

Dynamic imaging in pollen morphology

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Research

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Abstract

The performance of domestic agriculture and the food industry is constantly increasing. Demand for food is growing on the global market, with domestic food export growing by 10% in 2019. However, there are still reserves in exploiting the growth path and transforming it into income. Efficiency, revenue and profit growth of each product line depends on numerous factors; one of the key tools being the monitoring of the production process, getting to know the production environment accurately, data collecting, database building, development of applications that support automated interventions and decisions, and integration the above tools. The fundamental objective goal of the presented research is to approach the research of maize pollen cells as an agriculturally relevant model organism from a new direction, the long-term (Time-Lapse System) microscopic study of the growth dynamics of the pollen germ tube. In the scope of the research, two industrially and agriculturally important aspects taken into account, which also detected digitally: the growth of the maize pollen germ tube and the viability of the pollen. The research can provide a basis for the more accurate understanding and subsequent study of the effects of different biotic and abiotic stress factors on pollen growth, and may open up new possibilities in the field of digital agricultural biotechnology.

Introduction

Maize (*Zea mays* L.) is the third most important cereal in the world after rice and wheat in terms of production area and yield (32). Like most wind-pollinated plants, it has a high pollen-producing capacity, a property essential for hybrid maize seed production to achieve high yields and maintain the genetic purity of hybrids (4, 33). Assessing pollen viability is crucial to understanding plant reproduction and genetic improvement. Automatic pollen analysis viability in a wide range of hybrids Determined great genetic diversity for pollen, as well as differences in their sensitivity to the environment (31). Maize has spherical pollen with a diameter of 70–100 μm (13), the amount of which available for fertilization can be influenced by environmental factors, by changing the amount of pollen in a tassel and the synchronization of silking (4, 15). Pollen shedding lasts 2–14, most often 5–8 days, with the maximum amount of pollen dispersed reached on the 3rd day (20). Pollen-related studies should be performed within a short period, as under natural conditions maize pollen retains its viability in the open air for 1 to 4 hours after being dispersed from anthers (11). When mature pollen and the pistil interact, the water content of the pollen increases and the germ tube emerges from the pore on the located on the surface of the exine, the outer part of the pollen wall. In the case of grasses such as maize, pollen germination begins a few minutes after the pollen reaches the styles. After that, the germ tube grows at a relatively high rate, it can reach $1\text{cm}\cdot\text{h}^{-1}$ (3, 23). In most plant species, the pollen cell wall consists of two layers, an inner callus layer and an outer layer containing mainly pectin, cellulose, and hemicellulose. The pollen germ tube grows only in the apex of the tube, where has the newly synthesized cell wall constantly changing (30). The cell wall of this apex is composed of a simple layer of pectin, without callose and cellulose (10), which has a dual function, provides the necessary rigidity of the cell wall to maintain cell integrity and allows controlled growth of the germ tube with its flexibility (29). During the elongation

process of the germ tube, the cytoplasm, vegetative nucleus, and sperm of the pollen transferred to the germ tube, which under natural circumstances grows in the intercellular space within the pistil. The germ tube serves as a channel for sperm cells until they reach the eggs in the ovule (26, 27).

Successful fertilization has critically influenced by pollen fitness, pollen maturation dynamics, dispersion, germination, and growth rate of the germ tube (5, 12). Determining pollen viability provides an important tool for assessing plant growth potential and answering fundamental questions of pollen cell biology (20). Investigation of the viability and propagation of maize pollen is of practical importance in addition to basic biological research, as the distance of gene drift with the spread of genetically modified maize hybrids depends primarily on the time of pollen viability in addition to wind speed. Thus, the investigation of this is of paramount importance for the professional determination of the correct isolation distance (2).

In biotechnology, pollen primarily a sensitive biological examination tool for studying the effects of environmental pollutants and toxins, and on the other hand, pollen production can influence in improving the efficiency of plant breeding techniques (8). Pollens with different physiological properties, temperature, salt, and herbicide tolerance been successfully selected in many plant species (18, 24, 34).

There are various test methods for evaluating pollen vitality. Alexander's stain (AS), which distinguishes between living and inanimate (aborted) pollens by staining them red and turquoise that is a common method (1). The method for determining pollen tube extension using fluorescence, which created using tobacco pollen, must transferable to any pollen that can be quickly collected and examined in the laboratory. For example, robust protocols developed for growing tomatoes and maize, which make it possible to achieve comparative chemical plates (17). Another frequently handled method uses tetrazolium salts as an indicator to detect mitochondrial dehydrogenase activity. Such indicators include thiazolyl blue tetrazolium bromide 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 2,3,5-triphenyl tetrazolium chloride (TTC), which stain living pollens dark purple-black or orange (7). A significant disadvantage of these histochemical methods is that since they are cytotoxic, it is not possible to perform further studies requiring a living cell after their use (21). As technology advances, microscopic digital imaging techniques are also being used to various studies related to pollens (6, 13, 22, 28). These techniques generate new challenges to be solved due to the many biological and optical difficulties involved in the analysis of plant cells and tissues (9).

Materials And Methods

Our experiments performed at the Látókép Crop Production Experimental Plant of the University of Debrecen (47 ° 33 'N, 21 ° 26' E, 111 m asl). Maize (*Zea mays* L.) used as a test plant. Sampling performed during the pollen dispersal period of the maize tassels at a value of 5 on the HANWAY (16) scale. Sampling collected on the first and second days from the start of the pollen dispersal period of the given plant from fresh pollen to maintain viability. According to studies by KAEFER et al. (19), the viability

of maize pollen had highly dependent on environmental factors, including the relative humidity of the pollen dispersal period.

During sampling, an average sample prepared from pollen originating from the tassels of 10 plants. Pollen sampled at 9 a.m., after which the pollen grains immediately placed in a sterile Petri dish containing 5 ml of nutrient solution, as according to the research of AYLOR (2) viability of airborne pollens reduces by 50% in 4 h.

The pH of the solution 6 and it contained the following components: 15% sucrose, 0.01% boric acid, 0.025% CaCl₂, which solidified with 1% agar-agar for stationary analysis of pollen particles. Pollen cells incubated at 25 °C and 70% relative humidity for 24 hours and the development of germ tubes and loss of pollen cell viability observed by time-lapse imaging video microscopic system (TLS). Pollen grains can consider germinated if the length of the formed pollen germ tube is at least equal to the size of the pollen grain (14).

Description of equipment required for digital imaging of maize pollen grains

The time-lapse experiment took 24 hours and the method used three times. Important information filtered out of this time-lapse video. In the scope of the examinations, utilized the latest developments of the time-lapse video microscopic system.

These were the following: incubator type: SANYO MCO18-AC CO₂ cell culture incubator (Wood Dale, IL, US) equipped with four video microscopes. Olympus microscopes (Tokyo Japan) in an inverted position, with practical modifications: equipped with a light source in the turret and a CCD camera installed in the eyepiece. The lenses (x10: 0.25, achromat Carl Zeiss, Jena) located in front of the CCD camera. Properties of the CCD camera: 2 megapixel resolution UVC USB 2 webcam with modifications: camera body, lenses, and infrared filter removed. Light source: LED, emitting 960 nm wavelength, near-infrared light (5mm diameter; 1.2V; 50 mA). Phototoxicity minimized using infrared illumination (25).

images took every minute by averaging 11 frames to increase the signal-to-noise ratio for easier analysis in the entire duration of the experiments. The images captured in 1600 x 1200 pixel resolution and 24-bit RGB format. the used open-source Fiji (ImageJ) software developed by the National Health Institute used together with its additional plugins (<https://fiji.sc>) to analyze the images. Finally, the obtained results obtained presented in Excel. The experiments completed on 10 well-separable maize hybrid pollens in three replication.

