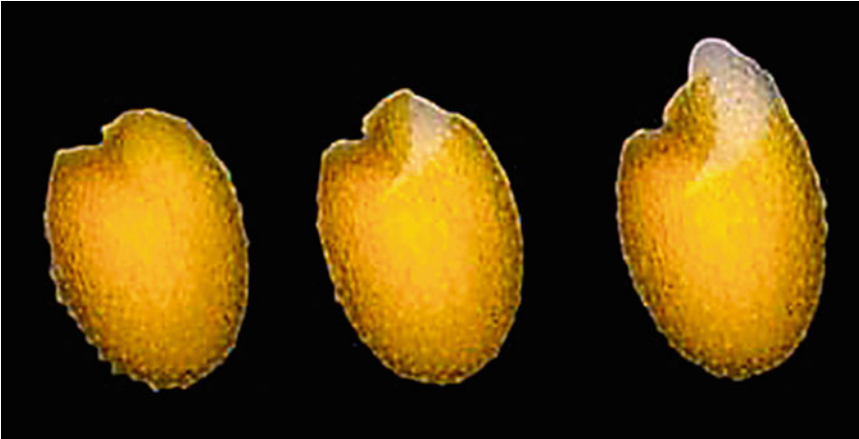
Fig. 1 Photographs of different stages of *Arabidopsis* seed germination. From *left* to *right*: fully imbibed seed (6 h after start of imbibition), seed coat rupture, and radicle protrusion

laborious task. This often hampers the collection of cumulative germination data in large-scale experiments and therefore often only the less informative percentage of germination after a nominated period of time is used to describe the germination performance of a seed lot. With this in mind, the GERMINATOR was developed, a cost-efficient and flexible procedure for high-throughput automatic scoring of germination that can be implemented without the use of complex robotics [3].

The uptake of water during seed imbibition is triphasic, and consists of a rapid initial uptake (phase I), followed by a plateau phase (phase II) and a further increase (phase III). During this last phase, the embryo axis elongates and breaks through the seed coat, which consists of dead tissue. The next step is the morphological completion of germination, which is characterized by the protrusion of the radicle through the endosperm (Fig. 1).

1.1 GERMINATOR   Most approaches to the automatic evaluation of germination based on image analysis extract information from a time series of images. For example, change in seed size over time can be used to evaluate the progress of germination very precisely. However, this analysis requires fully aligned images, which are often obtained by using fixed setups in specialized cabinets, flat-bed scanners, or camera systems over Jacobsen tables [4–7]. This type of setup has important consequences not only for the amount of samples that can be measured and the flexibility to follow germination under various environmental conditions, but also for the costs of the equipment. Especially this flexibility and level of throughput are essential characteristics of the GERMINATOR.

Evaluation of germination by using image analysis requires good contrast between background, seed, and protruding radicle. Therefore, a blue filter paper specifically designed for germination

tests is used. By using two different color threshold levels on a single image we can distinguish between background and seed coat, or background, seed coat, and radicle. This allows germinated and non-germinated seeds to be distinguished. To ensure the most accurate scoring, the number of non-germinated seeds to monitor germination is used. With this approach it is not necessary to obtain fully aligned images between the various time points at which germination is evaluated. The GERMINATOR package consists of three separate modules, based on the components of our procedure: (I) experiment design, (II) image analysis, and (III) curve fitting. Module I uses Microsoft Excel combined with Visual Basic scripts to create a database of experiments, and assists in experiment design. Germination is documented by photographs taken manually at various intervals during germination. Automated scripts were developed for Adobe Photoshop and ImageJ to automatically analyze the images and to minimize user input as much as possible. With the help of ImageJ, a two-color threshold analysis is performed on each individual image: first, a color threshold that selects the seed coat only, and second, a color threshold that selects both the seed coat and the protruding radicle. The specific settings for the color thresholds can be adjusted for each seed type during a system calibration. The output of the image analysis consists of four tables with information about the total number of seeds and the position and size of each individual seed that has been detected. In module II, these tables are then coupled with the experiment setup tables from module I, and cumulative germination curves are calculated. The calculation uses position and seed size in the various color threshold analyses to determine whether a seed has germinated. Module III performs a curve-fit analysis of the cumulative germination data. This module uses data generated by the image analysis of module II, or cumulative germination data of other sources (for example derived from manual counting). This module executes a curve fit using the four-parameter Hill function, which can accurately fit the typical sigmoid curve that describes the course of germination [8]. As soon as the best-fitted curve has been determined, typical germination parameters are extracted from the curve. Generally Gmax (maximum percentage of germination), t50 (time to reach 50% germination), tx (time to reach x% germination), uniformity (time interval between, e.g., 16 and 84% of viable seeds to germinate), and AUC (integration of area under the germination curve until a certain point of time) are determined. As described by El-Kassaby and co-workers [8] the AUC can also be used to calculate a dormancy index (DI), by extracting the area under the curve after dormancy release by cold stratification, by the area under the curve without cold stratification. By the same analogy the AUC can be used to measure the effect of any stress treatment and calculate a stress index (SI). The GERMINATOR script will summarize the results by calculating averages and

