Automated image analysis for quantification of histochemical detection of reactive oxygen species and necrotic infection symptoms in plant leaves

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RESEARCH ARTICLE

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In plants, the accelerated production of reactive oxygen species (ROS) is a common response to different stresses. We developed a computer method of leaf image analysis to automatically quantify the O_2^- and H_2O_2 staining patterns in leaves. The quantification of histochemically detected ROS is given as a percentage of stained area partitioned according to the staining intensity and hence O_2^-/H_2O_2 accumulation. Thus, this method expands the routine histochemical detection of ROS *in situ*. The same procedure can be also used to assess necrotic infection symptoms. In plants infected with necrotizing pathogens leaves can be simultaneously examined for pathogen-induced O_2^- and H_2O_2 accumulation and infection development. This procedure allows rapid and accurate examination of many leaves and provides an advantage over the routine visual assessment of *in situ* histochemical detection of ROS and infection symptom development.

Keywords: histochemistry; hydrogen peroxide; infection symptoms; leaf image analysis; superoxide anion radical

Introduction

In plants, the enhanced production of reactive oxygen species (ROS), especially superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2), is induced by numerous environmental factors, and ROS have been documented to play a profound role in pathogenesis and in acclimation to abiotic stress (Pitzschke et al. 2006; Bolwell & Daudi 2009). However, due to their high reactivity and extremely short lifetimes the study of ROS production in plants is very difficult. The quantitative biochemical and chemiluminescence methods developed for ROS determination do not provide information on their localization in plant tissues and the assessment by microscopy is limited to small tissue sections. Moreover, literature data on actual H_2O_2 concentrations show great variability (Cheeseman 2006). The histochemical detection of ROS provides the opportunity to identify the specific sites of their *in situ* production in plant tissues, but it imposes limitations on their quantitative analysis. The interpretation of results often relies on subjective visual estimation of stained areas yielding only qualitative data. However, to precisely describe the differences in staining patterns between experimental groups some form of quantification is required. The methods based on dye extraction from the stained tissue are time and labor consuming.

The assessment of pathogen-induced disease severity often presents a similar problem. The most popular method to quantify infection symptoms is based on visual estimation by using a rating scale.

This conventional assessment of infection development on leaves is subjective and its reproducibility is thus limited. Moreover, it may not always be sufficiently sensitive to distinguish discrete symptoms in the affected leaf areas that are difficult to quantify.

In recent years, imaging analysis has proven to be a useful tool in biology. Leaf image analysis is used as an objective and accurate alternative method to quantify the affected leaf areas. It enables to remove the element of human error from such assessments. However, leaf image analysis methods are often based on free available *Scion Image* application (Goodwin & Hsiang 2010) that needs a lot of time-consuming operations or a great level of knowledge in building appropriate macros to extract the region of interest. In this work, quantification of disease symptoms as well as nitro-blue tetrazolium (NBT)- and 3,3′-diaminobenzidine (DAB)-stained areas was performed by subjecting leaf images to the automatic analysis, and classification aimed to extract colored regions corresponding to pathogen-induced disease symptoms and stress-induced H_2O_2 and O_2^- accumulation, respectively. This method was tested on symptoms of angular leaf spot disease of cucumber as well as H_2O_2 and O_2^- staining patterns on leaves of cucumber and pumpkin plants exposed to infection or abiotic stress.

Materials and methods

Pumpkin (*Cucurbita maxima*) and cucumber (*Cucumis sativus*) plants were grown for three weeks in soil

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under growth chamber conditions (irradiance of 350 µmol m^{-2} s^{-1}, photoperiod 16/8 h, 23°C). Pumpkin plants were inoculated by touching *Erysiphe cichoracearum*-infected leaves to target plants or subjected to drought stress by withholding watering for seven days. *E. cichoracearum* isolate was kindly provided by Dr B. Dyki, Institute of Horticulture, Skierniewice, Poland. Cucumber plants were inoculated with *Pseudomonas syringae* pv. *lachrymans* (strain no. IOR 1990 from the Bank of Plant Pathogens, Poznań, Poland). Bacteria for inoculation were cultured for 24 h on King B medium at 28°C with vigorous shaking, and centrifuged at 3500*g* for 10 min. The bacterial pellet was washed twice and resuspended in sterile water, and adjusted to 10^7 cfu/ml. Three fully expanded leaves of cucumber were inoculated with bacteria or sterile distilled water (control) using a needleless hypodermic syringe. Alternatively, cucumber plants were treated with 50 mM NaCl for seven days. Control plants were irrigated with water. Based on the rate of relative water content decrease (data not shown) the water stress induced by drought and 50 mM NaCl was classified as moderate (Hsiao 1973). The third true leaves of stressed and control pumpkin and cucumber plants were harvested at various times following inoculation and abiotic stress treatment and used for histochemical detection of ROS.

