Electronic Journal of Polish Agricultural Universities is the very first Polish scientific journal published exclusively on the Internet, founded on January 1, 1998 by the following agricultural universities and higher schools of agriculture: University of Technology and Agriculture of Bydgoszcz, Agricultural University of Cracow, Agricultural University of Lublin, Agricultural University of Poznan, Higher School of Agriculture and Teacher Training Siedlce, Agricultural University of Szczecin, and Agricultural University of Wroclaw.

ELECTRONIC JOURNAL OF POLISH AGRICULTURAL UNIVERSITIES

2004
Volume 7
Issue 1
Series AGRICULTURAL ENGINEERING

ZDUNEK A., UMEDA M., KONSTANKIEWICZ K. 2004. METHOD OF PARENCHYMA CELLS PARAMETRISATION USING FLUORESCENCE IMAGES OBTAINED BY CONFOCAL SCANNING LASER MICROSCOPE **Electronic Journal of Polish Agricultural Universities**, Agricultural Engineering, Volume 7, Issue 1.
Available Online http://www.ejpau.media.pl

METHOD OF PARENCHYMA CELLS PARAMETRISATION USING FLUORESCENCE IMAGES OBTAINED BY CONFOCAL SCANNING LASER MICROSCOPE

Artur Zdunek[1], Mikio Umeda[2], Krystyna Konstankiewicz[1]
[1]*Institute of Agrophysics, Polish Academy of Sciences, Lublin, Poland*
[2]*Laboratory of Field Robotics, Division of Environmental Science and Technology, Graduate School of Agriculture, Sakyo-ku, Kyoto, Japan*

ABSTRACT

Fruits and vegetables are built mainly of thin walled parenchyma cells highly susceptible to mechanical damage. According to experimental investigation of relationship between cells geometrical parameters and mechanical properties of tissue a new efficient method of structure reconstruction and parametrisation is elaborated and presented in this paper. Special sample preparation and way of image taking by Confocal Scanning Laser Microscope with fluorescence mode allows obtaining images of any number of cells. Unique computer procedure based on morphological operators was created that allows analysing automatically any number of images with extracting geometrical parameters of each cell separately. The method was tested on potato and carrot tissue. Results show that the method is fast and gives proper cell reconstruction for both materials. In order to decrease error of incorrect reconstruction, possibility of manual correction was introduced to the procedure.

Key words: microscope image, image analysis, cell size, cell shape, plant tissue

INTRODUCTION

Fruits and vegetables are built mainly of thin walled parenchyma cells highly susceptible to mechanical damage. Therefore, agricultural machines or careless handling can cause irreversible changes in structure and next in colour and taste of the tissue. Mechanical properties of these soft plant tissues depend on external loading [1, 10, 14] and set of material properties, like turgor [6, 8, 9,10] and variety [14, 15]. One of the least known, but now intensively investigated, is influence of cells geometrical parameters on tissue mechanical properties.

In simplification, parenchyma tissue of plants is built of fluid filled cells connected by pectin bonds. Cell walls (internal – semipermeable plasmalemma and external – permeable wall) are relatively elastic with resistance for tension but weak for bending whereas the pectin bonds are amorphous and plastic [9]. The intracellular fluid causes tension of walls and certain level of tugor pressure. All mentioned elements create unique mechanical skeleton with characteristic mechanical properties. However, the cell is considered as a basic and the most important structural element of this structure.

A few successful attempts of theoretical and physical models of plant cell have been made. Pitt [9], Pitt and Chen [10], have created theoretical 2D model of single cell that partly explains influence of turgor and strain rate on tissue mechanical properties. The model was next extended to 3D and phenomena between the cells [2, 11]. Umeda et al. [12] have created physical model in the form of tetrakaidecahedron according to Kelvin's hypothesis that elements of this shape fill the space completely. Reaction forces in this model were measured experimentally and analysed with Finite Element Method. An influence of cell size on stress in cell walls was discussed by Haman et al. [3]. They have shown that stress in cell walls depends on their curvature and is higher for bigger cells at certain deformation. It means that big cells are more susceptible to mechanical damage. It is confirmed experimentally by Konstankiewicz et al. [4] that have shown that strength of the inner core of potato tuber, built of smaller cells is higher than strength of outer core built of bigger cells. According to experimental investigation of relationship between cells geometrical parameters and mechanical properties of tissue a special method was used in that research [5]. 2D images of potato tissue in natural state were obtained by Tandem Scanning Reflected Light Microscope. Images were outlined by hand over the walls and next automatically processed in order to obtain geometrical parameters of cells and their statistical distributions. This method gives satisfactory results, however the outlining requires much time and high attention.

The aim of this work is developing and testing a new efficient method of image obtaining and image analysis in order to determine geometrical parameters of any number of parenchyma tissue cells.

MATERIAL AND METHODS

Microscope. An efficient observation of any number of cells requires a microscope and camera system with relatively big field of observation and magnification around 10 times which is optimal for parenchyma tissue of fruits and vegetables. At this magnification a few dozen of parenchyma cells can be taken in one image. In this research Laser Scanning Confocal Microscope (Olympus Fluoview B50) was used. Confocality of the microscope ensures images with higher contrast comparing to standard light microscopes. An image is taken only from thin layer that thickness depends on aperture value of confocal diaphragms and can be adjusted. The microscope used in this research has fluorescence mode as well. An applying special fluorescence dye to cell walls increases amount of light coming from walls and contrast with objects inside the cells. The source of light used for excitation was Argon-Ion laser (450-515 nm). The observation was carried out with using UplanFI 10X/0.30Ph1 objective. Images were recorded by digital camera and next transferred to computer with resolution 512×512 pixels. At magnification 10 times it corresponds to field of observation of 1.4 mm × 1.4 mm. The magnification can be increased both by changing an objective and by changing field size of observation while resolution remains the same.