standard errors for repeated samples, performing a student-t test, and provide a clearly formatted output including graphs for the different germination parameters.

With the GERMINATOR package, a single person can easily handle more than 1000 germination tests at a time and monitor germination precisely over time. This greatly facilitates large-scale screens of mutant collections or genetic populations, such as recombinant inbred line populations [9, 10]. The package was tested and optimized for *Arabidopsis* seeds and it has been shown to also work for other species including *Brassica* spp. and rice. The whole GERMINATOR package, including a manual and video tutorials, is available from http://www.wageningenseedlab.nl/germinator.

2   Materials

2.1 Germination Assays

1. Germination trays (*see* **Note 1**).
2. Colored germination paper that fits in the germination trays (*see* **Note 2**).
3. A plastic flexible mold/template with the positions for the different germination tests in the same size as the germination paper (Fig. 2).
4. A germination cabinet (*see* **Note 3**).

2.2 For Image Acquisition

1. A digital single-lens reflex camera with more than ten million pixels and a macro lens.
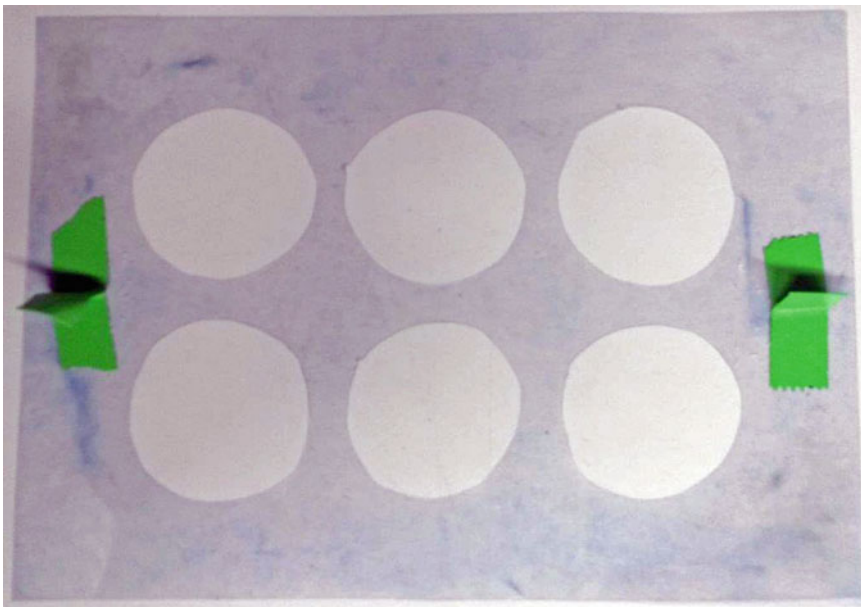

