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Application of novel image base estimation of invisible leaf injuries in relation to morphological and photosynthetic changes of *Phaseolus vulgaris* L. exposed to tropospheric ozone

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Abstract

This study aimed to evaluate the degree of *Phaseolus vulgaris* L. (bean) leaf tissue injury caused by tropospheric ozone. To validate O_3 symptoms at the microscopic level, Evans blue staining together with an image processing method for the removal of distortions and calculation of dead leaf areas was applied. Net photosynthetic rate (P_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined to evaluate leaf physiological responses to ozone. It was found that both resistant and sensitive varieties of bean were damaged by ozone; however, the size of necrotic and partially destroyed leaf area in the sensitive genotype (S156) was bigger (1.18%, 2.18%) than in the resistant genotype (R123), i.e. 0.02% and 0.50%. Values of net photosynthetic rates were lower in the sensitive genotype in ambient air conditions, than in the resistant genotype in ambient air conditions (Table 1). We further found that there was a correlation between physiological and anatomical injuries; net photosynthetic rate (P_N) was negatively correlated with percentage of necrotic area of both genotypes, while stomatal conductance (g_s), intercellular CO_2 concentration (C_i) were positively correlated with percentage of necrotic tissue of both genotypes. Moreover, visible injures in both genotypes were positively correlated with percentage of anatomical injures. In

conclusion, the presented combinations of morphological, anatomical and physiological markers allowed differential diagnosis of ozone injury.

Key words: tropospheric ozone, leaf injuries, image analysis, photosynthetic activity

Introduction

Tropospheric ozone is a widespread concern in the northern and southern hemisphere causing injury in numerous species which belong to different families of angio- and gymnosperms (Ashmore, 2004, Carvalheiro et al., 2013). The mechanism of oxidative stress in plants caused by ozone is based on its reactions with cell wall and membrane components and, as a result, production of reactive oxygen species (ROS) is observed (Pennell et al., 1997, Rao et al., 2000). If there is a huge amount of ROS in the apoplast, the latter one is supersensitive and responses causing fast, local cell deaths which appear as necrotic spots on leaf surfaces; in trees and herbs species, i.e. *Quercus ilex*, *Nicotiana tabacum* (Facuda, 2000, Gratani et al., 2000, Bandurska et al., 2009, Borowiak et al., 2010). Visible leaf injuries are widely used for determination the level of tropospheric ozone in many countries. Biomonitoring is a supplement of technical monitoring for further evaluation the negative effect of ozone to natural vegetation, crops as well as on tress (Klumpp et al. 2006). Moreover, bioindicators can be exposed in many locations which are not available for automatic equipment. Early necrotic changes, i.e. in *Populus alba*, *Nicotiana tabacum*, can be discovered using microscopic methods (Günthardt-Goerg et al. 2000, Vollenweider et al., 2003, Fares et al., 2006, Borowiak et al., 2010). Employing various dye factors, such as trypan blue, Evans blue (in *Hordeum vulgare*) (Huang et al., 1986), it is possible to observe certain stages of necrotic leisure creation caused by loss of cell membrane integrity at the leaf cell level using a light microscope. This dye is removed from living cells but penetrates cells with damaged membranes, dyeing them into an intense blue colour (Faoro and Iriti, 2005). Using a light microscope, we can observe varying intensity of colouring proportional to the degree of leaf cell membrane decomposition (Koegh et al., 1980). The existence of leaf damages invisible to the naked eye can be used as a useful tool to detect impacts in plants which are more resistant to ozone.

The main reason for the negative effect of ozone on plants concerns changes in proper functioning of the photosynthetic system. Most investigations are conducted in controlled or semi-controlled ozone conditions. However, these investigations revealed various and