Samples preparation and taking images in unbiased way. In order to develop and test the method potato tuber and carrot were chosen as examples of soft plant materials. Potato samples were taken from centre of tuber i.e. inner core and carrot samples were taken from outer part of root about 5 mm from the skin. These tissues have significant differences in cell size and textural parameters that are important from point of view of sample preparation, microscopic observation and image analysis. Thus they are suitable to test the method for reconstruction quality. From the other hand, these tissues contain only about 1% intercellular spaces of tissue volume, thus they will not influence on results significantly.

Common procedures used for fluorescence microscopes include cutting, fixing, staining and mounting. Each step of preparation can change geometrical parameters of the cells, especially fixing including dehydration can cause

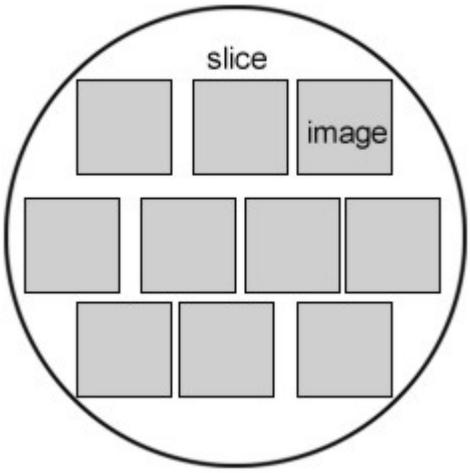
shrinking or swelling of cells. On the other hand, fixing can be avoided in this research, because confocal microscope allows observation of microstructure in natural state. In result, only cutting, staining and mounting have remained in the procedure. The best settings of each step have been found experimentally where the criterion was cells distinction as much clear as possible. Following settings of sample preparation have been found for parenchyma tissue:

1. Cutting cylindrical sample with dimensions 7 mm × 10 mm (diameter × height) and gluing to microslicer table.
2. Slicing by razor blade (using microslicer D.S.K., DTK-1000) into cylindrical slices with diameter equals 7mm and thickness 300 micrometers for carrot and 500 micrometers for potato. This thickness is optimal for samples in natural state maintaining high slice stiffness in order to preserve its folding and cell mechanical injuring during handling.
3. Staining in aqueous *Coriphosphine O* solution (excitation wavelength – 460 nm, emission wavelength – 575 nm) for 10 s and next washing in tap water for about 10 s. This dye is used for pectines staining that in the case of parenchyma tissue bond cell to cell. The time of dying and washing is the shortest as possible according to avoid slices swelling because of water transportation between tissue and external aqueous media.
4. Drying the slice and mounting on microscopy slide. The slice is placed on microscopic slide and carefully drained off by tissue paper that causes its sticking to the slide as well. Cover slides are not used thus maximal observation time cannot be longer than 5 minutes. After this time changes in geometry and size of the cells are visible under the microscope.

It should be underlined that above procedure takes no longer than 1 minute for one slice.

In order to test a quality of reconstruction, 10 slices of potato and 10 slices of carrot were analysed.

Fig. 1. Method of unbiased taking images from tissue slice. Rows are parallel, but distances between rows and between images in rows are random

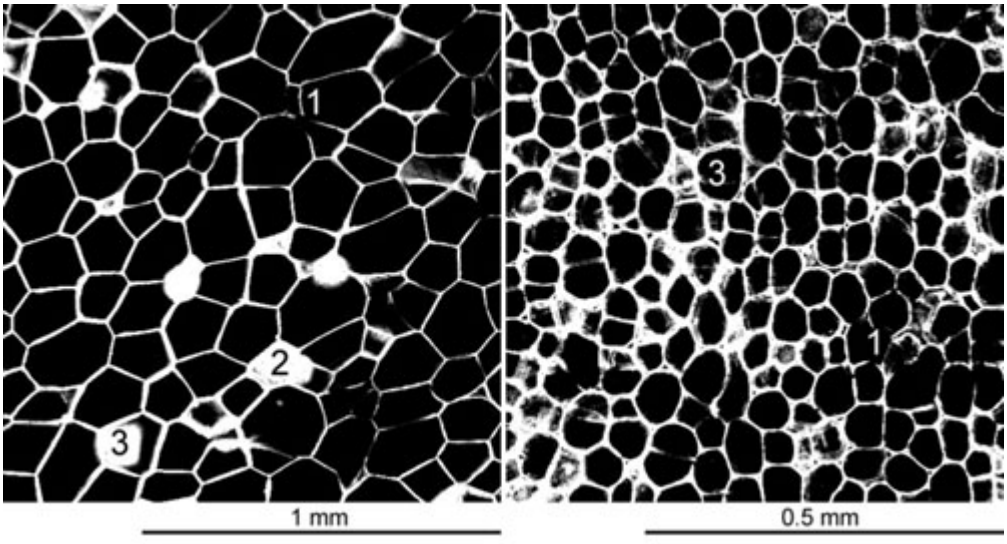


The Confocal Scanning Laser Microscope allows obtaining 2D grey (0-255) images from different layers, however images taken from the slice surface are the most suitable for automatic analysis. According to Stereology, images should be taken (sampled) in unbiassed way if we would like to describe 3D structure by 2D images [7]. Therefore in the present experiment, images are taken in 3 parallel rows, but distances between rows and between images in rows are random. 10 images are taken from each slice that takes about 4-5 minutes (fig. 1).