Fig. 2 Plastic mold/template to put in the germination trays to ensure that the different germination tests will be on the correct positions
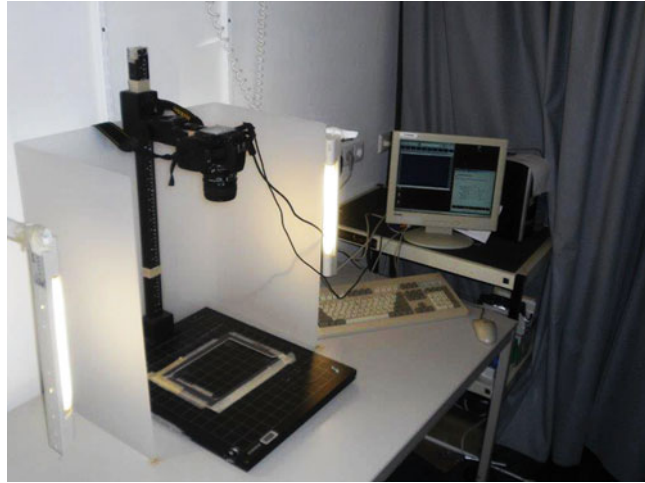
Fig. 3 Camera setup for the GERMINATOR

2. A power adaptor and USB connector cable for the camera.

3. A repro-stand.

4. Proper, preferably indirect, lightning. A Perspex screen is used around the camera setup to soften the light (Fig. 3) in a room without daylight in order to keep the light conditions as constant as possible. Our Perspex screen is 60 cm high, 55 cm wide, and 35 cm deep. As light source two fluorescent tubes (TL) of 30 cm long on both sides of the camera and one of 115 cm shining towards the ceiling of 115 cm are used (*see* **Note 4**).

5. A Windows-based PC (XP or Windows 7).

6. Software to control the camera. Nikon Control Pro version 2.0 is used in our setup.

2.3 For Image Analysis

1. A Windows-based PC.

2. Microsoft Excel (version 2003 or 2010 and English language version).

3. Adobe Photoshop CS3 or newer (English language version).

4. The GERMINATOR package (can be downloaded from www.wageningenseedlab.nl/germinator).

5. ImageJ version 1.40g with Montpellier RIO Imaging plug-ins (included in the GERMINATOR package).

3 Methods

3.1 Installing Scripts and Macros

1. Unzip the whole "Germinator.zip" file to a specific directory (keeping the folder structure of the file).

2. Adjust settings in Microsoft Excel to allow macros and install the Solver add-in.

For Microsoft Excel 2003:

1. Tools > add in > SOLVER ADD IN.

2. Tools > Macro > visual basic editor > tools > references > SOLVER.

3. Tools > options > security > macro security > trusted publishers > trust access to visual basic project.

For Microsoft Excel 2010:

1. Go to File > Options > Add-Ins. Choose Excel Add-ins and click Go. Select Solver Add-in and click OK.

2. Go to File > Options > Trust Center > Trust Center Settings > Macro Settings > Check "Enable all macros" and "Trust access to the VBA project object model" and click OK.

3. Adjust settings for Adobe Photoshop.

    (a) Select the window "Actions" with |Alt F9|. Open a menu on the right top of this window and select "load actions." Browse to the GERMINATOR package, select folder "Photoshop," and select "germinator.atn" (*see* **Note 5**).

    (b) In Photoshop, open one image of a typical seed germination test. In the "Actions" panel go to Germinator > crop and save and double-click the first "crop." Adjust the crop position that appears on the image to encompass the first germination test (*see* **Note 6**), and press enter and subsequently Ctrl + z to go back to the original image. Then do the same steps for the following five "crops" that are shown in the "Actions" panel and close Photoshop.

4. Install and adjust settings for Image J.

    (a) Install Image J 1.40g from the GERMINATOR package (*see* **Note 7**).

    (b) Extract Zip file "mri-plugins-base.zip" to the ImageJ 1.40g directory.

    (c) Extract Zip file "mri-all-plugins.zip" to the ImageJ 1.40g directory.

    (d) Copy the Germinator folder in the ImageJ folder of the Germinator.zip file to the ImageJ_applications directory.

    (e) Open ImageJ program, go to Plugins > Montpellier RIO Imaging > MRI VisualScripting. Go to Applications > Applications > Germinator > germination score + white, and click on the O. Make sure that the macro file points to the correct location of the "particle analysis macro. txt" file. Close the window, right click on the top white frame (just above the blue part), and click save. Close the script and do the same thing for germination score – white.