Results

Unnecessary sequences removed from the beginning until the end of the video as a first step, to examine the growth of pollens and assess their viability. This step greatly reduced the size of the sequences to analyse. Accelerated the process performed for analysis by Using destructive editing algorithms unnecessary information.

When the sequence loaded, the images converted to an 8-bit format (Figure 1), then image applied enhancement algorithms. Always the methods application and their extent depend on what information extracted from each recording. This affected by the quality of the images taken, so the analytic scheme may change depending on it. Usually, are applied the following macros, plugins, and filters:

8-bit conversion: The raw image sequence is in RGB 24 bit format. An eight-bit grayscale is created and the work proceeds in the intensity range from 0-255 (Figure 1). The amount of data decreases, but the image content does not change. *ImageJ converts 16-bit and 32-bit images and stacks to 8-bits by linearly scaling from min-max to 0-255.* <https://imagej.nih.gov/ij/docs/menus/image.html>

Histogram Smoothing: To reduce the dynamic differences between each image, the grayscale range containing useful image information interpreted on the histogram extended in each, allowing 0.3% saturated pixels (Figure 2) ling.

[https://imagej.net/Enhance_Local_Contrast_\(CLAHE\)](https://imagej.net/Enhance_Local_Contrast_(CLAHE))

Fast Fourier Transform, FFT: Using FFT, it is possible to apply a band-pass filter that removes image errors (pixel noise, background) below and above the given size range (Figure 3).

<https://imagej.nih.gov/ij/plugins/fftj2.htm>

Spatio-temporal reduction: reduction of the amount of data “in space and time”. That the keep proceeding with relevant information. Information on the recordings reduced to the event that is important for the study. By reducing the spatial and temporal size of the image sequence, the resource requirements of the analysis can significantly decrease.

a) FOV – reduction of the field of view

Reducing the FOV practically means separating the areas of the image that contain the processes relevant to the analysis (Figure 4). Reducing the FOV decreases the resource requirements of the analysis. After applying the procedure, further pre-processing operations are limited to the image content analysed and becomes more accurate parameterization of the additional procedures.

b) Temporal reduction

From the series of recordings (1422 frames), the interval (1-600 frames) covering the phenomenon to be studied is highlighted (Figure 5).

Segmentation of the prepared image sequence - separation of foreground and background

During the steps of image segmentation, a black and white image created that best describes the course of the examined event (binarization). In the course of binarization, these pixels brought to a value of common intensity different from the background by summing the contrasting extreme values. Then a softening following a Gaussian distribution with a radius of 5 pixels applied to the image (Gaussian blur).

Gaussian blur: Like all ImageJ convolution operations, it assumes that out-of-image pixels have a value equal to the nearest edge pixel. This gives higher weight to edge pixels than pixels inside the image, and higher weight to corner pixels than non-corner pixels at the edge. Thus, when smoothing with a very high blur radius, the output will be dominated by the edge pixels and especially the corner pixels.

<https://imagej.nih.gov/ij/docs/guide/146-29.html>

Sliding paraboloid: background subtraction

When removing the background of the softened image, typically a virtual sphere with an x-pixel radius attempted placed on the pixels, practically “rolling it through” the surface of the image. However, in the image with reduced data content, a smoother result (better following the outlines) obtained if a sliding parabola with the used same radius (in this case 10px) (Figure 7).

<https://imagej.nih.gov/ij/docs/guide/146-29.html#toc-Subsection-29.14>

Histogram Equalization: it is advisable to extend the values occupied by the image content in a gray area better suited to human perception before segmenting the reduced background image sequence with a threshold operation (Figure 8). the grayscale range containing useful image information interpreted on the histogram extended by image, allowing 0.3% saturated pixels to reduce the dynamic differences between each image. [https://imagej.net/Enhance_Local_Contrast_\(CLAHE\)](https://imagej.net/Enhance_Local_Contrast_(CLAHE))

Threshold - image segmentation:

In the process, the relevant information is separated from the background based on pixel intensity in a series of recordings of the prepared image sequence. The operation splits the 8-bit intensity range of the image into two values (Figure 9). *Use this tool to automatically or interactively set lower and upper threshold values, segmenting grayscale images into features of interest and background.*

<https://imagej.nih.gov/ij/docs/guide/146-28.html#toc-Subsection-28.2>

Duplication – *Creates a new window containing a copy of the active image or rectangular selection.*

<https://imagej.nih.gov/ij/docs/guide/146-28.html#toc-Subsection-28.9>

Image calculator: The next step subtracts the sequences so that in a third window the difference between them can be seen, i.e. in the present case the growth rate of the pollen germ tube. The third window that appears is the difference between the two sequences. The two original sequences will no longer be needed. *Performs arithmetic and logical operations between two images selected from popup menus described in the [Image operations](#) table. Image1 or both Image1 and Image2 can be stacks. If both are stacks, they must have the same number of slices. Image1 and Image2 do not have to be the same data type or the same size.*<https://imagej.nih.gov/ij/docs/guide/146-29.html#toc-Subsection-29.13>

Quantification: It performed with the ‘Analyse particles’ command. This command counts and measures objects in binary or thresholded images. The analysis performed on the existing area selection or the entire image if no selection present. <https://imagej.nih.gov/ij/docs/guide/146-30.html#toc-Subsection-30.2>

The measurement results are exported into a software suitable for evaluation, e.g. Excel and are represented in a chart.

Examination of germ tube growth

To examine the growth of pollens and evaluate their viability, irrelevant information removed from the video as mentioned above, and then the differences expressed in pixels by subtracting the areas of successive moving images. For the study of the digitally enhanced binary image sequence, examined the surface areas of pollens. In the case of the study of the surface area of each pollen, a drastic increase meant the onset of germ tube growth, and a drastic decrease meant critical water loss. A user-controlled spatial and temporal separation method used to avoid confluent germ tubes. The essence of the method was that the pollen examined in a square of 20,000 x 20,000 pixels, so the surface area of the germ tubes determined in units of time and space. For this reason, the only individual examined pollens that moved in the same focal plane. The test performed on 10 well-separable pollens, and then the growth profile of the given hybrid could be determined from the average of the germ tube growths and water losses.

Results of the study of the growth of maize pollen germ tube are shown in Figures 10 and 11.

Analysis of pollen viability

The results of the pollen viability analysis with a time-lapse microscope are shown in Figures 12 and 13.

Growth profile

The above analysis performed on 20 pollen grains, and then evaluated the average of the analyses. Consequently, the growth profile of the hybrid was determined. The total growth profile of the analysed maize hybrid pollen grains shown in Figures 14 and 15.

In the scope of monitoring the growth profile of maize pollen, the stages of the process became well separable. Germ tube growth started in approximately the first half-hour, followed by a long germ tube development of about 20 hours. The spores then lost water and then lost their viability.

Based on the analysis of the germination profile, it found that the formation of the germ tube started in the given maize hybrid 30 min after germination on average, and the pollens lost their viability on average 1257 min after germination (Fig. 15).

Discussion

In the presented study, a new method for digital morphological and quantitative analysis of maize pollen as a model organism demonstrated through the microscopic examination of maize pollen time lapse. The described algorithm allows the rapid and accurate study of the growth, germination, and viability strategy of the pollen. The created model being corrected and refined, but based on the presented analytic data obtained so far, has been proven the usability of the method. Another significant advantage of the

algorithm is that even large amounts of data created with high-throughput systems can be processed with it in a short time and accurately.

Conclusions

Given the growing importance of automated digital analysis of big-data systems in the agricultural biotechnology of the 21st century, there is a need to develop such and similar micro- and macroscopic image analysis techniques to obtain accurate and rapid determination methods for the measurement of essential features. Although enormous progress made in this area during recent years, most techniques specific to particular crops.

The presented image analysis method allows a high-resolution examination of the early development of any pollen from the time of germination. It hoped that the use of the technique will result in more rapid and routine detection of pollen growth kinetics and macro-morphological studies, thus significantly contributing to a rapid and accurate response to the challenges posed by climate change.