RESULTS AND DISCUSSION

Procedure of image analysis. Examples of obtained microscopy images of potato tuber and carrot root are shown in figure 2. In order to show problems during computer analysis presented images have average quality and they are relatively difficult for computer analysis.

Fig. 2. Images of potato tissue (left image) and carrot root (right image) obtained by Confocal Scanning Laser Microscope:
1 – "broken" walls, 2 – cells bottoms, 3 – nonparallel walls to observation axis



Dye used in the experiment stains pectines between walls, hence walls should be visible in images only. These walls create polygonal (for potato) or round objects (for carrot) that are cells or intercellular spaces. It is not possible to distinguish these two different structures in this method. However, potato and carrot contain small amount of intercellular spaces, thus we can assume that cells are visible in images, mainly. Most of the cell walls are clearly seen (grey level close to 255) and contrast with rest area (grey level close to 0) of the image is high. Unfortunately, there are walls that their grey level is close to background or they are completely invisible ("broken" walls shown in place No. 1 in fig. 2). It can be result of bad fluorescence or uneven slice surface in these places. Other difficult for computer analysis places are bottoms of cells (place No. 2 in fig. 2) or nonparallel walls to observation axis (place No. 3 in fig. 2). If cell bottom or nonparallel wall is located in focal layer these places will look in images as oval white areas or unusual thick walls. This problem appears especially for carrot tissue where cells are very small, thus even for thinnest focal layer there is high probability that nonparallel walls and cell bottoms will be visible in images.

Therefore, tasks of computer procedure are: 1 – to link "broken" walls, 2 – to remove oval white areas that are not cells but to remain adjacent walls, 3 – to separate cells and to measure their geometrical parameters. According to this a special computer procedure was developed containing series of morphological operators [13]. A combination of four operators was used in this research, in sequence: *DilateReconsClose*, *ThinSkeleton*, *Watershed.* Each operator is already defined and available in commercial software for image analysis. In this research Aphelion® image analysis software was used. *DilateReconsClose* (fig. 3) works similar to *Close* operator, but has the advantage of preserving shapes contours since the *Reconstruction* step recovers part of what was lost after *Dilation* [13]. In this operator, size and shape of structural element can be adjusted according to analysed images. The structural element is applied to each pixel and changes it according to conditions defined in *DilateReconsClose* operators. Thus, brakes between walls can be linked in this way if the structural element is big enough. Undesirable result of this operator is filling small cells too. We have found experimentally for tested images that square structural element with side equals 4 pixels is optimal for analysed images. *ThinSkeleton* (fig. 4) operator finds image skeleton of thickness 1 pixel [13]. The purpose of this operator is finding borders between cells and changing oval white areas into elements with branch appearance. Next, *Watershed* (fig. 5) operator detects only closed regions in images thus branches (previously oval white areas) disappear in this step. After this, each remaining object shown in figure 5 in different colour is labelled and is treated separately. Objects divided by image border are removed from further analysis. The procedure works in a loop thus it can be applied for any number of images automatically.

Fig. 3. Result of *DilateReconsClose* operator applying to image of potato (left image) and carrot (right image). The structural element is square with side equal 4 pixels

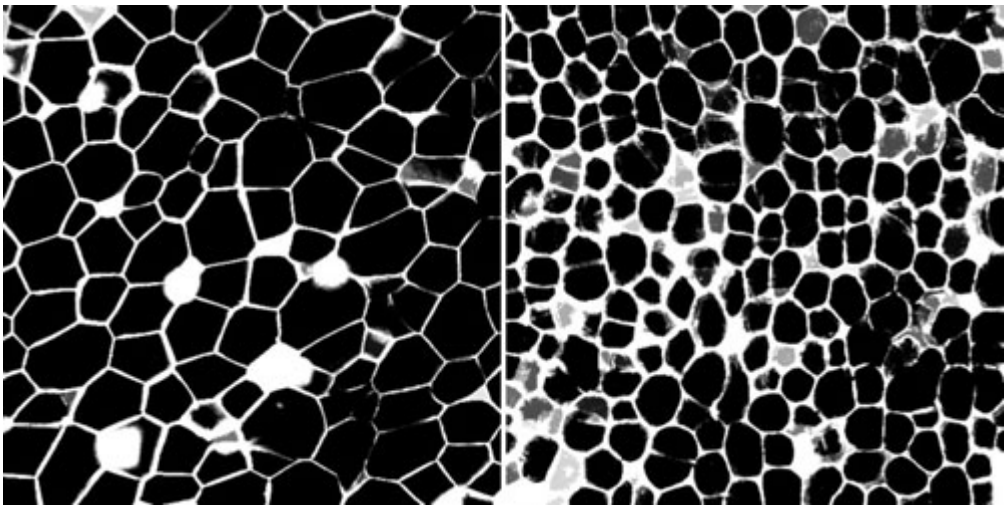


Fig. 4. Result of *ThinSkeleton* operator applying to image of potato (left image) and carrot (right image)

