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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. 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.

1. 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 and Lamb, 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*, *N. 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

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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 color (Faoro and Iriti, 2005). Using a light microscope, we can observe varying intensity of coloring 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 (*P. 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.

2. Material and methods

2.1. Experimental design

Well-known ozone-sensitive (S156) and ozone-resistant (R123) genotypes of *P. 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 km 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 m away from main roads and 50 m 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 fiber wicks placed in pots and trays with water.

Every 7th 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 analyzed, such as air temperature and humidity.

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