The presence of O_2^- and H_2O_2 in detached leaves was detected histochemically using NBT (Unger et al. 2005) and DAB (Thordal-Christensen et al. 1997), respectively. The quantification of ROS production given as a percentage of stained leaf area was achieved by counting the leaf image pixels with known colors corresponding to the respective reaction products. The images of stained leaves were scanned to JPEG files using a standard flatbed scanner with 200/300 dpi and 24 bit/pixel resolutions and then were analyzed by an algorithm run in MATLAB environment on a Windows computer (Gocławski et al. 2012). Briefly, each leaf was placed on a scanner glass in a fixed direction, with the background color differing from the colors found inside of a leaf blade, namely blue for DAB and red for NBT staining. The leaf images were subjected to the automatic analysis and classification aimed to extract colored regions of ROS-mediated histochemical reactions. These regions were then partitioned into three fractions of pixels based on the color saturation corresponding to the staining intensity and hence ROS accumulation, defined as weak, medium, and intense. Before the extraction of selected regions, the leaf area was separated from its background based on the background-specific color. The leaf petiole not considered at the classification stage was eliminated programmatically after the detection as the extreme leaf contour protrusion (Gonzalez & Woods 2007). The automatic extraction of ROS reaction regions has been performed by a feed-

forward type, two layer neural network (Figure 1). The network input data were hue and saturation of each pixel taken as the two components of image *Hue Saturation Value* color space derived from original RGB image. The first network layer represented a self-organizing competitive *Winner Takes All* (WTA) network (Masters 1993; Osowski 2006), which divided leaf image pixels into several H, S color subclasses. It was concatenated with a linear network
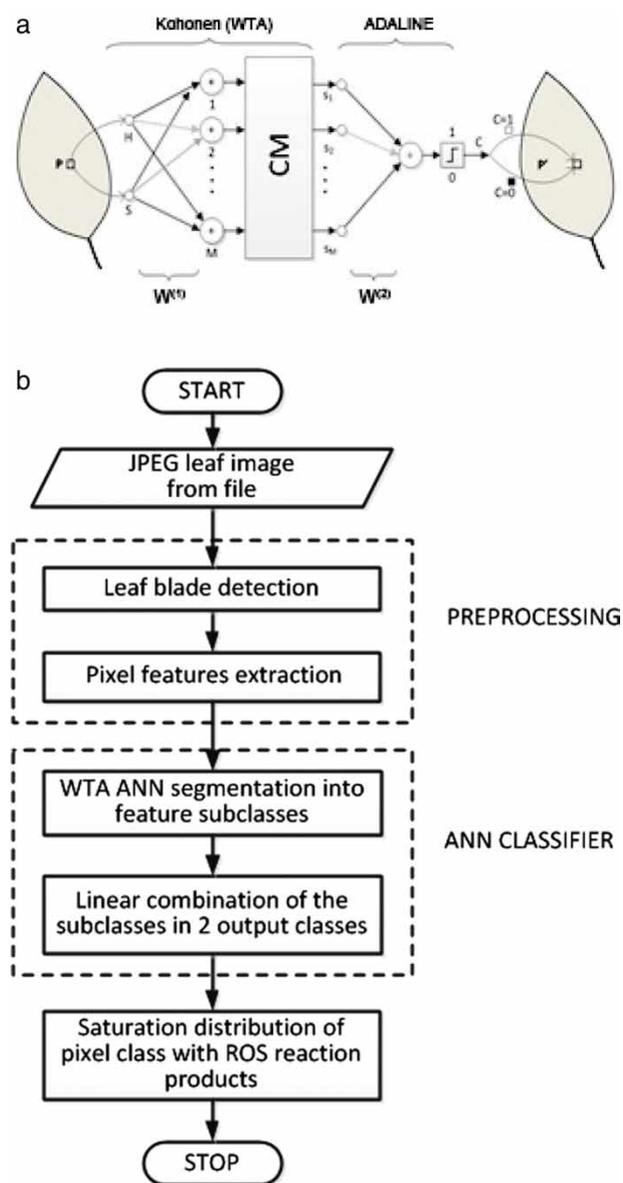
Figure 1. Leaf image analysis for the quantification of histochemically detected ROS: (a) schematic illustration of a two layer feed-forward network for the extraction of colored regions in a leaf blade. Kohonen (WTA [Winner Takes All]) – self-organized Kohonen network layer with M output subclasses, ADALINE – linear network layer with supervised learning, CM – WTA competitive module, W$^{(1)}$, W$^{(2)}$ – the weight arrays of the two network layers, H(P), S(P) – the network input data of each pixel Hue and Saturation, C(P') = 0 or 1 network output data; (b) the flowchart of the leaf image analysis algorithm. ANN, artificial neural network; ROS, reactive oxygen species.