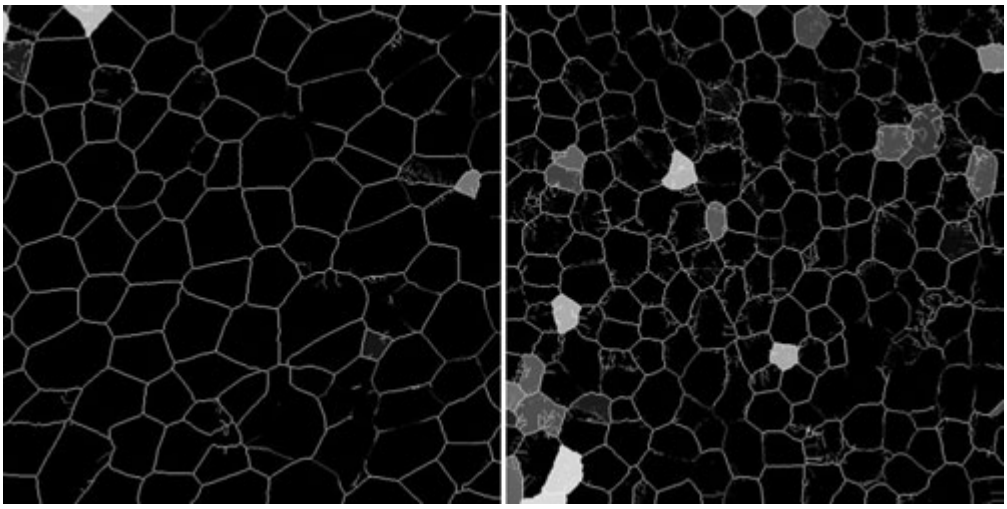
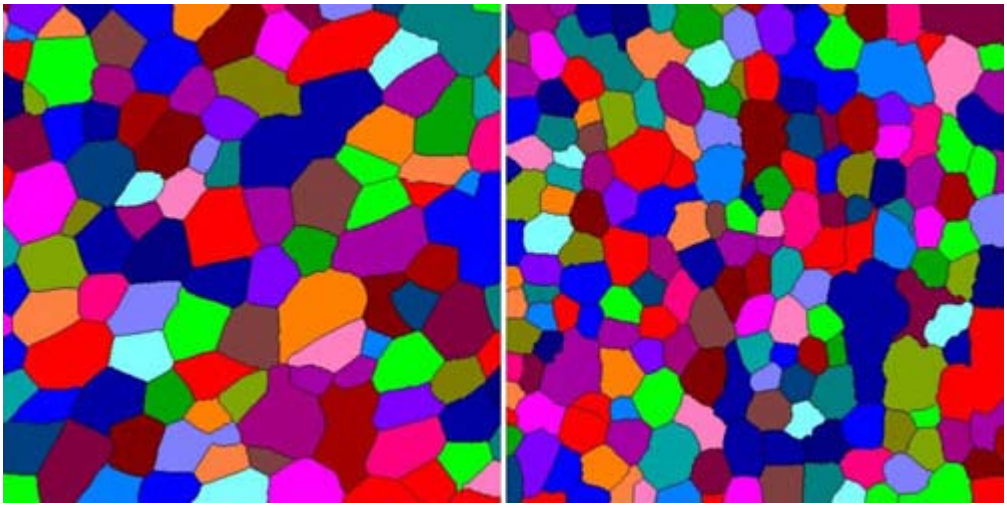


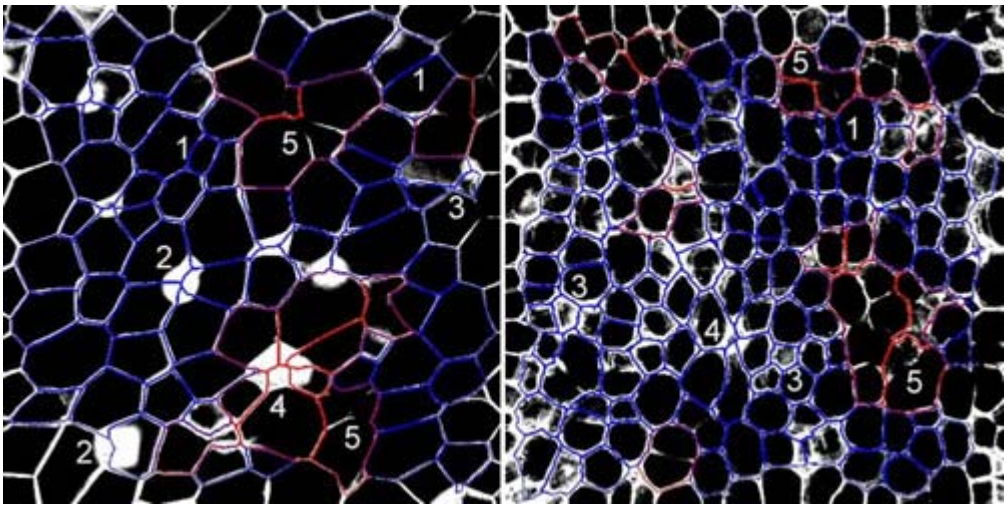
Fig. 5. Result of *Watershed* operator applying to image of potato (left image) and carrot (right image)



In figure 6 the final cell reconstruction sketch is shown. Most of cells are reconstructed correctly for both tested materials. Lines of the reconstruction lie on original walls and some of brakes are linked (example: No. 1 in figure 6, please compare with figure 2). Oval white areas like No. 2 in figure 6 are changed into lines lying around their middle. Other positive aspect of the procedure is correct cells separation filled by pixels of low grey level, seen in images as grey points (places No. 3 in figure 6).