interesting results. One of them was that stomatal closure as the first result of an ozone effect on plants resulting in a decrease of the net photosynthetic rate in *Populus tremulus*, *Phaseolus vulgaris*, *Liriodendron chinense* (Matyssek et al. 1993, Leipner et al., 2001, Zhang et al., 2010). Oksanen (2003a) suggested that ozone tolerance variability was related with the response of stomata. Hence, stomatal opening is treated as a very important regulator of ozone uptake by plants (Paoletti and Grulke, 2005, Heath, 2008, Mills et al., 2011). Novak et al. (2005) expanded this opinion to stomata acting as factors responsible for plant sensitivity to tropospheric ozone. Furthermore, Guidi et al. (2001) suggested that stomatal closure could be treated as part of the mechanism preventing further ozone injury in internal tissues. However, some investigators failed to find any correlation between stomatal closure and net photosynthetic decrease (Flagler et al., 1994, Paoletti et al., 2007). Lombardozzi et al. (2012) suggested that differences in the photosynthetic response might be due to non-stomatal factors, potentially driven by either photosystem oxidation, limiting energy of RuBP regeneration, or decreased efficiency of Rubisco due to direct enzyme oxidation or reduced CO_2 transport to the enzymes. Vahisalu et al. (2010) found that the ozone effect on plants was connected with rapid, but short-term g_s decrease. It seems that there are still many uncertainties concerning ozone effects on plant responses; even in controlled conditions, investigators found variable plant responses to ozone. Hence, it would be extremely important to evaluate plant responses in ambient air conditions for a variety of plant parameters. Previous studies on photosynthetic plant responses in ambient air revealed that they were related with levels of ozone concentrations during the duration of the experiment. If ozone concentrations were lower, plants responded quite differently and reached even higher P_N levels in locations with higher ozone concentrations. On the other hand, the experiment conducted in another growing season revealed a decrease of $P_{N,}$ especially in places with higher ozone concentrations. Moreover, the response was different for various plant species Borowiak (2013a, 2014). Hayes et al. (2007) also reported a range of responses of plant species to ozone.

Plant cell responses can affect other plant features, such as morphological parameters directly influencing their economic value. It was previously noted that ozone affects plant morphological parameters of many plant species, such as reduced leaf size in tree species (Dizengremel, 2001, Oksanen, 2003b, Riikonen et al., 2004, Riikonen et al., 2010), reduced leaf area and plant height of cotton plants (Zouzoulas et al., 2009), decreased leaf area ratio as well as

specific leaf area in soybean (Morgan et al., 2003), and reduced plant height in cucumber (Agrawal et al., 1993) and chickpea (Welfare et al., 2002). Our previous investigations on the cumulative ozone effect on plant morphology revealed that the more ozone-resistant tobacco cultivar showed higher mean plant growth and leaf growth than the ozone-sensitive one throughout the experimental period. However, at the exposure sites the ozone-sensitive cultivar showed plant growth similar to or higher than both cultivars of the controls, especially at the forest site where ozone concentrations were higher. This suggests a plant defense against reduction of leaf assimilation area (i.e. against leaf necrosis) (Borowiak, 2013b). It was also suggested that ozone can even accelerate plant growth due to faster creation of generative plant parts and faster seed production (Borowiak and Wujeska, 2012, Borowiak, 2013b).

We aimed to improve the knowledge about bean (*Phaseolus vulgaris* L.) response to ozone, describing and comparing the microscopic damages in leaves with and without visible symptoms exposed to controlled O_3. The presented investigations are a combination of the physiological, morphological and anatomical markers, which allows to a full diagnosis of invisible lesions detectable by microscope observations, especially in the early stages of the symptomatic progression and relation them to visible parameters. For this purpose an accurate estimation of the degree of death leaf tissue (mesophilic dead cells) image processing method was applied and subsequently for distortion removal and calculation of dead leaf areas. The obtained results were referred to the identified morphological and physiological changes (net photosynthesis rate, stomatal conductance, intercellular CO_2 concentration) of two genotypes of bean (ozone-sensitive S156 and ozone–resistant R123) under exposure to tropospheric ozone.

Material and methods

Experimental design

Well-known ozone-sensitive (S156) and ozone-resistant (R123) genotypes of *Phaseolus vulgaris* L. (bean) were used in this study. Genotypes were selected at the USDA-ARS Plant Science Unit field site near Raleigh, North Carolina, USA. The bean lines were developed from a genetic cross reported by Dick Reinert (described in Reinert and Eason (2000)). Individual sensitive (S) and tolerant (R) lines were identified, the S156 and R123 lines were selected, and then tested in a bioindicator experiment reported in Burkey et al. (2005). Plants were cultivated in the greenhouse for four weeks and then transferred to two exposure sites:

greenhouse (control conditions RK - resistant genotype in control conditions and SK – sensitive genotype in control conditions) and a suburban site - located 10 kilometers north of Poznań (N 52.486092, E 16.889669) (RE – resistant genotype in ambient air conditions and SE - sensitive genotype in ambient air conditions). Sites were chosen according to an Experimental Protocol, and were 200 meters away from main roads and 50 meters away from buildings (ICP Vegetation, 2012). Each site contained three samples from each cultivar in 1.5 L pots, filled with standard soil mixture, fertilized once with a slow release NAWOMIX fertilizer. Plants were continuously watered by fibre wicks placed in pots and trays with water.

Every 7^{th} day (from 18.08.2014 to 24.09.2014) the degree of leaf injury, morphological parameters and gas exchange parameters were measured. In addition, material for leaf tissue anatomical analyses was also collected from each plant.