layer merging the color subclasses in two pixel groups, where one of them included ROS-mediated reaction pixels. The self-organization of the first layer preceded the supervised training of linear layer by Widrow-Hoff method applying the binary pattern image data (Masters 1993; Osowski 2006). The pattern images were prepared manually in Corel Photo-Paint application using the source images representative for the whole tested image population. The image analyser can also be applied without previous training of the network output layer. Then the first layer pixel subclasses can be merged interactively in a dedicated dialog window to achieve final classification of regions stained with DAB and NBT. The algorithm ensured classification with high accuracy as shown by the leaf pixel classification error of 1.42% (Gocławski et al. 2012).

The algorithm execution is independent of specific color of leaf blade regions. Which color regions are of interest depends only on learning image patterns prepared by specialists or on the human operator selection of hue-saturation clusters recognized by network WTA layer. To avoid the generation of false positive effects, in the proposed algorithm selected leaf blade regions are excluded automatically or optionally from artificial neural network (ANN) color classification (Figure 1). This task is performed at the end of the pre-processing stage. Arbitrary selected areas to be removed should be individually marked as closed regions created by freehand drawing.

This software was also applicable to quantify *P. syringae* pv. *lachrymans* infection symptoms in cucumber leaves. Assessments were made 2, 5, 7, and 10 days after inoculation (T2, T5, T7, T10). First the blade area was separated from its background based on the background-specific color and quantified, followed by the identification and quantification of the area occupied by infection symptoms (Figure 2). Disease severity was expressed as a percentage of total leaf area affected by pathogen-induced spots.

Results and discussion

Among a wide variety of abiotic and biotic stressors, drought, salinity, and pathogens are important factors affecting plant health and productivity (Atkinson &