Declarations

Authors' contributions

János Nagy, László Tálás and Gábor Szemán-Nagy supervised the experiment, Csilla Bojté, Alexandra Kiss and Máté Szűcs made solutions for the germination, Árpád Illés and Csaba Bojtor performed the experiments and collected pollens in the field, Seyed Mohammad Nasir Mousavi and Márta Novák Hajós reviewed and prepared the manuscript to submit, Dávid Domonkos and Viktória Baksa made the figures and reviewed the manuscript.

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Availability of data and materials

All data supporting the conclusions of this article are included in this article

Ethics approval and consent to participate

Not applicable

Conflict of Interest Statement:

None

Consent for publication

Consent by the authors (János Nagy, László Tólas, Márta Novák Hajós, Csilla Bojté, Alexandra Kiss, Máté Szűcs, Árpád Illés, Csaba Bojtor, Seyed Mohammad Nasir Mousavi, Dávid Domonkos, Viktória Baksa Gábor Szemán-Nagy) were provided to publish his picture.

Competing interests

The authors declare that they have no competing interests.

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Figures

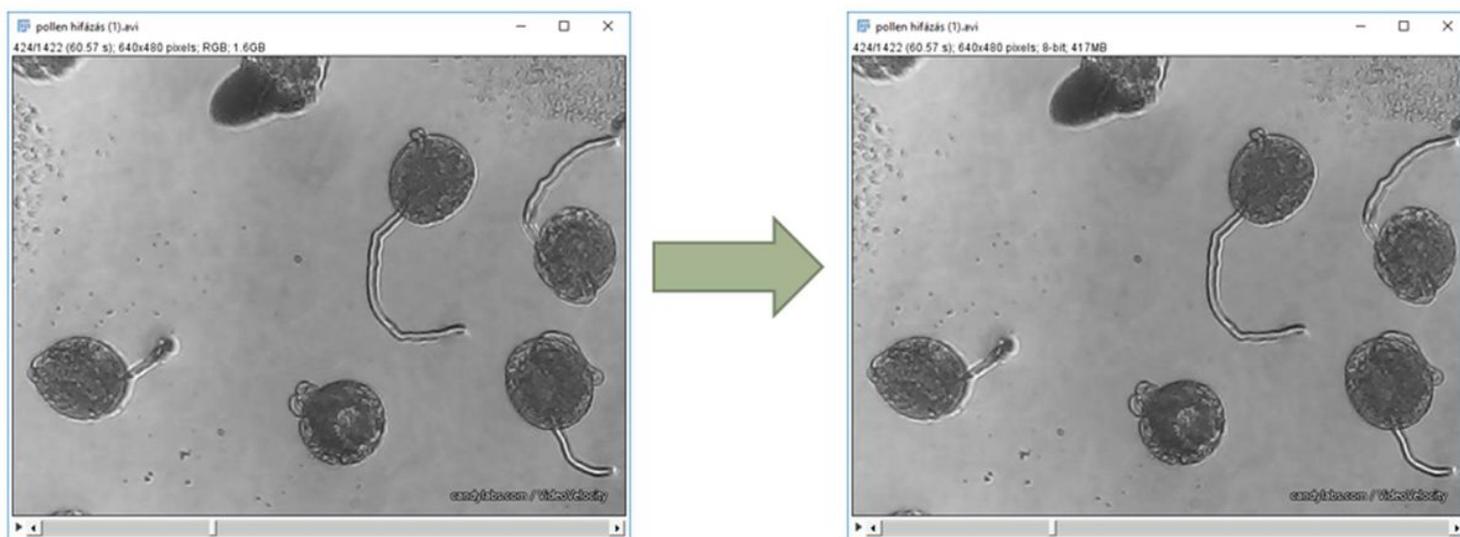


Figure 1

Conversion of RGB images to an 8-bit format to create a binary image of maize pollens.

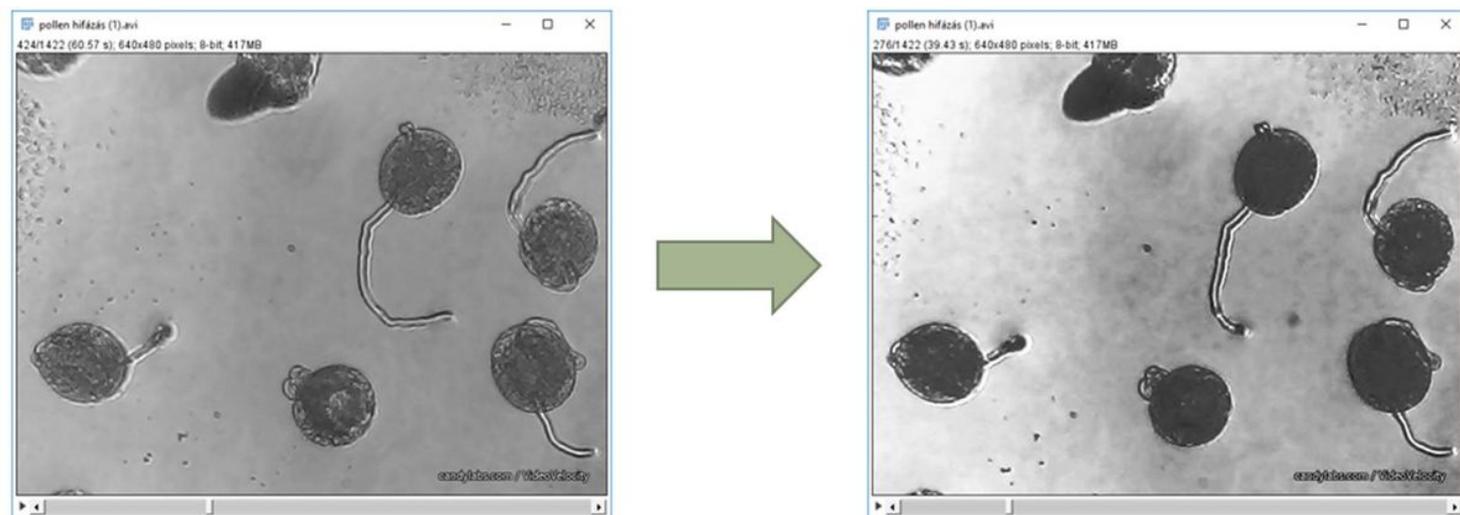


Figure 2

Improving the image of germinating maize pollens by histogram smoothing.