However, despite image processing and improving, there are still cells not correctly reconstructed. Big objects containing two or more cells being a result of long brakes in walls are the most important for result of analysis (No. 5 in figure 6). Other problematic places are objects completely white with a polygonal shape (No. 4 in figure 6). These objects are probably cells too but their bottom is placed in focal layer of the microscope. These objects are incorrect recognised as thick walls. Therefore, neighbour cells become bigger than in reality. Because the aim of this work is analysis as much automatic and efficient as possible, these problems cannot be completely avoided. Each image is different and taking into consideration all cases by changing operators or changing their sequence is very difficult. Therefore, we have decided to introduce into computer procedure possibility of manual checking and correcting of reconstruction quality. After cell labelling, reconstruction sketch is displayed over original image (like in figure 6). Now observer can decide which object is reconstructed incorrectly and delete it from analysis by clicking on object and choosing proper option. Examples of objects deleted in the case of presented images are marked in red in Fig. 6. It should be underlined here that not each image requires correction, but if any correction is necessary time of this operation is about 30 second per image for experienced observer.

Fig. 6. Cell reconstruction for potato (left image) and for carrot (right image) shown as blue lines. Objects in images not correctly reconstructed and deleted from further analysis are shown in red. Places in images correctly reconstructed:
1 – linked walls, 2 – oval white areas changed into lines, 3 – cells filled by pixels of low grey level. Places in images incorrectly reconstructed: 4 – polygonal shapes, 5 – objects containing more than one cell



Geometrical parameters distribution and estimation of reconstruction error. For remaining objects the procedure measures geometrical parameters. Similar to work of Konstankiewicz et. al. [4]: *cell area*, *elongation*, *Feret's Max.diameter* and *Feret's Min. diameters* were chosen as basic geometrical parameters of cells. *Elongation E* is given by equation $E = (a - b) / (a + b)$, where a and b are longer and shorter axis of ellipse of best fit inside the cell, respectively. *Feret's Max.* and *Min. diameters* are defined as longer and shorter sides of rectangle of best fit outside the cell, respectively. In order to estimate error of an incorrect cell reconstruction, measurements were done before correction and after correction. For potato number of reconstructed cells from 100 images (size 1.4 × 1.4 mm) is 5763 before correction and 5617 after correction. For carrot number of obtained cells from 100 images (size 0.7 × 0.7 mm) is 5144 before correction and 4520 after correction.

Results of measurements are shown in figure 7 for potato and figure 8 for carrot in a form of histograms. They have normal distribution: symmetric for *Feret's diameters* and little skewness to the left for *area* and *elongation*. Mean values of cell geometrical parameters before and after correction with standard deviations are shown in table 1. Comparing mean values from table 1 it is clearly visible that potato is built of bigger cells than carrot. Linear dimensions (*Feret's diameters*) of potato cells are more than twice longer. However shape parameter *elongation* of potato cells is similar to carrot cells.

Fig. 7. Histograms of cell area, elongation, Feret's Max. diameter and Feret's Min. diameter before and after correction obtained for potato