Ozone concentrations are given in AOT 40 units, a measurement standard adopted by the European Union. AOT 40 is the accumulated ozone concentration over a threshold of 40 ppb, the critical threshold for plants and ecosystems under Polish regulations, measured here between 8 a.m. and 8 p.m. It is useful for presenting cumulative ozone effects on plants during the growing season. The AOT 40 here presented was calculated based on Provincial Environmental Agency Monitoring station located nearby exposure site. The critical dose was evaluated for every week of experiment. For better understanding the plant response some meteorological data are here also presented and analysed, such as air temperature and humidity.

Physiological study, visible injuries and morphological parameters

The handheld photosynthetic system CI 340aa (CID Bioscience Inc., Camas, USA) was used to measure net photosynthetic rate (P_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i). To achieve comparable results of measurements, constant conditions in the leaf chamber were maintained: CO_2 inflow concentration (390 µmol (CO_2) mol^{-1}), photosynthetic photon flux density (PPFD) 1000 µmol (photon) $m^{-2}s^{-1}$, chamber temperature 23°C and relative humidity 50±3%. Investigations were conducted during midday light conditions, between 10 a.m. and 3 p.m.

Weekly measurements of leaf length/width growth of each trifoliate were made, according to the Ozone Experimental Protocol (ICP Vegetation, 2012), and the number of flowers, pods,

leaves with 1-5%, 5-25%, >25% damaged surface were measured. Subsequently, the correlation between the results of these observations and necrotic area was calculated.

Microscopic analysis

Samples of leaf tissue (without visible injury and with small, medium and large injures) were taken from both *Phaseolus vulgaris* genotypes. Twenty four leaf discs (20 mm in diameter) were randomly cut with a scalpel. In order to assess cell necrosis, a modified histo-cytochemical method of Faoro and Iriti (2005) was used. Leaf discs were boiled for 1 minute in methanol to remove chlorophyll and rinsed with distilled water. The prepared samples were then boiled for 1 minute in a mixture of: glycerol, phenol, lactic acid and distilled water containing 0.02 g/100 ml Evans Blue, prepared directly before use. After the staining process, samples were refrigerated overnight. Using Evan's blue, it was possible to observe in a light microscope individual stages of necrotic changes in leaf tissues. This type of staining is frequently applied to identify cells which lost integrity of cell membranes (Baker and Mock, 1994; Faoro and Iriti, 2009). The dye is removed from live cells but it enters and remains in cells with damaged membranes, staining them into intense blue color. Staining intensity, which is proportional to the degree of leaf cell membrane damage, is visible under a light microscope.

Image analysis

The input data were the leaf surface color images recorded with the digital camera (Canon A640) mounted on the binocular microscope eyepiece. Pictures were taken of all microscopic preparations and were magnified 40 and 200 times. The analyzed surfaces covered the area of 1 cm^2 for images enlarged 40 times, and the area of 0.5 cm^2 for images enlarged 200 times. The aim of image processing was to extract regions (segmentation), in which the tissue was completely destroyed, partially destroyed and or still alive, and to calculate their area.

The aim of image processing was also to remove noise in the form of incompletely discolored vascular bundles. An example image is shown in Fig. 1, in which the deep blue and blue portions are areas of dead tissue (necrosis) and the green parts are incompletely discolored vascular bundles (image noise). In order to remove noise, a threshold was used in the RGB color space; however, satisfactory results were not obtained, because together with

the noise, part of the relevant information was removed. A better solution was to transform images from RGB to CIE Lab color space (Hoffmann, 2003) and apply a threshold in this space. CIE Lab space separates the luminance (L component) from the chrominance (a and b) (Fig. 2). Mathematical transformation formulas from RGB to CIE Lab can be found, e.g. in the OpenCV Manual (The OpenCV Reference Manual 2014) and a detailed description in the work of Hoffmann, (2003). Using a threshold in this space allows for effective removal of noise from incompletely stained leaf (Fig. 3). Next, proportions of the area consisting of partially destroyed and necrotic tissue were calculated for each surface.

After the segmentation process, when only damaged areas of tissue were visible on the image, boundaries between areas of cells completely and partially dead were experimentally determined (Fig. 6). The surface area of each one was then determined. Image processing was carried out using software written in C ++ developed by the authors using OpenCV library. The program is available on line at: https://www.dropbox.com/s/4n4z6jwgr2q1zwv/CDA.zip?dl=0. The method is also less sensitive to local lighting conditions during image acquisition.

Statistical analysis

Descriptive statistics were calculated (arithmetic average, standard deviation, minimum and maximum). In order to determine statistical significance of average values of traits of the samples in question, the factor variance ANOVA F-statistics was used. To investigate the correlation between injury assessments by eye and the amount of necrosis and between anatomical and physiological parameters, Pearson correlation coefficients were calculated. Statistical analysis were made using STATISTICA 10 for Windows software (Stat Soft, Inc. 2014).