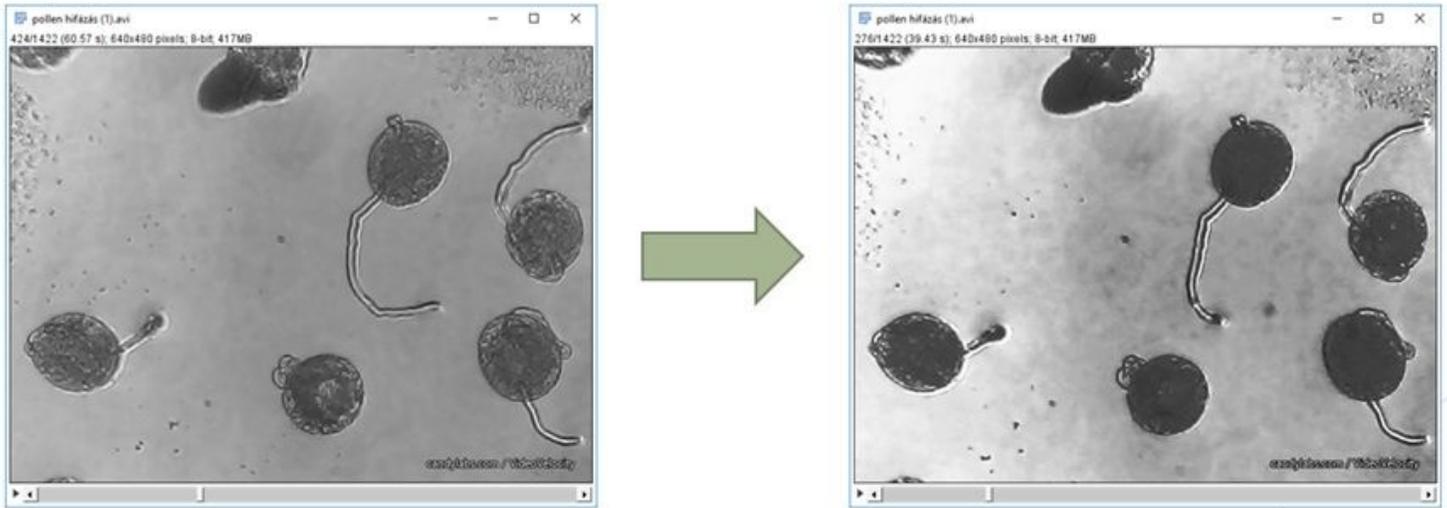


Figure 3

Removal of image errors by means of FFT

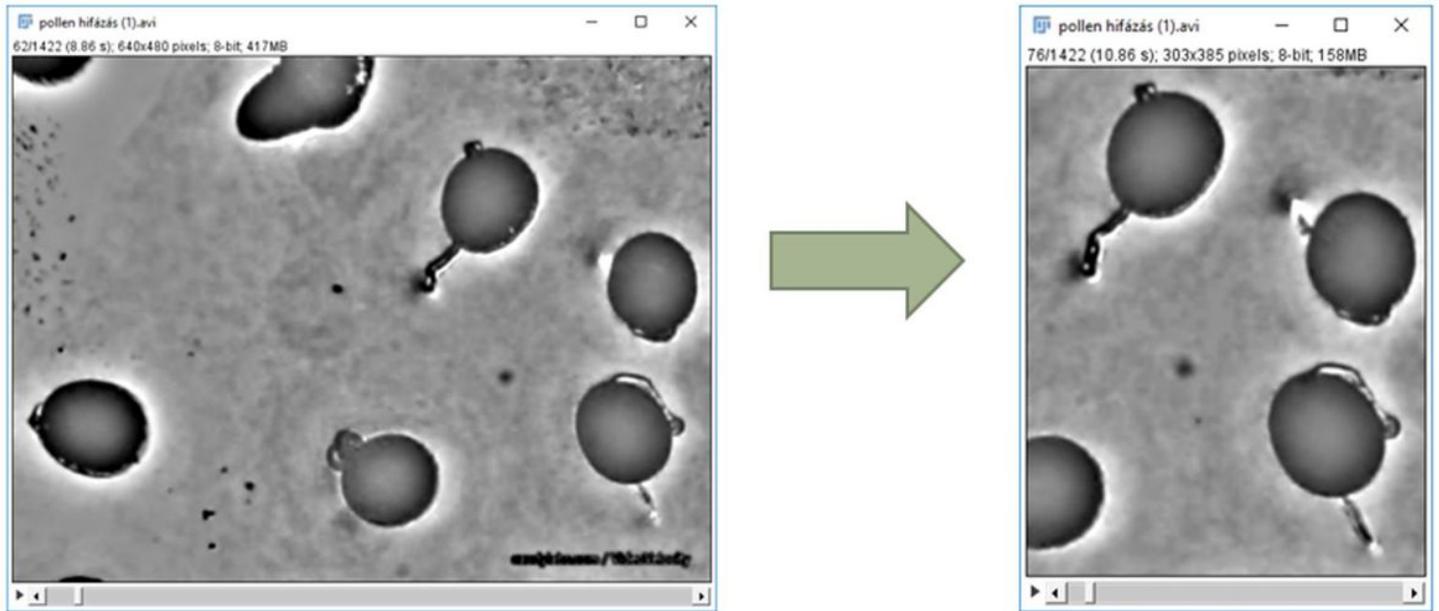


Figure 4

Reduction of field of view in Time Lapse video microscopic examination of maize pollens