Urwin 2012). Cucumber and pumpkin studied here are economically important vegetables of the *Cucurbitaceae* family, and both are sensitive to salinity and drought which impose water deficit followed by the secondary oxidative stress (Munns 2002). *P. syringae* pv. *lachrymans*, causing the angular leaf spot disease and the powdery mildew pathogen *E. cichoracearum* are important pathogens of *Cucurbitaceae* crops causing significant losses in cucurbits yield (Olczak-Woltman et al. 2008; Cook & Braun 2009). We used the pumpkin/*E. cichoracearum*/drought and cucumber/ *P. syringae* pv. *lachrymans*/salinity interactions as models to automatically quantify the histochemically detected ROS and disease development. In pumpkin and cucumber leaves, the NBT and DAB staining procedures led to a weak background staining that was eliminated by the program. Five days after *E. cichoracearum* infection a marked increase in weak (18-fold) and medium (19-fold) NBT-stained area was observed, and O_2^- was detected over the whole infected leaf (Figure 3a). In control pumpkin leaves the production of O_2^- was nearly absent. Although drought stress applied to pumpkin plants was sensed by roots it also induced ROS accumulation in leaves confirming that ROS can act as both local and systemic signals during environmental stress response (Pitzschke et al. 2006; Torres 2010). However, the NBT-staining intensity and the total stained area in leaves of drought-treated plants were reduced by more than 50% when compared to the infected ones (Figure 3a). As to H_2O_2, we found that in control it concentrated around the vascular bundles, whereas in *E. cichoracearum*-infected pumpkin leaves DAB-stained spots scattered over the leaf surface corresponding to the infection sites (data not shown). The percentage of leaf area showing DAB staining increased after infection. It was due to the 40% increase in medium DAB-stained surface and the simultaneously observed 3.3-fold increase in intense stained area (Figure 3b). The accumulation of ROS, especially H_2O_2 is usually recognized as a component of effective defense strategy against biotrophic pathogens (Glazebrook 2005). Drought-induced H_2O_2 generation was associated with vascular bundles as DAB-stained spots located almost exclusively along leaf veins. According to visual assessment the extent
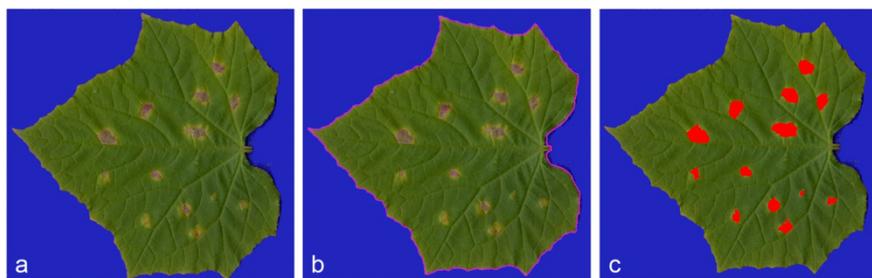
Figure 2. The example cucumber leaf images: (a) scanned image before segmentation with necrotic symptoms of *P. syringae* pv. *lachrymans* infection; (b) the leaf area separated from the background (pink line) based on the background-specific color; (c) the result of classification shown as red areas overlapped with the necrotized leaf regions.
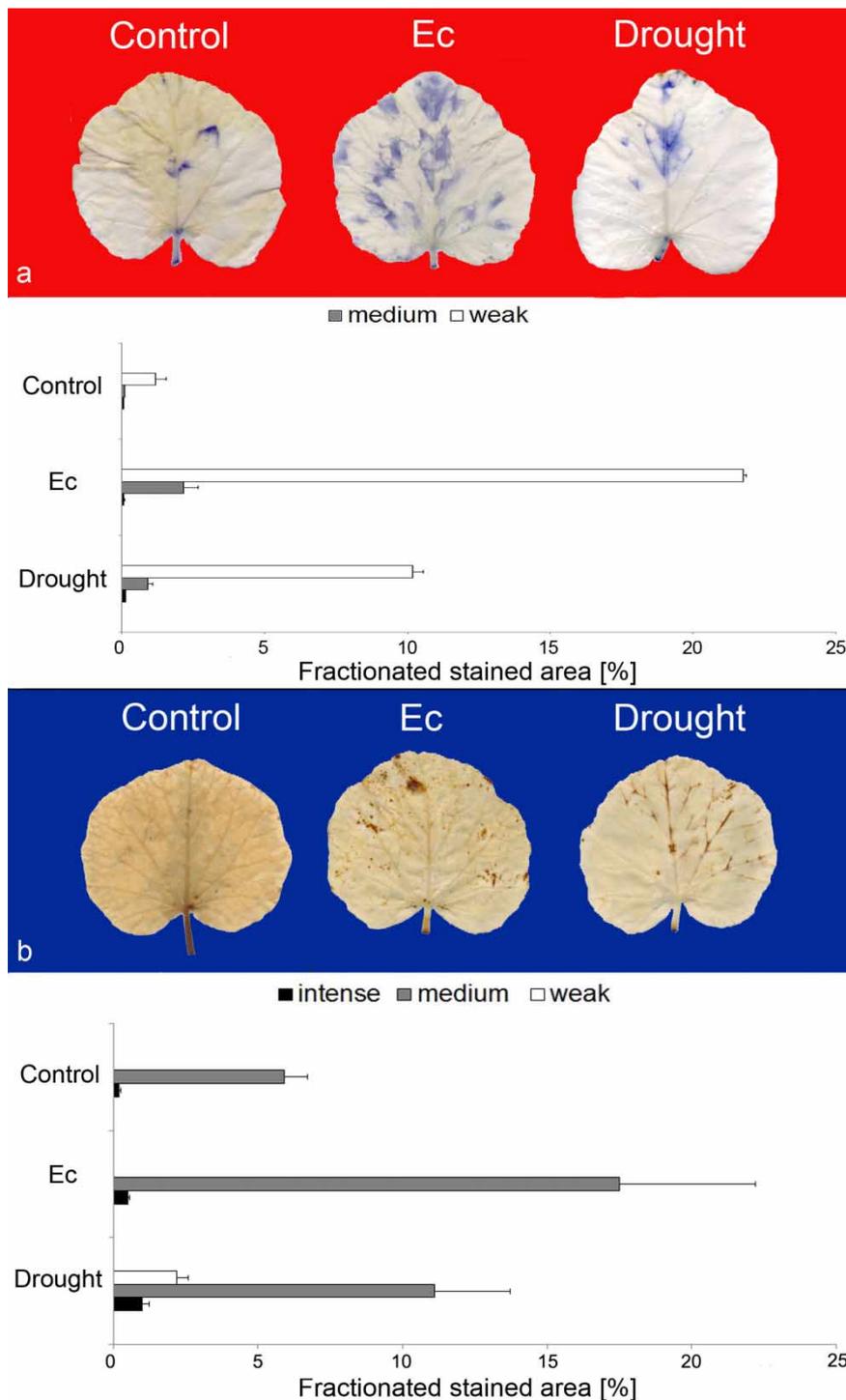
Figure 3. Histochemical detection of ROS in pumpkin leaves. Images of control, drought-stressed, and *E. cichoracearum*-infected (Ec) pumpkin leaves infiltrated with NBT for O_2^- detection (a) and stained with DAB to detect H_2O_2 (b) and quantification of the stained leaf areas. The blue coloration (a) indicates the formation of insoluble formazan deposits when O_2^- reacts with NBT. The brown staining (b) indicates the formation of polymerization product when H_2O_2 reacts with DAB. The pictures show representative leaf images. The graphs represent means $\pm$ SD of three independent experiments.

