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Automated image analysis for quantification of reactive oxygen species in plant leaves

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ABSTRACT

The paper presents an image processing method for the quantitative assessment of ROS accumulation areas in leaves stained with DAB or NBT for H_2O_2 and $O_2^{\cdot-}$ detection, respectively. Three types of images determined by the combination of staining method and background color are considered. The method is based on the principle of supervised machine learning with manually labeled image patterns used for training. The method's algorithm is developed as a JavaScript macro in the public domain Fiji (ImageJ) environment. It allows to select the stained regions of ROS-mediated histochemical reactions, subsequently fractionated according to the weak, medium and intense staining intensity and thus ROS accumulation. It also evaluates total leaf blade area. The precision of ROS accumulation area detection is validated by the Dice Similarity Coefficient in the case of manual patterns. The proposed framework reduces the computation complexity, once prepared, requires less image processing expertise than the competitive methods and represents a routine quantitative imaging assay for a general histochemical image classification.

1. Introduction

Reactive oxygen species (ROS) were initially recognized as unavoidable, potentially damaging by-products of the aerobic metabolism which accumulate in stressed cells. In fact, oxidative stress, defined as an imbalance between ROS generation and detoxification by the antioxidant system, is a common feature of the plant response to most abiotic and biotic stresses. In recent years it has become clear that ROS, especially hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\cdot-}$), also act as versatile signaling molecules in the processes of plant growth and development as well as in the response to abiotic and biotic stresses. The outcome of ROS signaling depends on the ROS production site, intensity and duration. Therefore, the spatial and temporal analysis of ROS generation in plant tissues, especially in green leaves which are considered the major targets of environmental stresses, is of great interest.

Detection and quantification of ROS can be performed by different methods but it still remains challenging due to the low steady-state level, short lifetime and reactivity of these molecules [1]. The conventional, invasive biochemical assays are likely to overestimate H_2O_2 when applied to plant leaves [2] and the analysis is restricted to a small leaf area taken to prepare the sample. The fluorescence-based methods, routinely used to study changes at the cellular and subcellular levels, meet extra challenge in leaves due to the production of singlet oxygen ($^1O_2$) upon illumination and the accumulation of light-scattering starch grains. Moreover, the fluorochromes have been shown to be ROS generators themselves [3,4]. The histochemical detection of ROS provides precise information about the *in situ* distribution and accumulation of ROS in different cells and tissues over a relatively large area, e.g. whole leaf blade. Routinely, 3,3′-diaminobenzidine (DAB) and nitro blue tetrazolium chloride (NBT) are used as chromogens for the assessment of H_2O_2 and $O_2^{\cdot-}$, respectively [5,6]. The ROS staining methods become increasingly important in plant stress biology as non-invasive tools for mapping oxidative stress in plants before the appearance of any visible damage symptoms. However, the quantification of the results of histochemical analysis still remains difficult, especially when a detailed analysis of the differences in the patterns and gradients of staining between experimental plant groups or tissue sets is required. The manual staining assessment is subjective and prone to error. By contrast, computer-assisted image analysis referring to the field of using algorithms to extract quantitative information from images, offers an objective

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automated method for quantification of the histochemical data quickly and efficiently [7,8].

Image analysis can be performed using a variety of commercial software, open-source bio-imaging application, as well as with special tools dedicated to the resolution of individual problems. A set of specialized applications has been developed by Regent Instruments Inc., and *WinFOLIA* is among the most suitable commercial software for the analysis of leaf morphology and quantification of different areas distinguished against the leaf background. *WinCAM NDVI*, with a similar scope of applications. is not as specialized for a group of objects as the *WinFOLIA* software, but it offers a wide variety of implementations which may functionally overlap with them. For example, it can measure the color area on fruits, leaves, flowers or any other plant material that shows a minimum level of color contrast. Assess 2.0 is a commercial software dedicated to direct plant disease quantification [9]. These applications provide benefit of automation and reproducibility, but they also have a number of limitations. Commercial software is related to the purchase costs and often has a closed environment. Moreover, these tools may not be applicable to atypical probes.

Atypical plant materials or staining patterns require a custom approach to extract relevant data from the analyzed image. Systems designed individually are often characterized by a hybrid structure, e.g. the system for automated pomegranate disease recognition and grading uses fuzzy logic, back propagation neural networks and support vector machines [10]. Those methods often use classifiers and generally work well only under conditions and situations to which they were trained. This group includes the algorithms previously developed by the authors, concerning the classification and quantification of ROS reaction areas in the leaves of pumpkin and cucumber, which used *MWTA* and *LVQ* neural networks [11–13].