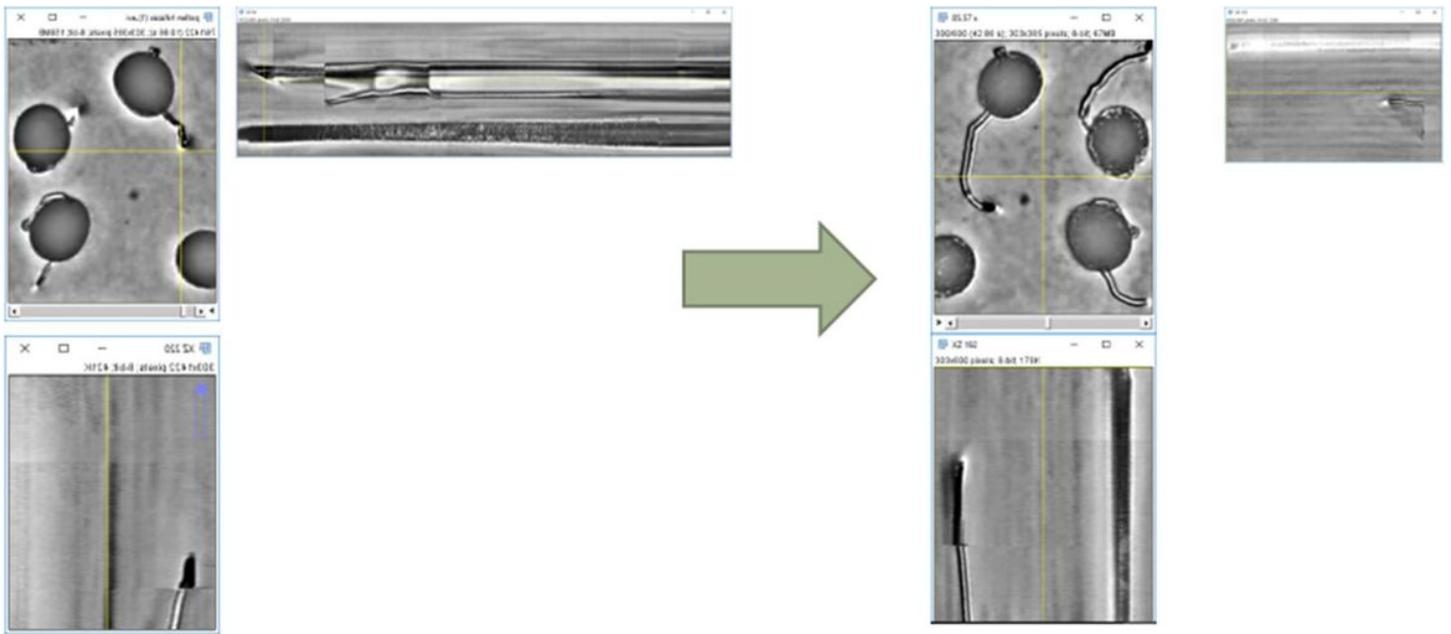


Figure 5

Temporal reduction of the image sequence of maize pollen grains

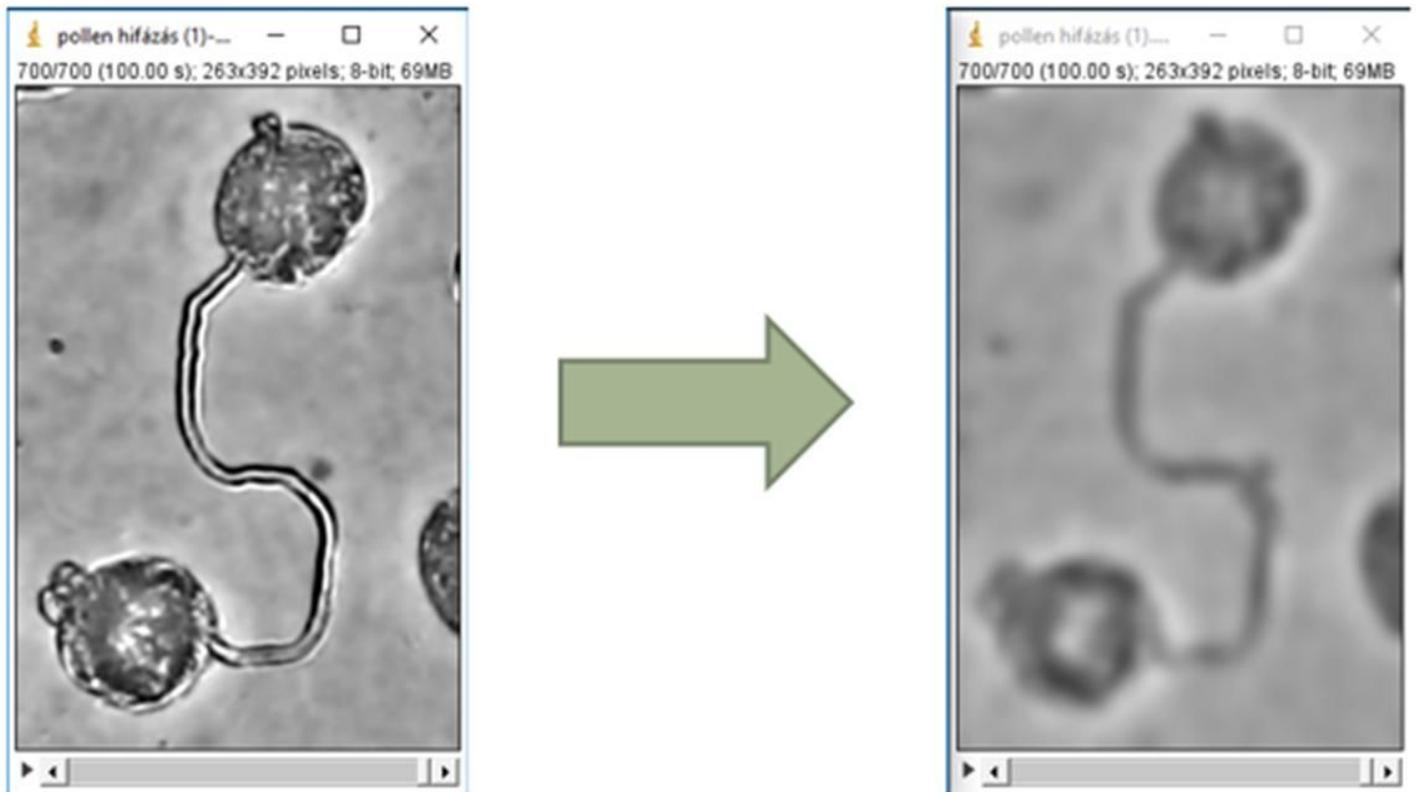


Figure 6

Image of germinating maize pollens before and after Gaussian blur

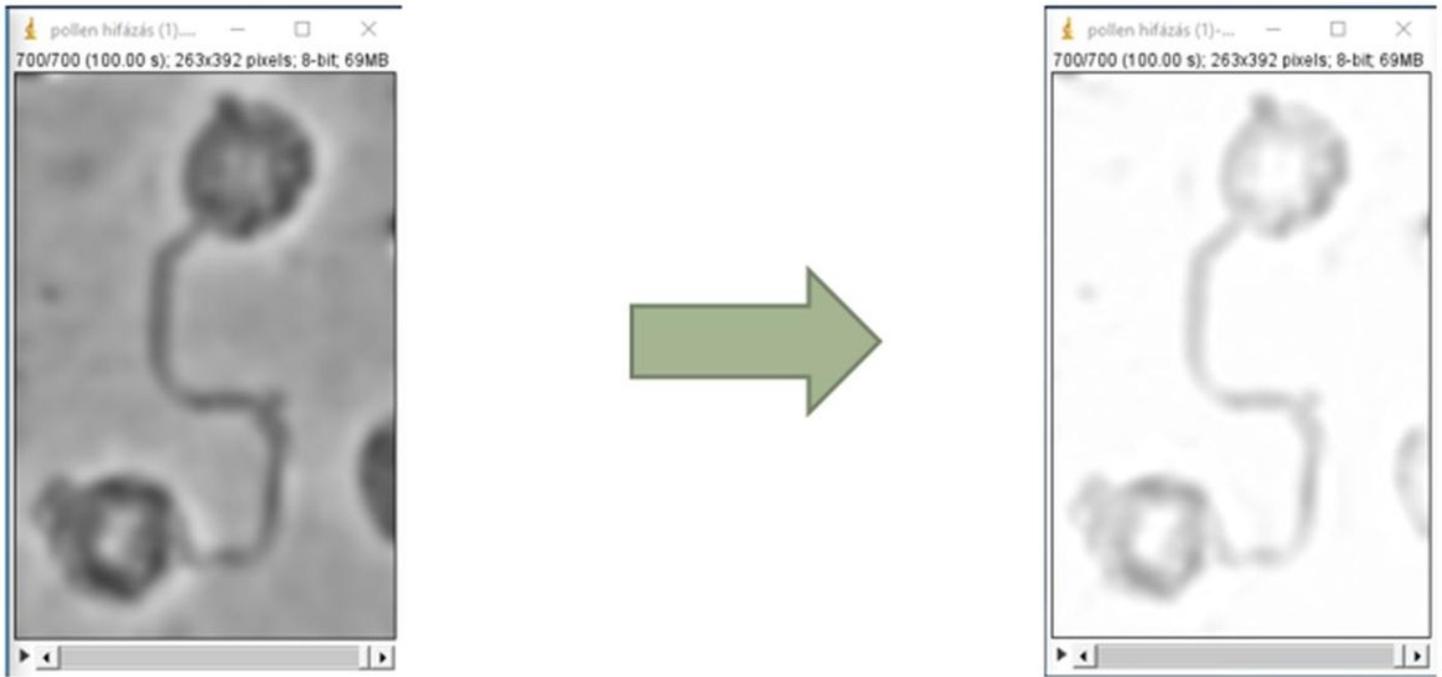


Figure 7

The softened image after subtraction of the background.

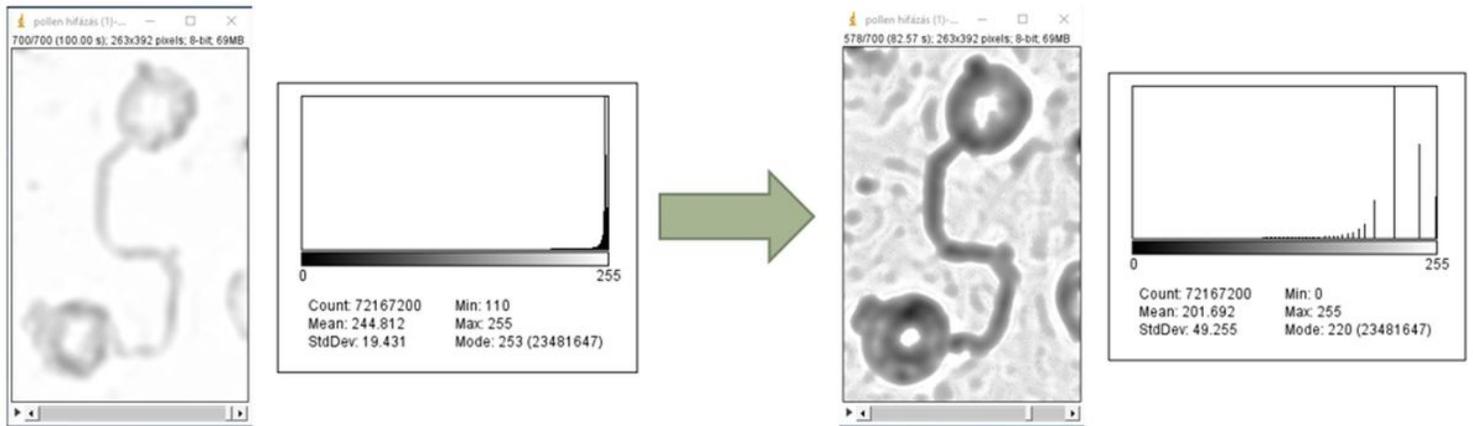


Figure 8

Sequence equalization of maize pollen with reduced background.