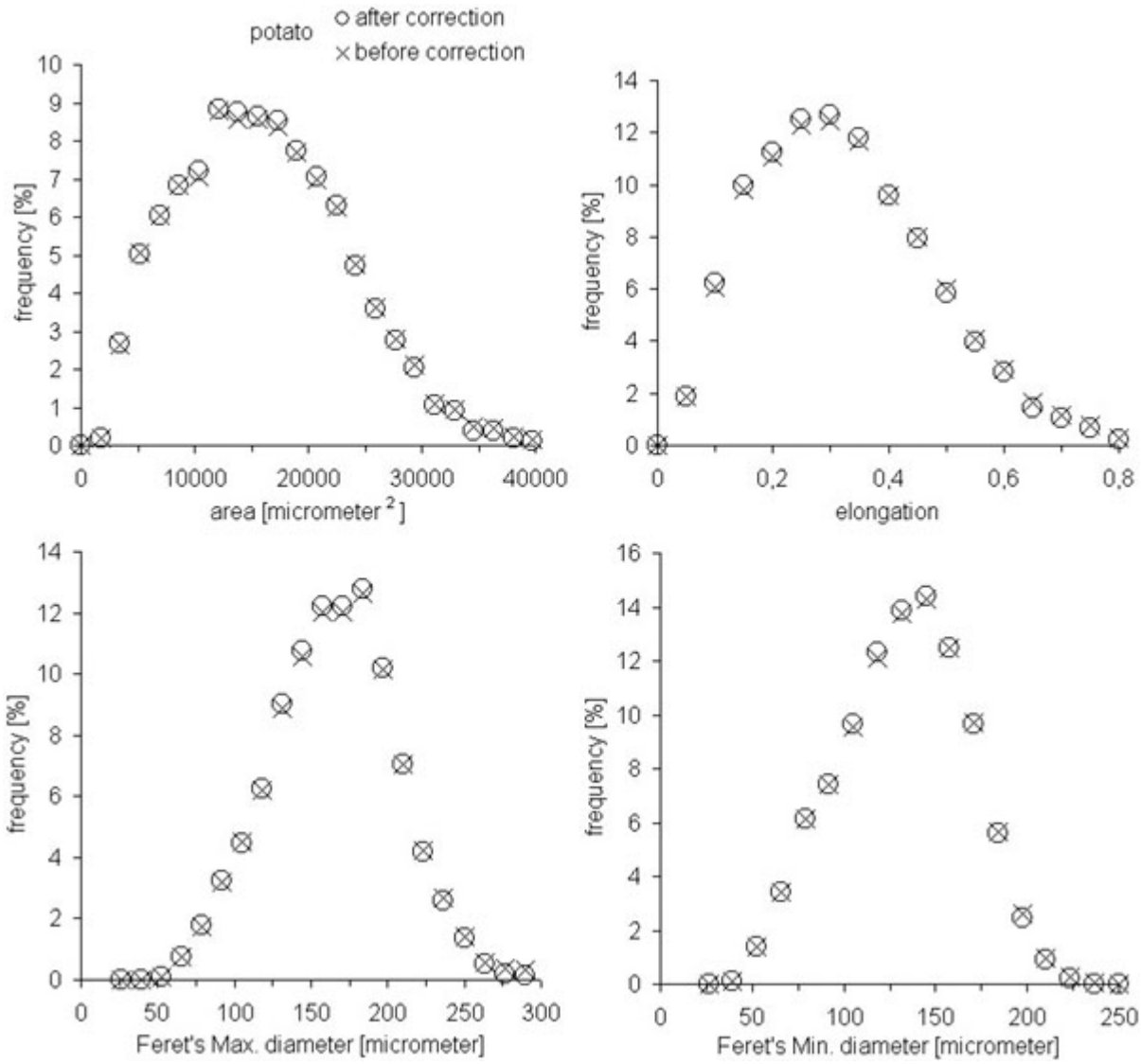


Fig. 8. Histograms of cell area, elongation, Feret's Max. diameter and Feret's Min. diameter before and after correction obtained for carrot

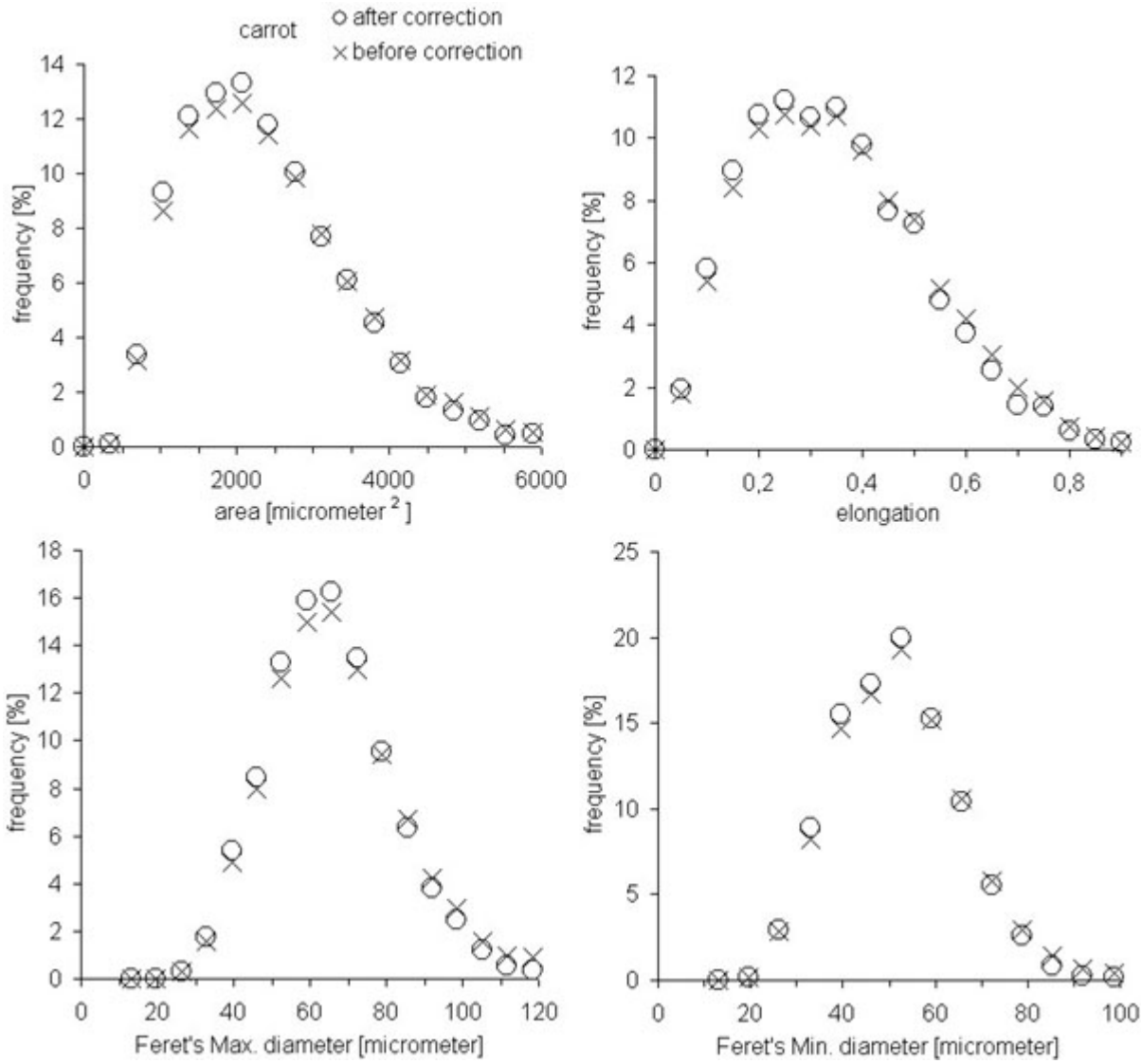
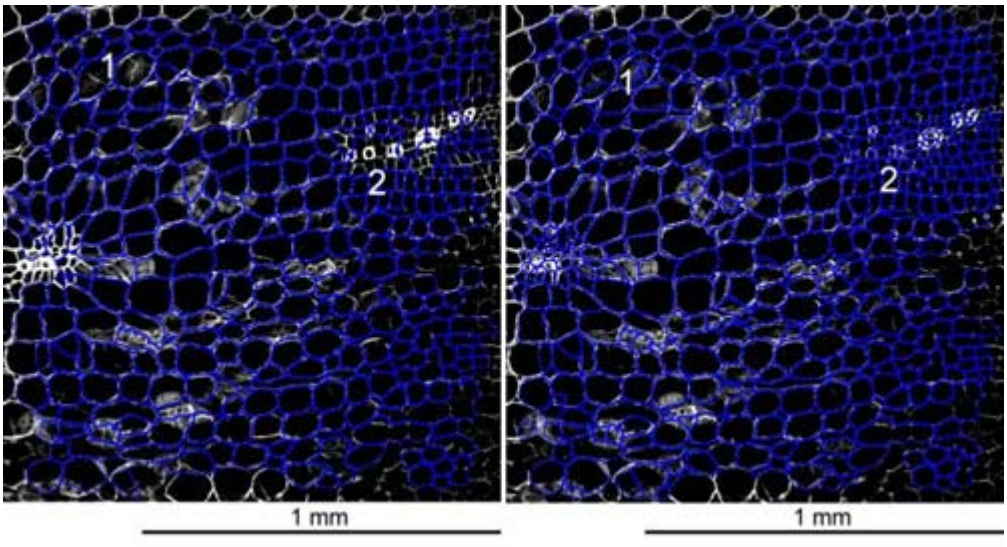