of DAB staining in infected and drought-treated pumpkin leaves was similar. However, the rigorous automated quantification revealed that the treatments had different effects on H_2O_2 production. In drought-treated leaves the total DAB-stained area was smaller by 25% than in the infected ones. Moreover, under abiotic stress the production of H_2O_2 was more heterogeneous as weak, medium,

and intense DAB staining could be distinguished, whereas pathogenesis-related local H_2O_2 generation was stronger and the medium DAB staining which indicated a considerable accumulation of H_2O_2 accounted for 97% of the total stained area (Figure 3b).

Similarly, quantitative evaluation of NBT- and DAB-stained leaf areas in cucumber revealed the heterogenicity of staining for infection and abiotic

Figure 4. Visualization of O_2^- detected by NBT in control, *P. syringae* pv. *lachrymans* (Psl)-infected (a) and 50 mM NaCl-treated (b) cucumber leaves and quantification of the stained leaf areas. The pictures show representative leaf images. The graphs represent means ± SD of three independent experiments.
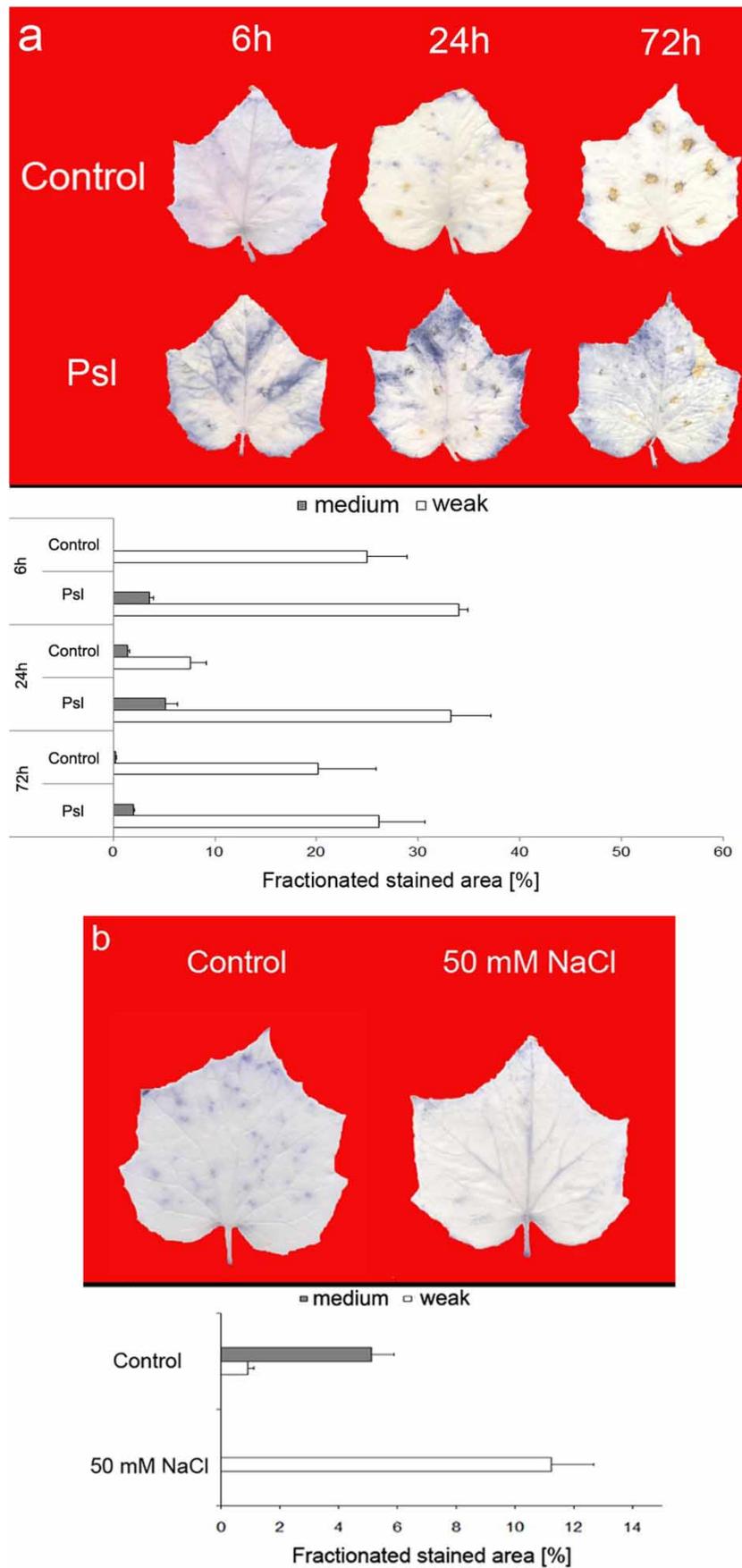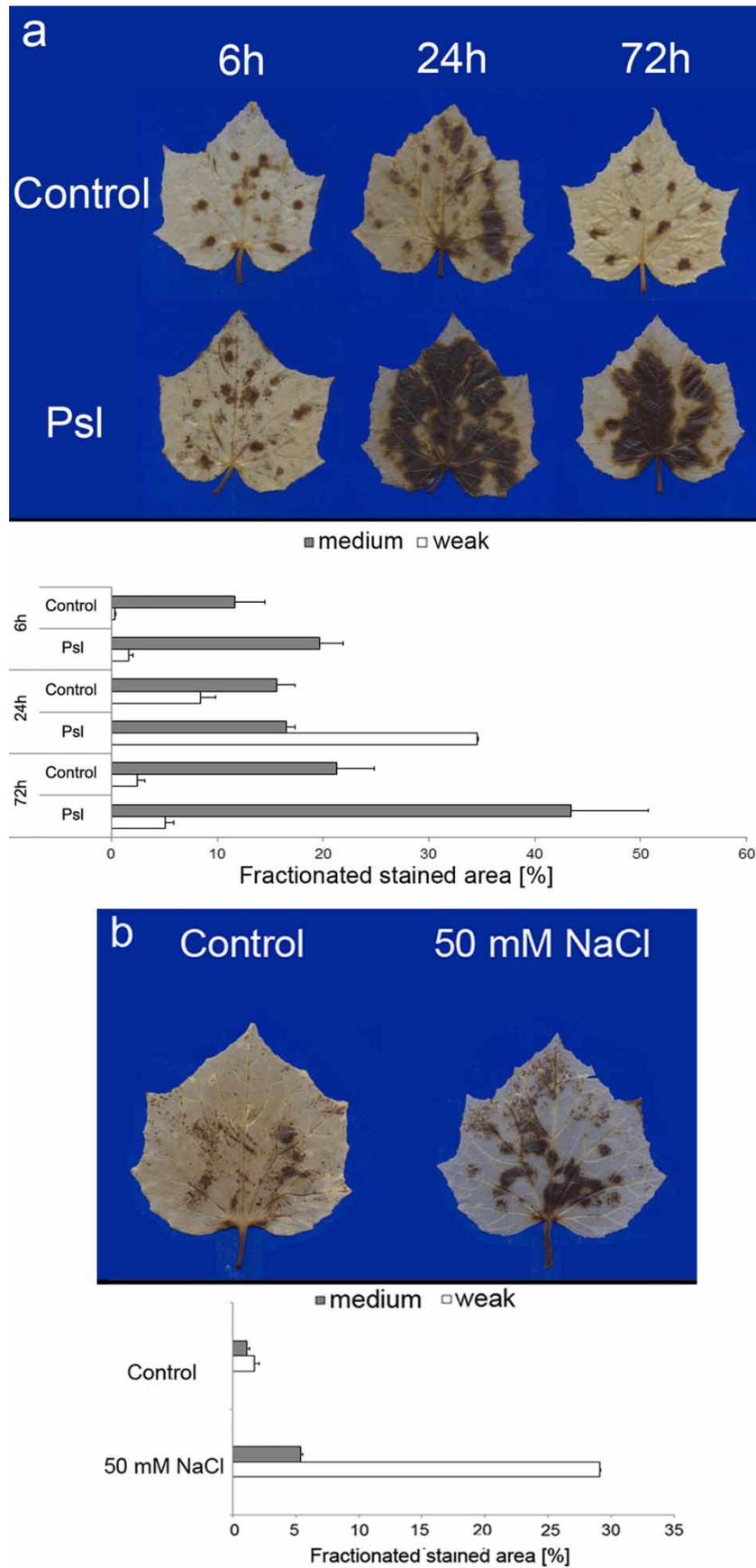
Figure 5. Visualization of H_2O_2 detected by DAB in control, *P. syringae* pv. *lachrymans* (Psl)-infected (a) and 50 mM NaCl-treated (b) cucumber leaves and quantification of the stained leaf areas. The pictures show representative leaf images. The graphs represent means $\pm$ SD of three independent experiments.
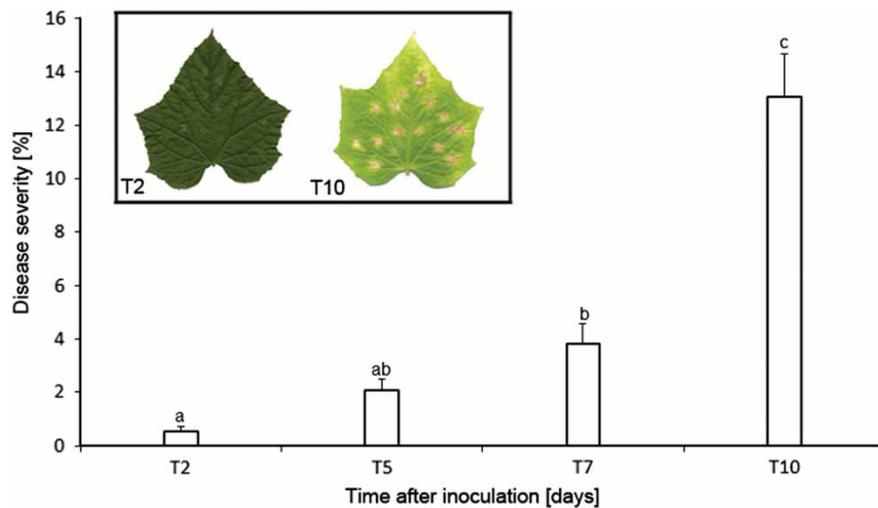
Figure 6. Angular leaf spot development on cucumber leaves. The pictures show representative leaves with disease symptoms caused by *P. syringae* pv. *lachrymans* recorded 2 and 10 days after inoculation (T2, T10). Severity of angular leaf spot disease is expressed as the percentage of leaf area affected by necrotic lesions. Data represent the mean values ($\pm$SD) from four to seven replicates ($n = 4–7$). Data were statistically analyzed using Kruskal–Wallis one-way analysis of variance and followed up by comparisons of mean ranks with P-values < 0.05 considered significantly different.