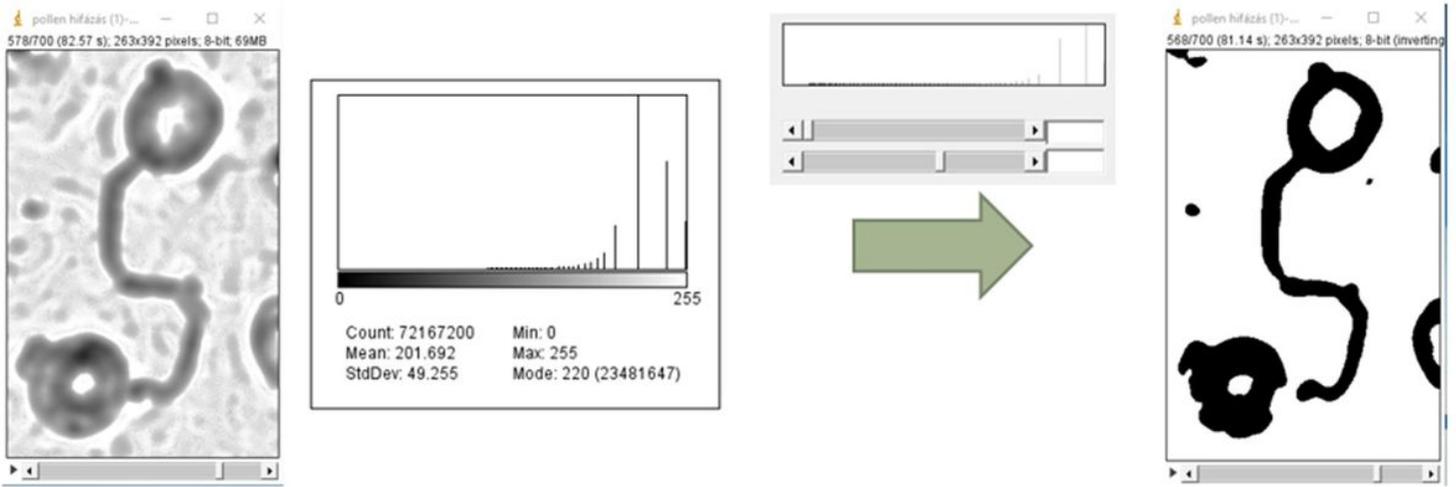


Figure 9

Binary image of a maize pollen ready for evaluation

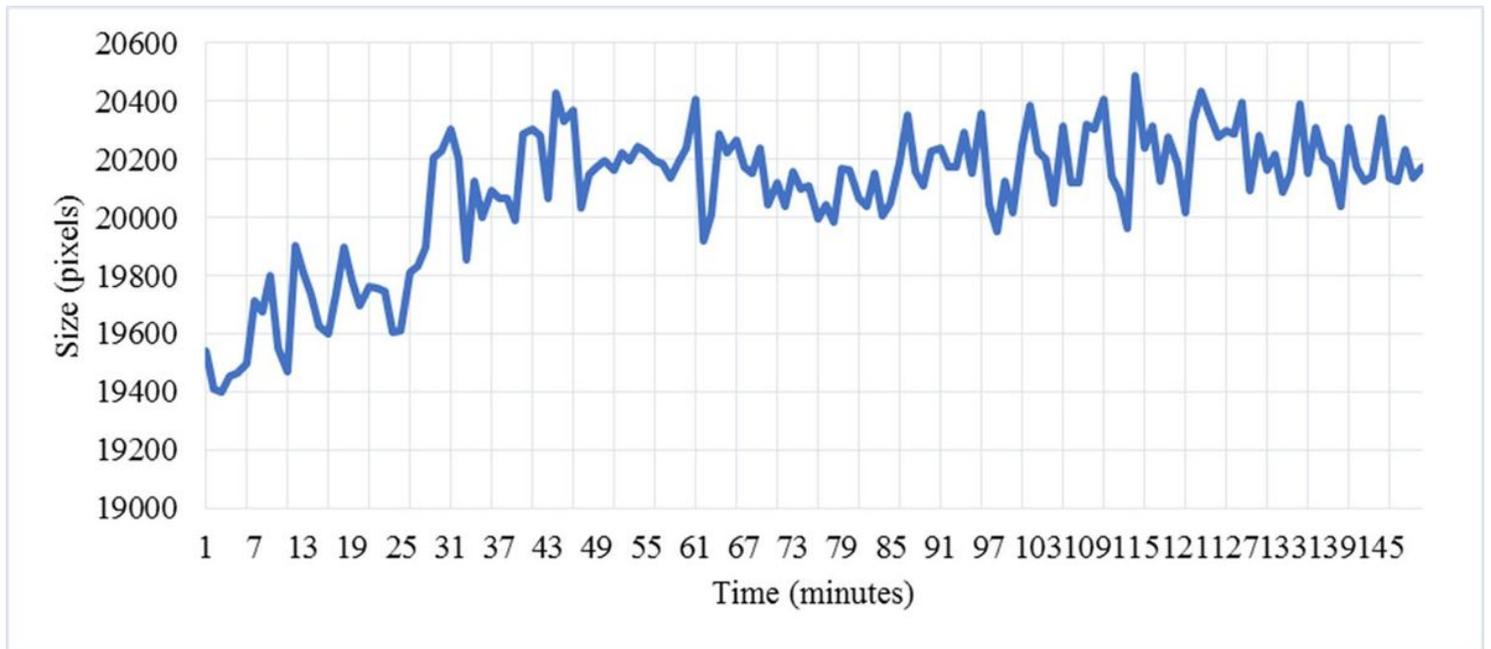


Figure 10

Examination of the growth of a pollen germ tube. The change in the size of a pollen germ tube was detected every minute during the incubation period. A drastic increase in the surface area of the germ tube (25 min) shows the burst of the outer shell and the onset of germ.