Results

Gas exchange and morphological parameters

An AOT 40 critical value of ozone concentrations calculated for each one week period during plant exposure ranged from 43 to 304 ppm h. The highest air temperature was recorded in the second week of experiment, while the lowest in the third. The air relative humidity was higher in the last two weeks experiment (Table 1). The results showed variability in plant response to tropospheric ozone, depending on their genotypes. Mean values of net photosynthetic rates

(P_N) in exposed individuals of the sensitive genotype were significant smaller, amounting to P_N = 9.43-12.98 μmol(CO_2) m^{-2} s^{-1}, than those observed in the case of the resistant genotype - P_N = 9.57-15.35 μmol(CO_2) m^{-2} s^{-1}. On the other hand, no significant differences were recorded between the examined genotypes with regard to the intercellular CO_2 concentrations (C_i) and stomatal conductance (g_s) (Table 1).

Morphological examinations revealed more visible injuries on leaves of the sensitive genotypes caused by tropospheric ozone; furthermore, growth and size of leaves were much smaller than in resistant ones (Fig. 4). There was no significant difference for the effect of ozone between the examined genotypes with respect to the number of flowers and pods.

Image analysis based on microscopic images

After 28 days of experiment, dead cells in leaf tissues of both ozone sensitive and resistant beans were stained from dark to light blue, depending on the stage of cell membrane degradation (Figs 1, 5). Measurements of injures visible on microscopic preparations stained with Evan's blue demonstrated that the size of partially destroyed area damaged by ozone (green color in Fig. 6) and necrotic areas (red color in Fig. 6) in the case of the sensitive genotype (SE) was bigger and amounted respectively 2.18% and 1.18%, while in the resistant genotype (RE), partially destroyed area and necrotic area reached 0.5% and 0.02% respectively (Fig. 6, 7). The degree of necrotic changes of the measured parameters within individual genotypes varied.

Pearson correlation coefficients showed that percentage of necrotic areas of both genotypes was negatively correlated with net photosynthetic rate (p=-0.378; r=0.332) and were positively correlated with stomatal conductance (p=0.667; r=0.324), intercellular CO_2 concentration (p=0.278; r=0.665)respectively. Furthermore, we found a tendency for a positive correlation between percentage of necrotic area and the number of leaves with visible symptoms.he data obtained in the course of the performed experiments showed that the scale of sustained damages overlapped; both resistant and sensitive plants were damaged by ozone.

Discussion

Ambient tropospheric ozone concentration were at the level causing visible leaf injuries of the ozone-sensitive bean genotype at the exposure site, while both cultivars revealed a small amount of injuries in control conditions. The resistant genotype at the exposure site appeared

with comparable injuries to both control genotypes. Differences between genotypes were mainly observed for P_N, where statistically significant ($p<0.05$) lower levels were observed for the ozone-sensitive genotype at the exposure site. As has been previously reported, the decrease of P_N was not always related to stomatal closure. Nali et al. (2009) reported higher stomatal conductance in sensitive white clover biotype in comparison to resistant one. In some cases, the stomatal closure was very rapid and short-term, while the response of the photosynthetic apparatus was much slower and longer term (Vahisalu et al., 2010). Similar results were noted by Feng et al. (2011), when they reported the g_s fluctuations during the growing season in the case of one winter wheat variety, while another one revealed a consistent decrease in g_s. Lombardozzi et al. (2012) found a higher decrease of P_N than g_s. However, the stomatal closure can be a defence mechanism against high ozone exposure (Heath et al., 2008) and it is possible that in our case, the ozone concentration was not sufficiently high to cause stomatal closure, or the effect of P_N decrease was caused by other factors such as a decrease in the amount and activity of primary carboxylation enzymes Rubisco (Fiscus et al. 2005, Francini et al., 2007, Galmés et al. 2013), or the direct effect of oxidative stress through other mechanisms, such as suppression of the Calvin cycle (Guidi and Degl'lnnocenti, 2008, Heath, 2008). Ozone can reduce the biochemical capacity to fix CO_2, hence a higher decrease of P_N can be noted than of g_s. Tropospheric ozone effects on photosynthetic activity can influence morphology and crop and fruit plants production by reducing growth of plants (Morgan et al., 2003, Biswas et al., 2008, Tresmondi and Alves 2011). Our investigations confirmed a negative effect of ozone on plant growth for the ozone-sensitive genotype. Similar results were observed in an experiment with a common bean *Phaseolus vulgaris* "Nerina" conducted several years ago, where direct relations with ozone concentrations were observed only for this cultivar, even when low ozone concentrations occurred (Borowiak, 2013b, 2014).