stress-induced H_2O_2 and O_2^- providing an advantage of the devised method over the routine visual assessment of *in situ* histochemical detection of ROS. The insoluble formazan deposits and DAB precipitates found in control (mock-inoculated) cucumber leaves indicated the wound-induced production of O_2^- and H_2O_2, respectively (Figures 4 and 5). After *P. syringae* pv. *lachrymans* inoculation, a marked increase in O_2^- content was observed at 6 h and 24 h and the NBT-stained surface accounted for 38% and 37% of the total leaf area, respectively. Thereafter (72 h), the increase in formazan deposits indicating O_2^- accumulation was not visible in inoculated cucumber leaves (Figure 4a). These results confirmed the well-known temporal pattern of oxidative burst after infection (Torres 2010). In contrast, salt stress caused approximately two-fold increase in H_2O_2 concentration relative to control plants (Figure 4b). However, it is worth noting that this response was characterized by weak NBT-staining only, indicating that the salt-induced accumulation of O_2^- remained low. The application of biotic stress also provoked an enhanced generation of H_2O_2 (Figure 5a). The DAB-stained area and intensity of staining progressively increased and reached the maximum values, 72 h after *P. syringae* pv. *lachrymans* inoculation when 48% of the total leaf area was stained brown indicating H_2O_2 accumulation. As at 72 h medium staining accounted for 43% of the total DAB-stained leaf area, the production of H_2O_2 could be considered significant. These data indicate that the generation of both ROS was not restricted to the infection sites; however, the bacterial pathogen-induced O_2^- and H_2O_2 did not accumulate at the same rate and to the same extent. Abiotic stress-induced generation of H_2O_2 in cucumber leaves was significantly enhanced and the DAB-stained area accounted for 34% of the total leaf area;