3.2 Prepare for a Germination Experiment

1. Open Germinator_menu 1.01.xls and set defaults: Provide a name for the overview and click Browse to select a default directory in to store the germination results. Click Save twice.

2. Click Browse and select the germinator table file from the GERMINATOR package (Germinator_table 1.05.xls) by double-clicking and subsequently click save (*see* **Note 8**).

3. Click on the "add new experiment" button, fill in the different parameters for the germination experiment that should be performed, and finish by clicking Add (*see* **Note 9**).

4. Click button "Add tables," select "New tables," and click the button "Make Tables" in the newly opened Excel file.

5. Fill in the parameters for the germination experiment like details, the number of samples that should be analyzed, and the number repetitions and treatments. The number of treatment option is there for when a germination test should be performed at, e.g., 22 and 25 °C or in water and in a certain concentration of ABA.

6. By checking the "Optional sample file name" box, sample names can be extracted from an Excel file (according to the format as shown in the "sample file name example.xls" file in the Excel directory of the GERMINATOR package). If biological replicates should have different names, these can be placed in different columns in this file. Select "separate biological replicates" to have the biological replicates in separate trays in the experiment setup.

7. After clicking start, the tables that show the experiment setup in which for each sample the tray number and position in the tray are indicated can be printed (*see* **Note 10**). The Excel file will be saved in the default directory under the experiment's name.

8. The printed table is brought to the lab to start the germination experiment.

3.3 Start a Germination Experiment

1. Take a tray and insert two sheets of blue blotter paper (*see* **Note 11**).

2. Mark the sheets with a plate number (depending on the amount of germination tests that will be performed) in the upper-left corner with a pencil.

3. Add 50 ml of demi water (*see* **Notes 12** and **13**).

4. Place the plastic mold/template in the tray to ensure that the different germination tests will be on the correct positions (Fig. 2) (*see* **Notes 14** and **15**).

5. Add around 50 *A. thaliana* seeds in each well (Fig. 4, *see* **Note 16**) according to the printed Germinator table and note the time of the start of imbibition in the table.
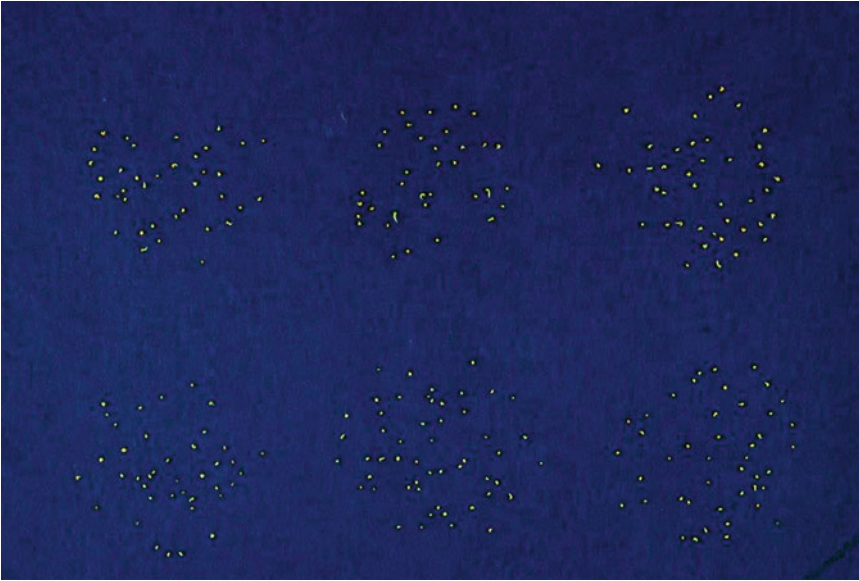
Fig. 4 A picture of a typical germination experiment with *A. thaliana* seeds

6. Pile up to 17 filled trays, add 1 tray with filter paper and water at the bottom of the pile and 2 at the top + a lid, and pack the whole pile in a big plastic bag (*see* **Note 17**).