Table 1. Mean cell geometrical parameters, standard deviations and λ values of Kolmogorow-Smirnow test. Not significant differences of results distributions are marked as "*" when $\lambda < \lambda_\alpha$, $\lambda_\alpha = 1.358$ ($\alpha = 0.05$)

Cell parameter	Mean		Std. Dev.		λ value of K-S test
	before correction	after correction	before correction	after correction	
Potato					
Area, μm^2	15383	15188	7655	7253	0.419*
Elongation	0.302	0.297	0.155	0.152	0.470*
Feret's Max.diameter, μm	160	159	43.2	40.5	0.280*
Feret's Min.diameter, μm	126	126	35.5	34.7	0.562*
Carrot					
Area, μm^2	2462	2230	2099	1151	1.266*
Elongation	0.332	0.320	0.174	0.168	1.384
Feret's Max.diameter, μm	66.0	62.8	24.1	17.5	1.729
Feret's Min.diameter, μm^2	50.1	48.4	16.5	13.2	1.284*

Figures 7 and 8 show that histograms after correction are similar to histograms before correction and it is difficult to estimate any changes. Especially potato reconstruction looks correct; only few points of histograms are not covered completely (fig. 7). For carrot, the influence of correction is more significant. Points of left side of histograms obtained after correction lie higher and some points of right side lie lower than before correction (fig. 9).

Fig. 9. Reconstruction of carrot tissue taken from centre of root with *DilateReconsClos* square structural element with side equals 4 pixels (left image, 1 – cell reconstructed correctly, 2 – cells reconstructed incorrectly) and side equals 2 pixels (right image, 1 – cell reconstructed incorrectly, 2 – cells reconstructed correctly)



In order to test hypothesis that cells geometrical parameters before and after correction have the same distributions Kolmogorow-Smirnow λ test at critical value $\lambda_\alpha = 1.358$ (where $\alpha = 0.05$) was used. In table 1, λ values are shown and not significant differences of distributions are marked as "*" when $\lambda < \lambda_\alpha$. Analysis has shown that differences of histograms are not significant for all potato cells parameters, however for carrot *elongation* and *Feret's Max. diameter* have been changed significantly after correction.

Table 1 shows that mean values of all geometrical parameters decrease after correction for both potato and carrot, even if Kolmogorow-Smirnow test does not show significant differences of histograms. It means that in correction process mainly big objects were deleted being result of "broken walls". However, changes for carrot are bigger. For example, mean cell area has decreased about 10% after correction comparing to 1% for potato. This is result of overall quality of carrot images that are worse than potato images. If we compare the number of cells after and before correction, we will see that number of deleted objects for carrot is much higher: 624 (12%) deleted objects for carrot and only 146 (2.5%) for potato. Above result shows that correction would be not necessary when source images have relatively good quality like in the case of potato tuber inner core where we change results after correction only about 1%. On the other hand, results after correction are shifted always into smaller value, therefore it can be considered as a systematic error of the method that is about 1% for potato tuber inner core and lest than 10% for carrot tissue of external part of root. However, this error is different for different tissues thus should be estimated for every kind of tissue individually if we decide to not correct images.