Ozone generation is strongly affected by meteorological conditions (Tarasova et al., 2003). The analysis of obtained results of gas exchange parameters did not clearly relate to meteorological conditions. There was only possible to find relation between ozone cumulative concentrations and air temperature and relative humidity. The lower ozone concentrations were related to lower temperature and higher air humidity. High relative humidity is connected with the low ozone levels provided by wet ozone deposition on the water droplets (Tarasova et al., 2003; Kovać-Andrić et al., 2009, Quansah et al. 2012).

Validation based on microscopic studies has numerous advantages, i.ncluding possibilities of scaling down from plant tissue responses to cell reactions. Moreover, it can be used for early stage detection of injuries invisible for the naked eye (Vollenweider et al., 2003, Moura et al. 2011). Leaf surface analysis based on their images divided into three areas, is described in the work (Faoro and Iriti, 2005). Removing the veins was done "by hand" using an image editor. Division of the image into areas was done based on the histogram of the component B (Blue) using Adobe Photoshop 6.0. According to research conducted in this study, the assessment of cell damage performed only on the basis of the component B of the image is insufficient and subject to significant error. We proposed a method of image processing which gives more accurate results of leaf surface damage assessment. This method allows to distinguish and assess the percentage of damaged tissue, also including partially death areas, which is not possible with using the available software for image processing. Moreover, morphological and anatomical damages were positively correlated. The results presented here show early identification of the extent of tissue degradation of both bean genotypes in the glasshouse as well as in exposure conditions. The presented results are very promising, however require further validation with other common ozone-sensitive plant species.

Conclusions

Implementing image processing into the software developed by the authors made it possible to identify early tissue micro-damages both in sensitive and resistant genotypes of *Phaseolus vulgaris*. The method allows to distinguished partially and totally destroyed and necrotic areas. Proportions of tissue damage in the sensitive genotype were higher in comparison with plants of the resistance genotype. Moreover, a correlation of visible injuries, physiological and anatomical injures of *Phaseolus vulgaris* was found.

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Table 1. Photosynthesis parameters in ozone-exposed genotypes of *Phaseolus vulgaris* (net photosynthetic rate (P_N - µmol(CO_2) m^{-2} s^{-1}), stomatal conductance (g_s - µmol (H_2O) m^{-2} s^{-1}), intercellular CO_2 concentration (C_i µmol (CO_2) mol^{-1}) and one-week AOT40 (ppb h), mean week air temperature (ºC), air relative humidity (%). Means; n = 6; different letters denote significant differences at level $p \leq 0.05$ among exposure series and parameters.

Date	25.08			1.09		
AOT 40 [ppb h]	52			304		
Air temperature [ºC]	19.7			22.0		
Air relative humidity [%]	53.4			60.3		
Sample	P_N [µmol(CO_2) m^{-2} s^{-1}]	g_s [µmol (H_2O) m^{-2} s^{-1}]	C_i [µmol (CO_2) mol^{-1}]	P_N [µmol(CO_2) m^{-2} s^{-1}]	g_s [µmol (H_2O) m^{-2} s^{-1}]	C_i [µmol (CO_2) mol^{-1}]
RK	10.93a	78.99a	264.83a	8.47a	44.27a	108.52a
SK	14.04b	96.54b	224.66b	14.30c	69.72b	244.71b
RE	12.82c	124.06c	222.94b	13.62b	108.48c	247.22b
SE	11.65a	141.16d	256.86c	12.98b	103.85d	243.17b

Date	8.09			15.09		
AOT 40 [ppb h]	43			104		
Air temperature [ºC]	14.6			16.2		
Air relative humidity [%]	73.6			74.2		
Sample	P_N [µmol(CO_2) m^{-2} s^{-1}]	g_s [µmol (H_2O) m^{-2} s^{-1}]	C_i [µmol (CO_2) mol^{-1}]	P_N [µmol(CO_2) m^{-2} s^{-1}]	g_s [µmol (H_2O) m^{-2} s^{-1}]	C_i [µmol (CO_2) mol^{-1}]
RK	13.62a	125.19a	367.46a	13.62a	125.19a	347.15a
SK	17.26b	112.31b	312.8b	6.52b	72.08b	413.88b
RE	15.35c	92.39c	275.42c	9.57c	135.84c	355.95a
SE	9.43d	133.53d	383.8a	12.14a	123.83a	385.33c

RK - resistant genotype in control conditions, SK – sensitive genotype in control conditions;

RE – resistant genotype in ambient air conditions, SE - sensitive genotype in ambient air conditions