7. Place the pile in a germination cabinet and set the desired temperature (*see* **Note 18**).

3.4 Optimize Image Acquisition

1. Attach the camera in a repro-stand and adjust height to ~53 cm (*see* **Note 19**).

2. Switch camera to full manual control and connect with USB to a PC.

3. Place the Perspex screen around the camera setup to soften the light, place the lights, and try to avoid interference of other, unstable, light sources (Fig. 3).

4. Place a mask that fits the germination trays underneath the camera to fix the imaging position (Fig. 3) (*see* **Note 20**).

5. Put a germination tray as described in Subheading 3.1 under the camera and focus.

6. Use Nikon camera Control Pro software to set resolution to maximum and file format to jpg with normal compression.

7. Adjust camera settings (aperture, ISO, and shutter speed) with Nikon camera Control Pro software and if necessary light conditions to optimize the pictures with respect to contrast between germination paper, seed coat, and radicle (*see* **Note 21**).

8. Save the optimal settings via Settings > Save Control Settings (as *****.ncc file) (*see* **Note 22**). Also the tone compensation

curves can be adjusted manually to fine-tune the pictures via Camera > Edit Camera Curves. Optimal tone compensation curves can be saved by clicking Save… under the curve (as *.ntc file).

3.5 Take Pictures of the Germination Experiment

1. Open the Camera Control Pro program, select settings > settings > load control settings, and select the previously saved optimal settings (*.ncc file). Go to image processing > tone comp > edit > load and select previously saved optimal settings for tone compensation curves (*.ntc file).

2. Go to tools > download options and select a folder to store the pictures. Subsequently go to edit file name. In prefix, first month, then the day followed by the year and time that the pictures are taken are indicated in a mmddyyyy-hhmm format followed by # (e.g., 10212015-0923#). Set start numbering at 1 and length of number at 3 digits. Finish by clicking OK.

3. Start taking pictures of all germination trays in the experiment starting with tray number 1 and adjust time settings every 5–10 min as indicated under Subheading 3.5, **step 2**. An experienced user can easily take pictures of 30 plates in 10 min (*see* **Note 23**).

4. Repeat taking pictures in certain intervals. For *Arabidopsis* routinely pictures are taken two or three times a day (*see* **Note 24**).

3.6 Determining the Optimal ImageJ Settings to Score Germination

1. Open a photo of germinating seeds (some already germinated, others not) with ImageJ (*see* Fig. 4 for an example).

2. Go to Process > Enhance Contrast and adjust contrast of the picture to 0.2 % saturated pixels and click OK (*see* **Note 25**).

3. Go to Plugins > Segmentation > Threshold Colour, select YUV (at the bottom of the window) (*see* **Note 26**) and adjust color settings of the three channels until settings are found that selects all seeds and all radicles but no background (Fig. 5, Filter +).
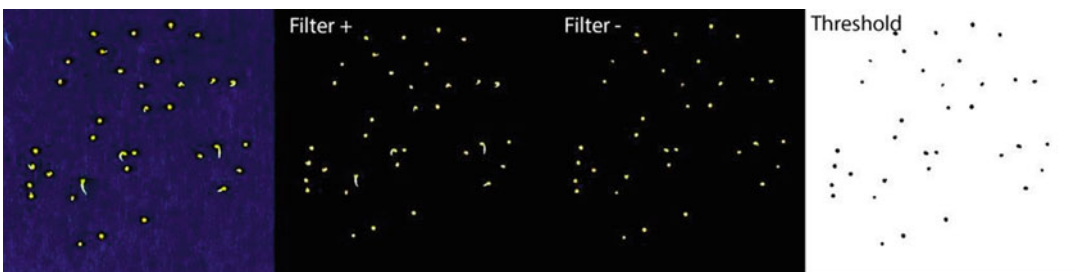

Fig. 5 A photograph of a typical germination experiment and the two color thresholds used to count seed germination. Filter +: an example of filtering everything but the background. Filter −: an example of filtering only for the seed coat color. Threshold: example of the Filter—picture after binarization and inversion and ready for particle analysis

Write these settings down. For the example image, these settings are Y 50-255, U 0-130, and V 0-255.