Reconstruction of highly heterogeneous parts of tissue. Tests with different types of tissues have shown that properties of investigated materials play important role for reconstruction. When tissue is relatively homogenous slicing in natural state by razor blade gives good results. Unfortunately, when tissue consists of objects like vascular bundles with different mechanical properties for example, cutting does not give smooth surface. In this case, it is difficult to obtain proper image to analysis. Additionally, having big difference between sizes of objects in the same image or among group of images it is difficult to choose universal structural element of *DilateReconsClose* operator. Thus, images or even their fragments should be analysed separately. Example is shown in figure 9 for carrot tissue taken from centre of root where structure is very heterogeneous. When structural elements of *DilateReconsClos* operator equals 4 – big cells are reconstructed correctly (No. 1 in figure 9), but small object like vascular bundles in left side of image (No. 2 in figure 9) are badly reconstructed. On the other hand applying the same operator with structural element equals 2 improves reconstruction of small elements (No. 2 in figure 9) but some bigger cells are divided into smaller objects (No. 1 in figure 9).

CONCLUSIONS

5. A new method was developed in order to describing geometrical parameters of any number of parenchyma cells. The reconstruction and parametrisation method is a unique combination of sample preparation, image obtaining and image analysis. Advantages of the developed method are:
6. Correct cell reconstruction because of proper combination of image quality and automatic computer image analysis.
7. Error of automatic cell reconstruction is about 1% for potato tissue taken from inner core of tuber and 10% for carrot tissue taken 5 mm under the skin of root. Manual correction of reconstruction allows avoiding this error.
8. Method is universal for materials with different structure like potato and carrot. Only one parameter is adjusted to improve reconstruction of more heterogeneous parts of tissues.
9. Fast image obtaining. It takes only about 1 minute for slice preparation and about 4-5 min per one slice to take 10 images.
10. Automatic image analysis of high number of images. Analysis of 100 images and obtaining parameters of about 5000 cells takes no more than 40 minutes with correction and only about 5 minutes without correction for computer P4 class.

ACKNOWLEDGEMENTS

Paper is supported by Japan Society for the Promotion of Science.

REFERENCES

1. Bajema R. W., Hyde G. M., Baritelle A. L., 1998. Temperature and strain rate effects on the dynamic failure properties of potato tissue. Trans. ASAE 41(3), 733-740.
2. Gao Q., Pitt R. E., 1991. Mechanics of parenchyma tissue based on cell orientation and microstructure. Trans. ASAE 34(1), 232-238.
3. Haman J., Konstankiewicz K., 2000. Destruction Processes in the Cellular Medium of a Plant – Theoretical Approach. Int. Agrophysics 14, 37-42.
4. Konstankiewicz K., Pawlak K., ZdunekA., 2001a. Influence of structural parameters of potato tuber cells on their mechanical properties. Int. Agrophysics 15, 243-246.
5. Konstankiewicz K., Pawlak K., ZdunekA., 2001b. Quantitative method for determining cell structural parameters of plant tissues. Int. Agrophysics 15, 161-164.
6. Konstankiewicz K., Zdunek A., 2001. Influence of turgor and cell size on the cracking of potato tissue, Int. Agrophysics 15, 27-30.
7. Mouton P. R., 2002. Principles and Practices of Unbiased Stereology: An Introduction for Bioscientists. The Johns Hopkins University Press, Baltimore, Maryland.
8. Murase H., Merva G. E., Segerling L. J., 1980. Variation of Young's modulus of potato as a function of water potential. Trans. ASAE 23(3), 794-800.
9. Pitt R. E., 1982. Models for the rheology and statistical strength of uniformly stressed vegetative tissue. Trans. ASAE 25(6), 1776-1784.
10. Pitt R. E., Chen H. L., 1983. Time-dependent aspects of the strength and rheology of vegetative tissue. Trans. ASAE 26(6), 1275-1280.
11. Pitt R. E., Davis D. C., 1984. Finite element analysis of fluid – filled cell response to external loading. Trans. ASAE, 1976-1983.
12. Umeda M., Namikava K., Nagasaka Y., Motomura T., Nakahara T., 1997. Mechanical Properties of Tetrakaidecahedron Model Cell (Part 1). Analysis of Reaction Force of a Single Model Cell. Journal of the Japanese Society of Agricultural Machinery 59, 5, 55-62.
13. Wojnar L., Majorek M., 1994. Komputerowa analiza obrazu. Fotobit-Design.
14. Zdunek A., Konstankiewicz K., Gancarz M., 2001. Failure and acoustic emission of potato tuber tissue of selected varieties in compression tests at various strain rates. Electronic Journal of Polish Agricultural Universities. Agricultural Engineering, volume 4, issue 2. http://www.ejpau.media.pl/series/volume4/issue2/engineering/art-07.html
15. Zgórska K., 1989. Biologiczne i ekologiczne czynniki warunkujące podatność bulw ziemniaka na powstawanie ciemnej plamistości pouszkodzeniowej [Biological and ecological factors determining susceptibility of potato tubers to blackspot bruising]. Pracownia Małej Poligrafii Instytutu Ziemniaka, Bonin [in Polish].

Artur Zdunek, Krystyna Konstankiewicz
Institute of Agrophysics, Polish Academy of Sciences
4 Doświadczalna Street, 20-290 Lublin, Poland
tel. (+48 81) 744 50 61, fax (+48 81) 744 50 67
e-mail: azdunek@demeter.ipan.lublin.pl

Mikio Umeda
Laboratory of Field Robotics, Division of Environmental Science and Technology
Graduate School of Agriculture, Sakyo-ku, Kyoto 606-8502, Japan
tel., fax: (+81 75) 753 61 68
e-mail: umeda@elam.kais.kyoto-u.ac.co.jp

Responses to this article, comments are invited and should be submitted within three months of the publication of the article. If accepted for publication, they will be published in the chapter headed 'Discussions' in each series and hyperlinked to the article.