however, the majority of this area was light brown (Figure 5b). As similar results were found for NaCl-induced O_2^- production it appears that salt stress is a rather weak inducer of ROS in cucumber leaves. These results are in line with the observations that plants differentially enhance the generation of ROS depending on the quality of environmental stress (op den Camp et al. 2003).

The computer method was also successfully applied to monitor the development of angular leaf spot disease caused by *P. syringae* pv. *lachrymans* on cucumber leaves. The use of leaf area covered with infection symptoms was considered an appropriate measure of disease severity as under the experimental conditions disease development resulted in larger necrotic lesions rather than greater necrotic spotting. The quantification of angular leaf spot revealed a progressive increase in the leaf area covered by disease symptoms. The necrotic area increased from 0.5% two days after inoculation to 13% eight days later (Figure 6). In the course of infection a slower symptom development phase from two to seven days after inoculation and a faster one thereafter were detected. When disease development was assessed, leaf tip and/or margin necrosis of non-pathogenic origin were not considered. They were marked as excluded for neural network classification by the interactive erosion of binary leaf blade mask (Figure 2b).

This study has shown that the developed leaf image analysis method is a versatile tool for quantifying both necrotic symptoms and histochemically detected ROS accumulation in plants under biotic and abiotic stresses. The proposed procedure is a general-purpose leaf imaging method that can be used for many other plant species and can be adapted for simultaneous quantification of other responses, for

example, stress-induced cell death detected histo-chemically using Evans blue and GUS staining.

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