4. Adjust color settings until the settings are found that select the seed coat, but not the radicles and the background (Fig. 5, Filter −). For the example image, these settings are Y 50-255, U 0-90, and V 0-255. Write these settings down (*see* **Note 27**).

5. Select Threshold and close the Colour Threshold window.

6. Go to Process > Binary > Make Binary followed by Edit > Invert. This results in a black-and-white image with black seeds on a white background (Fig. 5, Threshold).

7. To count the black spots on such a photo go to Analyse > Set Measurements and select Area, Circularity, Centroid, Perimeter and click OK. Go to Analyse > Analyse Particles, select size 0–Infinity, Circularity 0.00–1.00, Show Outlines, Display Results, Summarize, Exclude on Edges, and Include Holes, and click OK.

8. In the Summary table that will appear the total number of seeds that are counted can be found (Count) and in the Results table parameters for the individual seeds are shown. The Area is a measure for size of the individual seeds. This area will be used in the Excel Germinator script.

9. To prevent counting of clustered seeds or other artifacts, such as remnants of siliques, a limit needs to be set for the size of particles that are still measured and considered as single seeds. The lower size limit is typically set at approximately $0.5 \times$ the smallest seed that is counted and the upper limit at approximately $2 \times$ the biggest seed that is counted (*see* **Note 28**).

10. Open the "particle analysis macro.txt" file (from Subheading 3.1, **step 4e**). Adjust the line "size = **40–175**" by replacing the bold numbers with the limits obtained under Subheading 3.6, **step 9**, and save the file.

11. Open the visual scripting tool of ImageJ by going to Plugins > Montpellier RIO Imaging > MRI VisualScripting. Go to Applications > Applications > Germinator > germination score + white and click on the O. Fill in the values obtained under Subheading 3.6, **step 3**, for the three different channels in the fields min/max channel 1–3. Close the window, right click on the top white frame (just above the blue part), and click save.

12. Close the script and do the same thing for germination score – white with the settings obtained under Subheading 3.6, **step 4**.

3.7 Analyze a Germination Experiment

1. When germination is completed pictures are analyzed for automatic scoring of germination in batch. The first step is to divide the pictures into the separate germination tests. To do so, first

Adobe Bridge is opened, all pics to be analyzed are selected, and if the pictures are upside down they are rotated first: go to Edit > Rotate 180° (*see* **Note 29**). Subsequently go to Tools > Photoshop > Batch and select the following settings: Set: Germinator; Action: Crop and Save; Source: Bridge; Destination: Folder. Click Source and select the folder in which the cropped separate pictures should be saved. Click override action "save as" and click OK (*see* **Note 30**).

2. Open ImageJ and go to Plugins > Montpellier RIO Imaging > MRI VisualScripting. Go to Applications > Applications > Germinator > germination score + white and click the big blue button. Select file type as all images and select all images to be analyzed. Click Open. When ImageJ is finished, there are two windows. One named Summary, which should be saved as summary+.txt, and one named Results, which should be saved as results+.txt (*see* **Note 31**). Perform the same steps on the same pictures for Applications > Applications > Germinator > germination score – white (*see* **Note 32**) and save the two windows as summary-.txt and results-.txt, respectively.

3. The first time the GERMINATOR is used, the optimal settings for variances that are allowed for the position of seeds and differences in seed area will have to be determined and thereby the performance of the system will be optimized. To do so first count the number of germinated seeds from some samples and save them in an Excel file according to the format as shown in the "manualcount-example file.xls" file in the Excel directory of the GERMINATOR package.

4. Open the Excel table that was made under Subheading 3.2, **step 7**, click Optimize parameters, and select the txt files that were created under Subheading 3.7, **step 2** (*see* **Note 33**). Under Name manual results file, select the Excel file created under Subheading 3.7, **step 3**. Select Area and Absolute (*see* **Note 34**) and select the range in which to look for the optimal settings for both the variance in Area and XY position. Use first a rough screen (e.g., for our system with *Arabidopsis* for Area from 10 to 100 in steps of 10 and for *XY* variance from 0 to 2 in steps of 0.2) to get an idea about the optimal range and do subsequent analyses to zoom in to the best settings (*see* **Notes 35** and **36**).