Figures

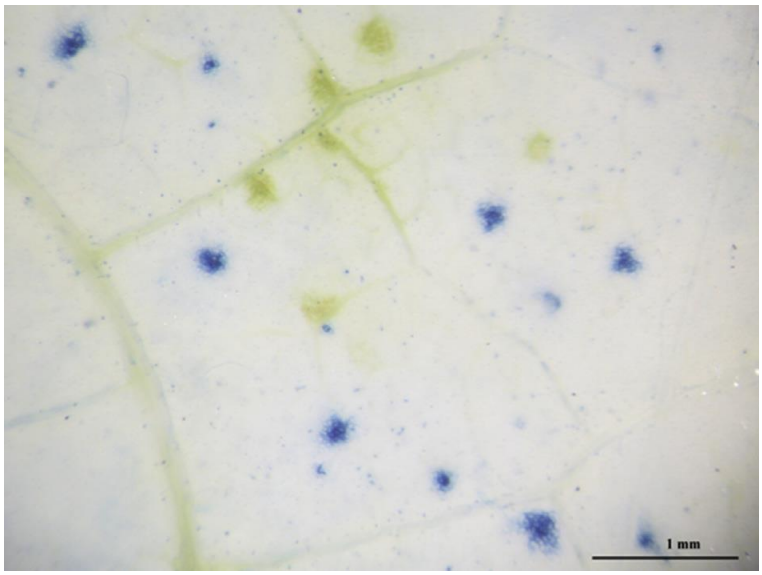

Fig. 1. Visible symptoms in leaf fragments of sensitive genotype (S156) *Phaseolus vulgaris*, dead cells are stained in blue.

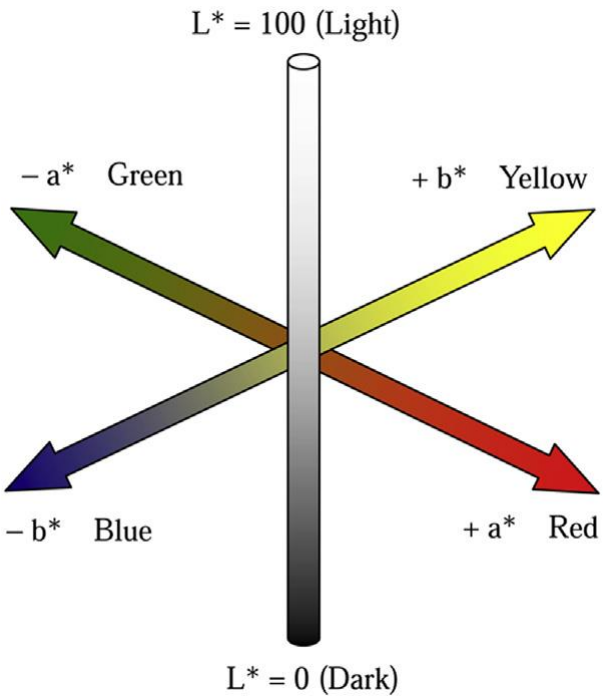

Fig. 2. CIE Lab color space (Technical Guides).

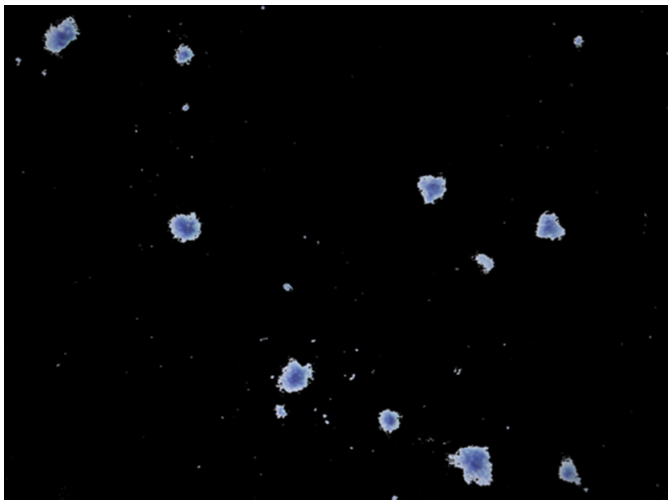

Fig. 3. Segmentation of image in leaf fragments of sensitive genotype (S156) *Phaseolus vulgaris* depicted in Fig. 1.