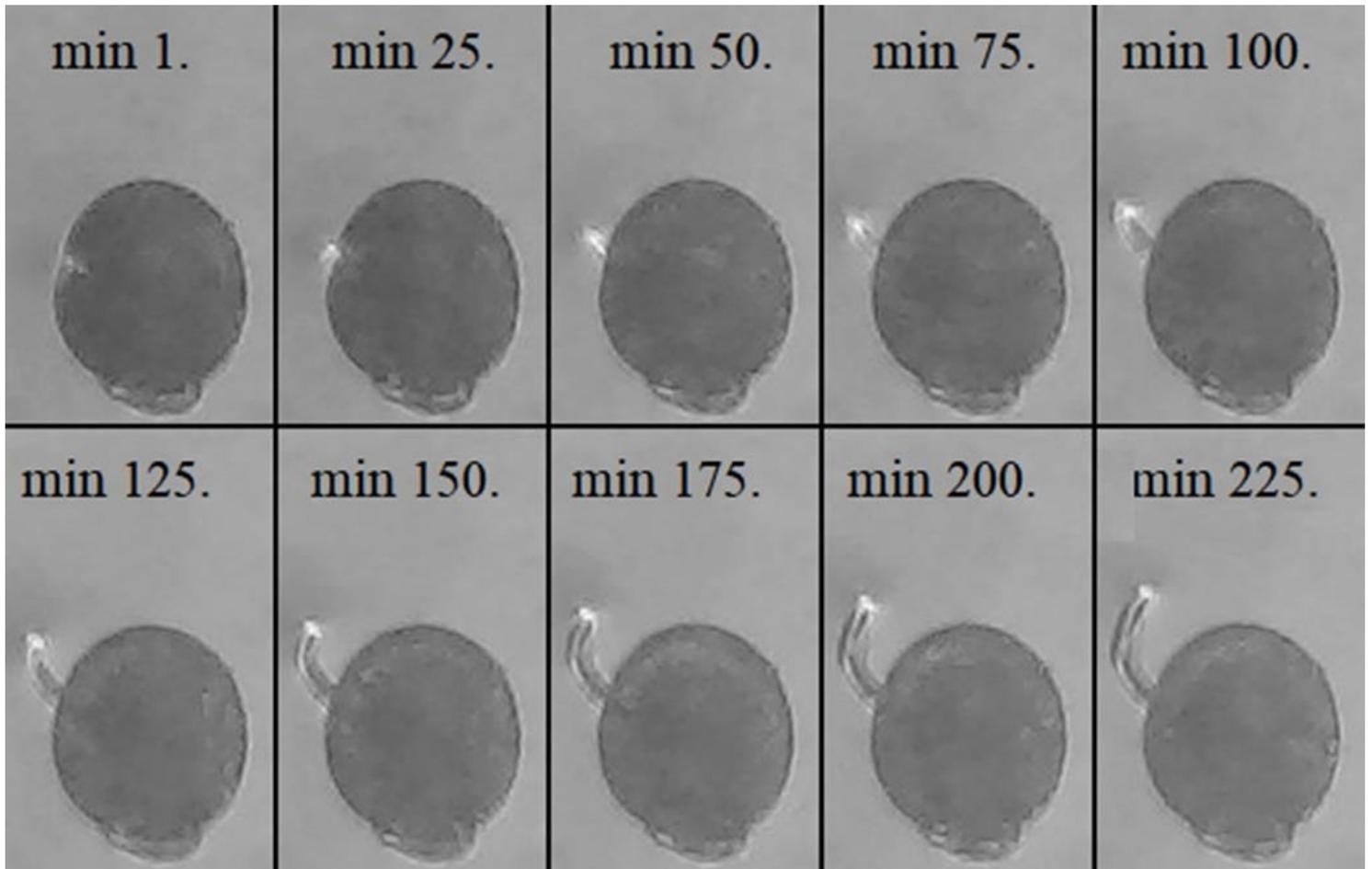


Figure 11

Growth of the pollen germ tube (10x magnification)

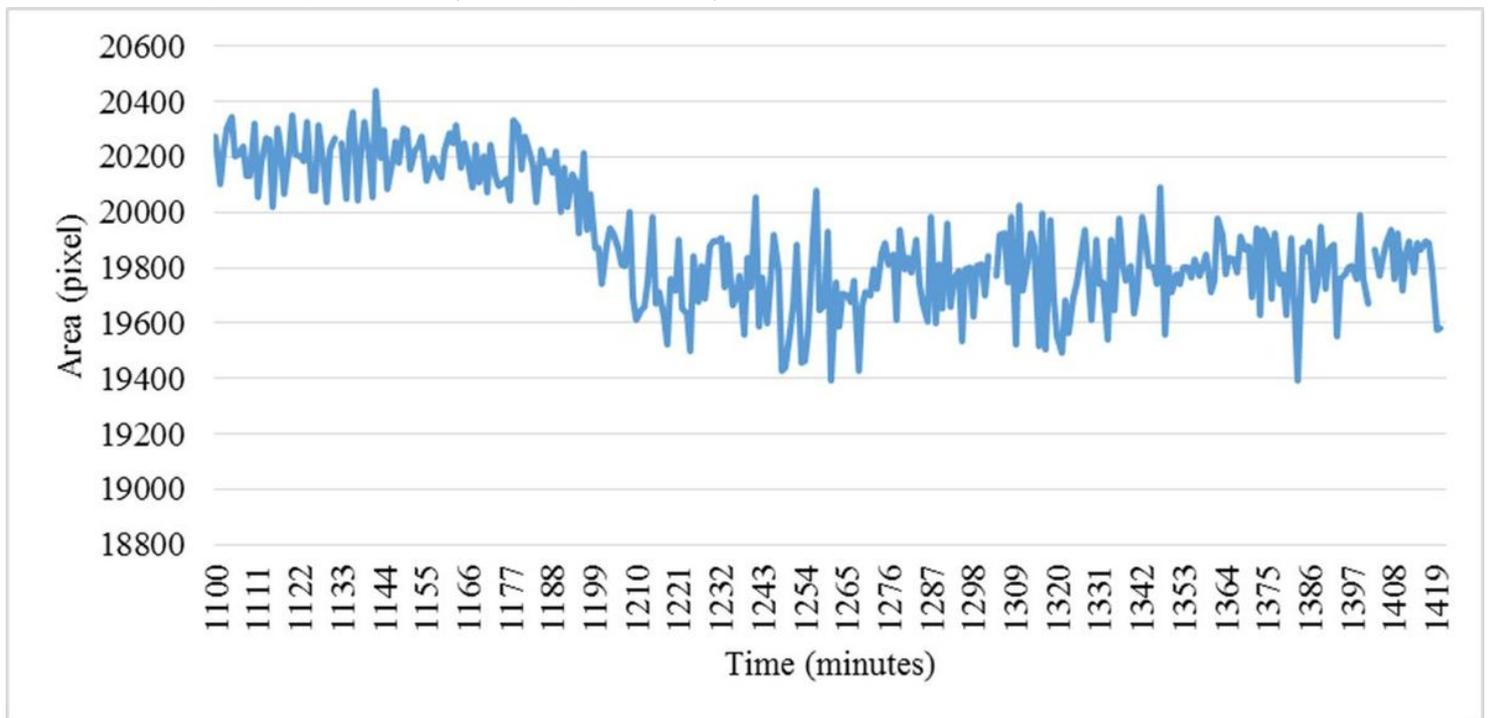


Figure 12

Development of maize pollen growth as a function of surface area and time change. Changes in terms of the size of the pollen and the germ tube were detected continuously every minute during the incubation period. A drastic decrease in the surface area of the germ tube (1200 min) indicates the end of germ tube growth and spore viability.