5. To measure germination for the whole germination experiment with the optimal settings, click Calculate; select the txt files that were created under Subheading 3.7, **step 2**; fill in the optimized parameters as obtained under Subheading 3.7, **step 4**; and click Calculate. The results file with the cumulative germination table of the experiment will be automatically saved in the default directory.

3.8   Curve Fitting and Interpretation of the Data

1. A cumulative germination table in itself does not provide much information. Therefore the GERMINATOR also has a curve-fitting module to convert the cumulative germination data into useful parameters that can be used to compare germination characteristics of seed batches. After the cumulative germination table has been calculated, there will be an option to fit curves. Click Yes and select the Germinator_curve-fitting 1 29.xls file in the Excel directory of the GERMINATOR package and click Save.

2. In the OUTPUT sheet of the curve-fitting file, several parameters for the curve fitting can be adjusted: the minimal germination at which a germination curve will be fitted through the data, the number of hours until which the area under the curve (AUC) should be calculated (*see* **Note** 37), the critical *p*-value that should be used for the statistical analysis (default on 0.05), which additional parameter should be calculated apart from t50 (e.g., t10 or t90), a lower limit for the *r*-square of the fit, the uniformity parameter that should be calculated (e.g., u8416 or u7525), and if letters should be added above the bars in the graphs to indicate statistical differences between the different measurements.

3. Click GO in the OUTPUT or INPUT sheet to fit all germination curves and calculate the different parameters per curve. These will be shown in the OUTPUT sheet. By placing 1 in column R behind a specific germination sample and clicking Graph, the curves of the selected samples will be plotted.

4. For germination samples with the same names, averages will be calculated with standard errors and statistical differences between samples will be calculated and shown in the statistics sheet.

5. Also after the curves are fitted, some parameters as mentioned under Subheading 3.8, **step 2**, can be adjusted and statistical analysis will be repeated after clicking the "recalculate statistics" button in the OUTPUT sheet.

4   Notes

1. Our system is optimized for stackable 15×21 cm trays (ref 109, DBP plastics, Belgium. www.dbp.be), but any other germination tray can also be used. The advantage of the DBP trays is that they are stackable and allow for convenient storage in germination incubators in which each tray functions as the lid of the tray below and as such avoids evaporation as much as possible.

2. The germination paper should have a color (in wet conditions) which is sufficiently contrasting to the color of the seed coat

and radicle. We use 14×19.5 cm blue blotter germination paper from Anchor (Anchor paper company, St Paul, MN, USA; www.seedpaper.com). It can be ordered in any desired size if it should be used in different size germination trays.

3. Any germination cabinet that is used for normal germination tests can be used. For working with *Arabidopsis* it is important to have light and preferably from the side, so that all trays get equal exposure to light.

4. As alternative, A3 paper sheets can be placed between the light and the trays to get a similar effect of indirect lightning and also the strength and position of the lights can be changed to optimize the light conditions.

5. There are also specific Photoshop action files for use with 3, 20, and 24 germination tests per tray. Other formats can be requested from the author.

6. It is wise to select the cropping areas as broad as possible with as little as possible chance to select seeds of the neighboring germination tests.

7. It is important to use ImageJ version 1.40. Newer versions unfortunately do not support Visual Scripting.

8. Select Germinator_table 2.01.xls if a different number of germination tests on one tray is used. Version 2.01 allows the formation of germination tables with any number of germination tests per tray.

9. The content of the drop-down selectors can be changed or items can be added to it in the "Experiment descriptors" sheet.

10. Make sure that for the date format in cell B10 "-" is used as separator instead of "/". This can be changed in the Region and Language control panel of the used Windows system under the Short date format.

11. It is important to use two sheets of germination paper to obtain a big enough buffer of solution. This will make the system less sensitive to influences on germination due to evaporation.

12. The amount of solution added to the trays is very important. Too little will have influence on the germination, but too much will hamper the image analysis: too much water will cause a light-reflecting meniscus around the seed that will be mistakenly detected as a radicle by the system.