An alternative to the foregoing possibilities are free software packages, characterized by the open structure and having the possibility of personal extension with the essential auxiliary tools [14,15]. Popular freeware applications, widely used throughout the scientific community, are NIH Image (developed at the U.S. National Institute of Health) and Scion Image (Scion Corporation, www.scioncorp.com) [16,17]. NIH has been superseded by Java based environment ImageJ [18,19] with its extended version known as Fiji [20] available with many additional components and large number of plugins in Java language, including advanced image processing algorithms.

Typically, public domain applications mentioned above are built from elementary image processing procedures and also contain image acquisition methods, that cooperate with cameras or simple scanners. They are mostly developed as a cross-platform software (for Windows, Mac or Linux). To create more complex image analyzer or classifier with public domain software, the user should find a plugin ready to use for this purpose or arrange different elementary processing procedures in a proper macro form [17]. This, however, requires some understanding of basic image processing operations.

Plant leaves usually show variability in DAB/NBT staining quality and stresses produce a range of staining patterns, making the quantitative assessment of ROS distribution and accumulation difficult and contributing to the discrepancies of results between studies. Therefore, an extremely sensitive and, on the other hand, possibly universal approach to the segmentation of leaf images with visible effects of histochemical ROS staining is required.

In this article, the authors proposed a novel cost free method for the automatic quantification of histochemical ROS reaction products in leaves stained with DAB [5] and NBT [6], using image segmentation performed in the public domain Fiji software. The pixel classification was performed using the *Trainable Weka Segmentation* (TWS) plugin (former *Advanced Weka Segmentation*), that is designed for segmentation via interactive learning [21]. This image analysis system uses a *Fast Random Forest* (FRF) learning method for classification, which is a multithreaded implementation of the *Random Forest* classifier for Java [22]. It is a highly effective machine learning technique that generates classifiers in the form of an ensemble ("forest") of decision trees.

In biological applications, the segmentation of image data typically requires applying a sequence of algorithms to many images separately or in a so-called pipeline. In the proposed Fiji segmentation method, a fast prototyping of whole multi-step procedure has been facilitated via scripting, including image preparation, FRF learning and prediction process as well as calculation of the various degrees of staining. It involved writing simple programming commands to define sequences of operations, that can be applied to images of a certain type set accepted by the program.

The effectiveness of the proposed method for the assessment of ROS production has been verified on pumpkin, cucumber and ice plant leaf images with different histochemical staining patterns and its accuracy has been compared to the method that was developed in the Matlab environment.

2. Material and methods

2.1. Plant material

Common ice plant (*Mesembryanthemum crystallinum*, collection of the Botanical Garden of the Technical University of Darmstadt, Germany), pumpkin (*Cucurbita maxima)* and cucumber (*Cucumis sativus*) plants were grown in soil, in a growth chamber under irradiance of 350 μmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night) and temperature 23 °C. Pumpkin was infected with biotrophic fungus *Erysiphe cichoracearum* (kindly provided by Barbara Dyki, Research Institute of Horticulture, Skierniewice, Poland) by touching *E. cichoracearum*-infected leaves to target plants or subjected to drought stress by withholding watering for 7 days. Cucumber plants were subjected to salt stress and irrigated with 50 mM NaCl for seven days. Thereafter the salt-pretreated plants were inoculated with *Pseudomonas syringae* pv *lachrymans* (strain No IOR 1990 from the Bank of Plant Pathogens, Poznań, Poland). The fully expanded leaves of cucumber were infiltrated with bacterial suspension (10^7 cfu ml^{-1}) or sterile distilled water (control) using a needle-less hypodermic syringe. *M. crystallinum* plants were inoculated with *Botrytis cinerea* (isolate 1631 provided by the Bank of Plant Pathogens, Poznań, Poland). The conidial suspension (1×10^6 spores ml^{-1}), supplemented with 5 mM glucose and 2.5 mM KH_2PO_4, was infiltrated into the abaxial surface of adult leaves of the second pair using a syringe without a needle. Control plants were infiltrated with 5 mM glucose and 2.5 mM KH_2PO_4.

The leaves of pumpkin, cucumber and common ice plants were harvested at various time points following inoculation and abiotic stress treatment and used for histochemical detection of ROS.

2.2. Histochemical detection of ROS

The presence of H_2O_2 in leaves was detected by DAB staining method [5]. Leaves were submerged in 1 mg ml^{-1} DAB–HCl (pH 3.8), under light conditions, for 12 h. In the presence of endogenous peroxidase, polymerization of DAB at the sites of H_2O_2 accumulation generates a brown DAB-polymer that is macroscopically visible. For *in situ* detection of O_2^- the procedure described by Unger et al. [6] was followed. Leaves were immersed in 10 mM K/Na phosphate buffer (pH 7.8) containing 0.1 mM NBT, 0.1 mM EDTA and 10 mM NaN_3. The solution was vacuum infiltrated for 15 min. The leaves were then held at room temperature until the blue precipitates of formazan that is produced as a result of the reduction of NBT by O_2^- became visible.