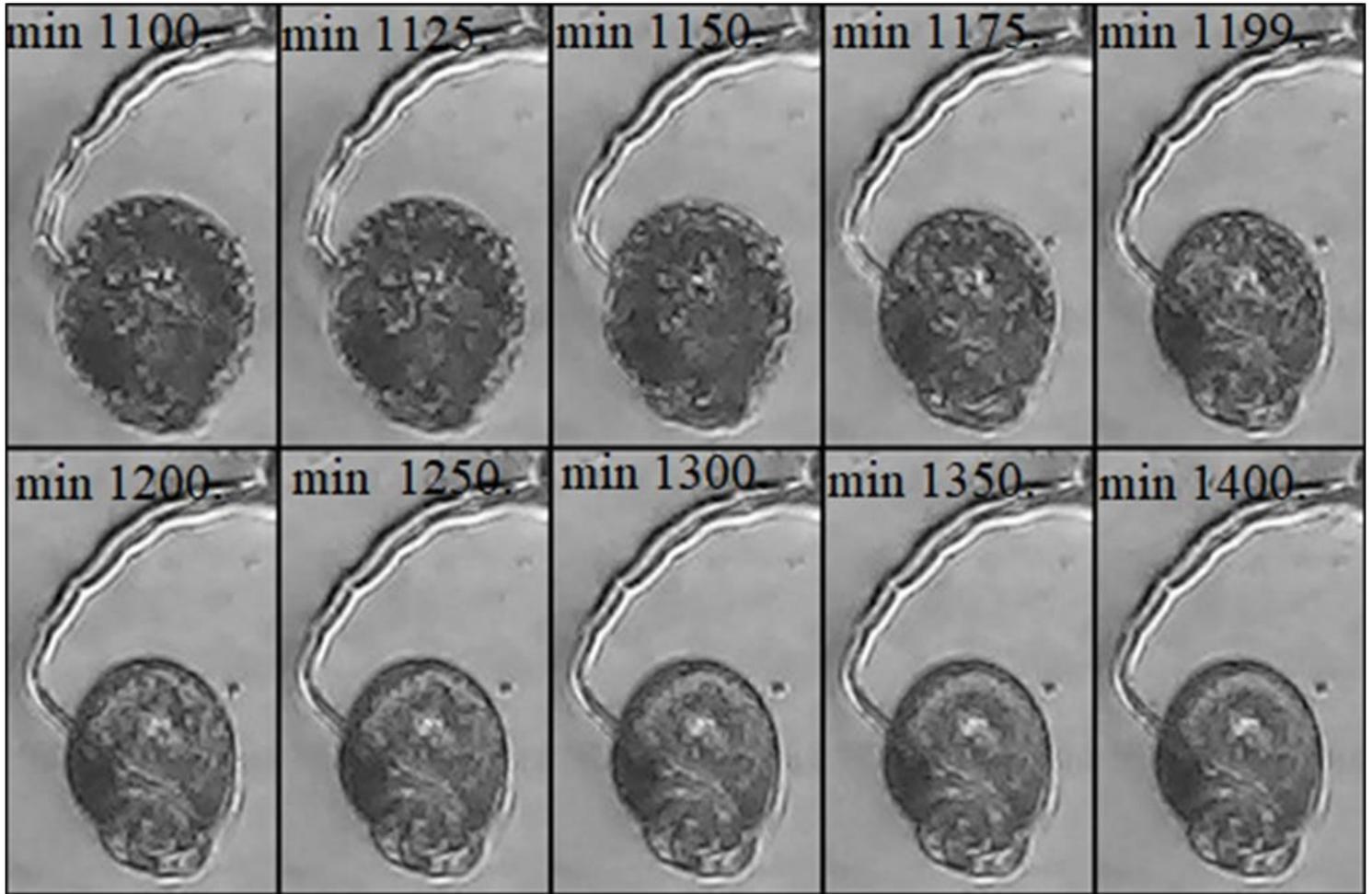


Figure 13

Analysis of Pollen viability (10x magnification)

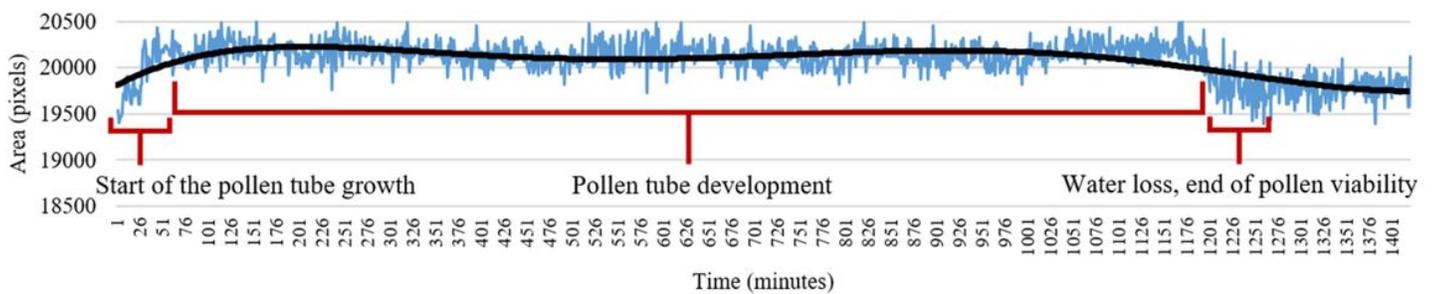


Figure 14

Analysis of the total growth profile of the examined maize pollen.

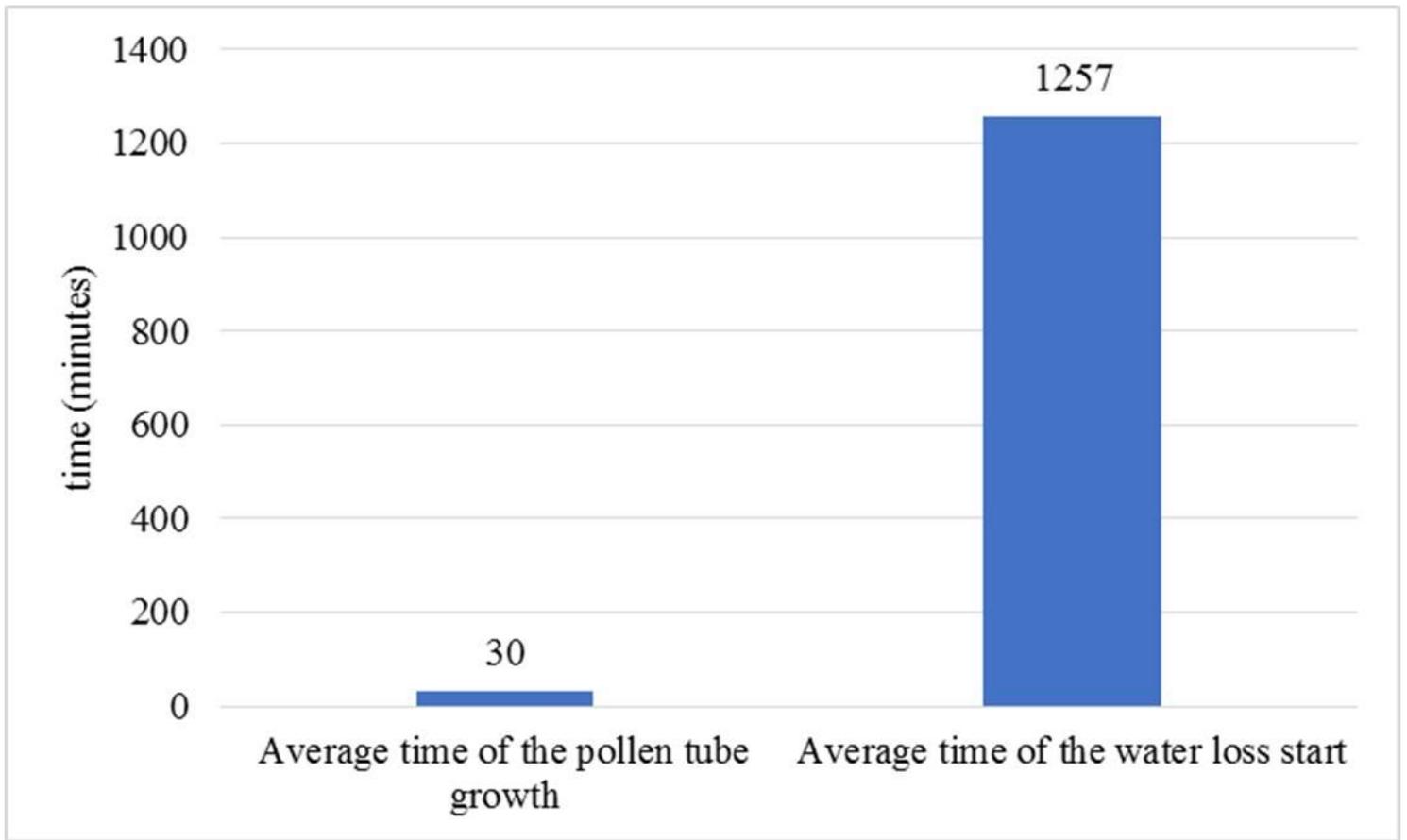


Figure 15

Average germination profile of the analysed maize pollen grains.