13. Instead of adding water, also salt, mannitol, PEG, ABA, or any other compound can be added to the trays to analyze the effect of these compounds on seed germination.

14. The mold is made manually by cutting holes in a plastic sheet. In our system, a mold for 6 germination tests per tray is used, but as mentioned before, also molds for, e.g., 2, 3, or 24 germination tests per tray can be made.

15. The mold is reused for a next tray, after sowing one tray.

16. The amount of seeds depends on the size of the used seeds and of the holes in the used mold. Take care to have the seeds as evenly distributed as possible since too many seeds too close together will hamper reliable automatic scoring of germination.

17. The trays on top and bottom of the pile are there to prevent unequal evaporation as much as possible.

18. For dormancy breaking purposes, the piles can also be incubated at 4 °C for 3–4 days before incubation in the germination cabinet.

19. The 53 cm is the optimal distance for the 15×21 cm trays, but should obviously be adapted for trays with different sizes.

20. The mask does not need to fit the trays very accurately, since our system is not sensitive to slight changes in position of the trays at the different time points. Changes up till 5 mm will not have an effect on the automatic scoring.

21. Low light with longer shutter speed works in general very well to enhance contrast.

22. In our setup, ISO 400, F/18, and 1/3 s are used, but these settings have to be defined depending on the specific light conditions. It is advised to be very punctilious in optimizing the camera settings. The better the pictures, the better the automatic scoring will perform.

23. The first picture should be taken after the initial imbibition, but before the first seeds begin to germinate, since taking a picture before initial imbibition (imbibition phase I) is completed will have an effect on the size of the seeds and the count of non-germinated seeds on the first picture will determine the total amount of seeds that is counted in the germination test.

24. The frequency of taken pictures depends on the germination speed. In general it is advised to have at least three pictures in the steep part of the germination curve. After all germination tests within an experiment have reached t50, the frequency can also go down. In general, pictures are taken till the moment that no further germination is expected.

25. In our setup 0.2 % saturated pixels is used to enhance contrast, but slightly other settings might be optimal for other setups.

26. In our setup the best results are obtained with YUV channels, but HSB, RGB, or CIE channels might work better in other setups.

27. Make sure that there is no, or as little as possible, size difference between both settings for non-germinated seeds, but that there is an obvious size difference for germinated seeds.

28. The upper size limit will also filter out seeds with long radicles, but this will not hamper the analysis, because seeds not present in the + white filter will be automatically counted as germinated seeds.

29. In our setup pictures are automatically taken upside down due to the orientation of the camera on the repro-stand.

30. The system is designed for the Photoshop version in the English language. The names of the files for the cropped separate germination tests should end with "copy" followed by a number (e.g., 10212015-0923#001 copy 4.jpg).

31. It is important to save the files with the .txt extension and not with the default .xls extension due to compatibility issues with the subsequent analysis by the Excel scripts.

32. Both the germination score + white and germination score – white analysis can be performed simultaneously, which saves PC calculation time. To do so, ImageJ has to be started twice and the subsequent steps have to be followed in each open instance of ImageJ for germination score + white and germination score – white, respectively. In this case it is important to keep the two appearing Summary and Results windows apart. One way to do so is to drag the two windows of the germination score – white analysis to the left of the desktop and the two windows of the germination score + white to the right of the desktop.

33. When the suggested naming of the txt files is used, only the first txt file (summary+.txt) has to be selected and Excel automatically will fill in the other three files.

34. In our system with *Arabidopsis* the Area variance and absolute variances are used. For other setups and seed types, other settings might work better. Also the Perimeter can be used instead of the Area of the seeds, or relative differences (%) as compared to absolute differences.

35. The optimal settings for our system with *Arabidopsis* seeds are 48 for Area and 0.8 for *xy* variance.

36. The maximum number of combinations that can be tested at one time is 255.

37. The AUC is determined until a user-defined time point. This time point is preferably chosen shortly after most germination tests have just reached maximum germination. When using an earlier time point, the AUC will mainly correlate with the t50 and using a much later time point will result in AUCs that are highly correlated with the maximum germination. If chosen at the right position, AUC will be a combinational measurement reflecting aspects of both the t50 and Gmax.

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