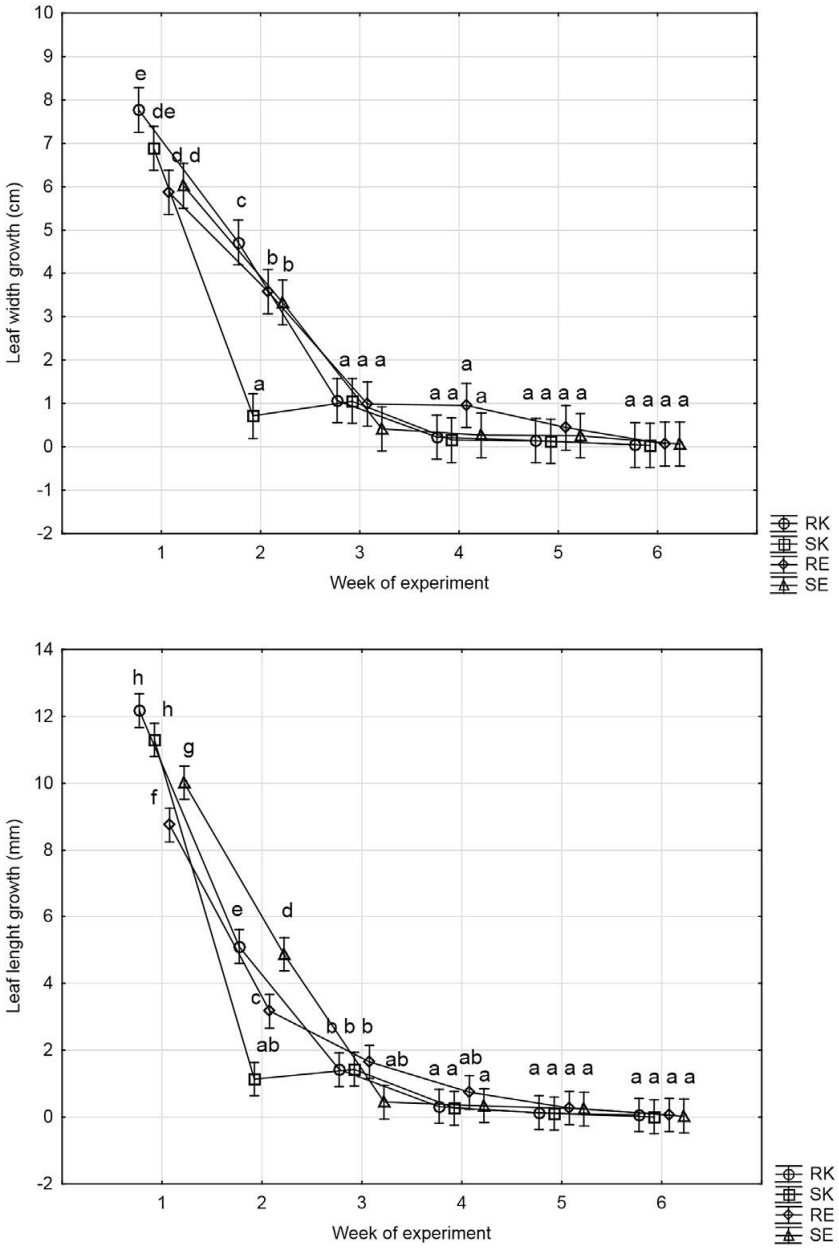

Fig. 4. Mean (± SD; *n* = 6) values leaves dimensions – leaf width and length growth (SE – sensitive genotype in ambient air conditions, SK - sensitive genotype in control conditions, RE – resistant genotype in ambient air conditions, RK – resistant genotype in control conditions.1- 6 terms of weekly measurements). Different letters denote significant differences at level p≤0.05 among exposure series and parameters.

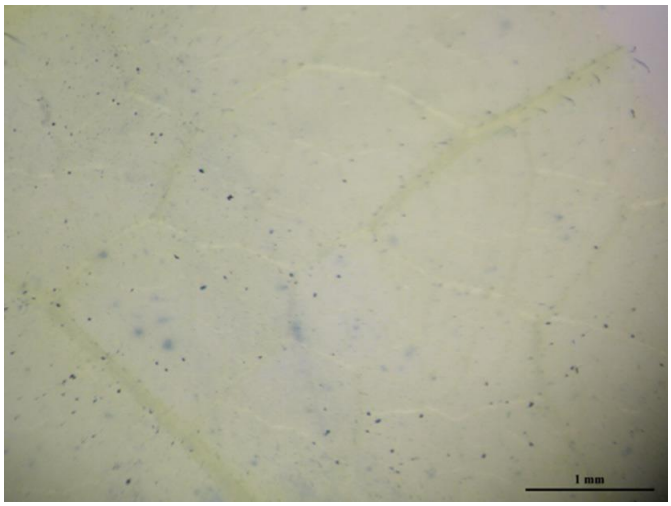

Fig. 5. Small amount of dead cells are (stained in blue) in leaf fragments of resistant genotype (R123).

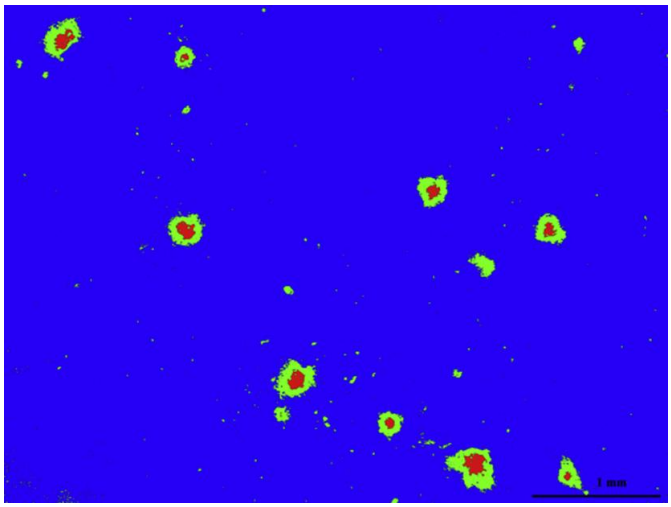

Fig. 6. Separation of different image areas: red – area totally destroyed, green – area partially destroyed, blue - living tissue.

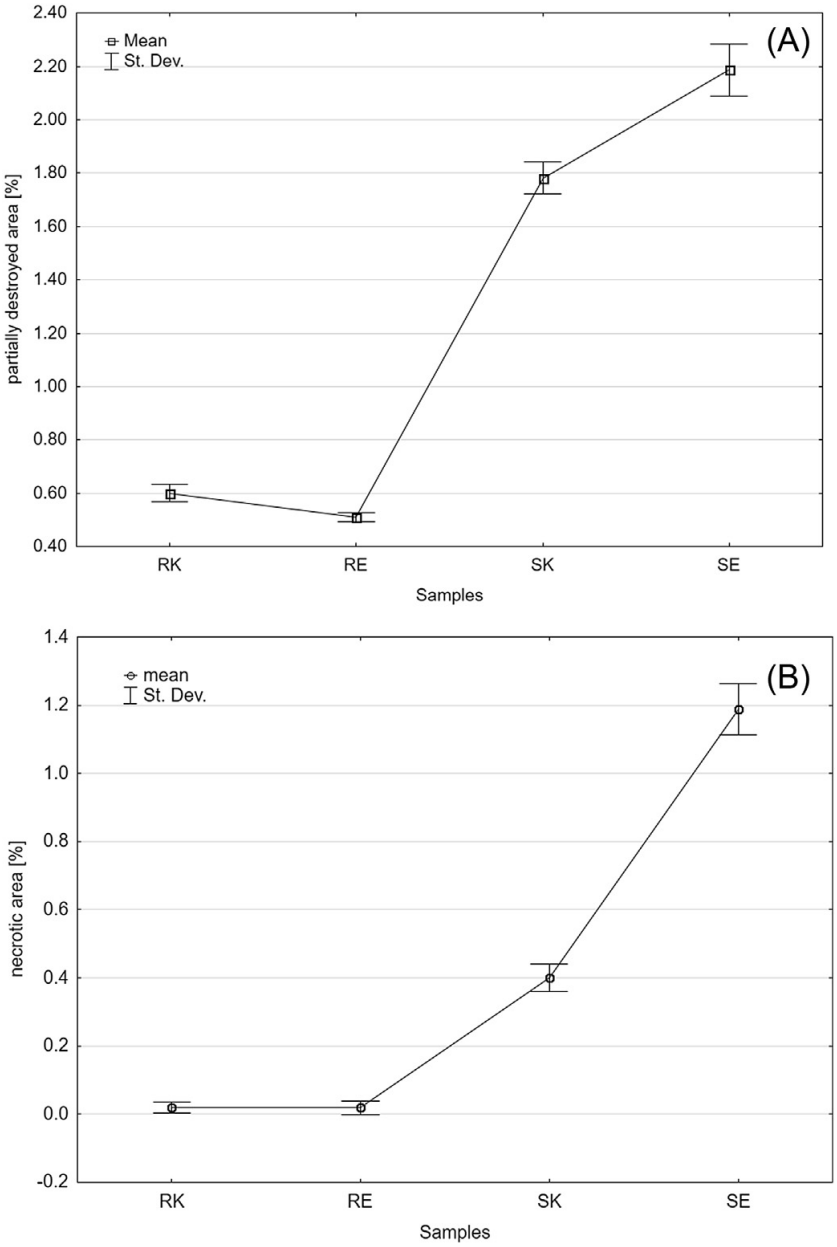

Fig. 7. Mean percentage (±SD) of partially destroyed area (A) and totally destroyed area (B) (%) of both genotypes based on image analysis method, (SE – sensitive genotype in ambient air conditions, SK - sensitive genotype in control conditions, RE – resistant genotype in ambient air conditions, RK – resistant genotype in control conditions).