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Ozone fumigation results in accelerated growth and persistent changes in the antioxidant system of *Brassica oleracea* L. var. *capitata* f. *alba*

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ABSTRACT

The growth response and antioxidant capacity of *Brassica oleracea* var. *capitata* f. *alba* plants treated with 70 ppb of ozone was examined. Four week old cabbage seedlings were fumigated with O_3 for 3 days before being transplanted into the growing field. The effect of O_3 treatment was determined directly after fumigation and over the course of field cultivation. Plants subjected to O_3 treatment had an increased diameter of rosettes and number of leaves after 3 and 7 weeks in agriculture, respectively. In addition, the vast majority of fumigated plants reached marketable quality faster than control plants, indicating a positive role of episodes of increased O_3 concentrations during vegetation on growth and yielding.

Our analysis revealed that by fumigating juvenile white cabbage plants with moderate doses of O_3 the activity of catalases (CAT) and peroxidases was elevated. The activity of the examined enzymes was not affected directly after fumigation, but it increased after several weeks in the experimental field. Increased CAT activity was accompanied by changes in 2 out of the 3 CAT genes *CAT1* and *CAT2*, where *CAT2* seemed to be responsible for the induced CAT activity.

The biosynthesis of low-molecular stress protectants – tocopherols and the glucosinolate (GLS) sinigrin was transiently affected by ozone. γ-Tocopherol (γ-toc) content significantly increased directly after fumigation, but after 3 weeks of vegetation in the field its concentration reached values similar to control. The biosynthesis of α-tocopherol (α-toc) and sinigrin seemed to be upregulated in fumigated plants. However, the response was delayed; no differences were registered directly after treatment, but 3 weeks after transplanting the concentration of sinigrin and α-toc was elevated.

Introduction

The oxidation–reduction equilibrium plays a pivotal role in regulation of plant metabolism. Changes in the environment alter the plants redox homeostasis, which in turn fine-tunes many aspects of plant metabolism (Apel and Hirt, 2004; Foyer and Shigeoka, 2011). At the cellular level various abiotic and biotic stress factors e.g. UV radiation, ozone (O_3), drought, low temperature, pathogens etc. elicit a similar response – they enhance the production of reactive oxygen species (ROS) and can cause oxidative stress (Torres et al., 2006; Ślesak et al., 2007). Oxidative stress occurs when the production of ROS exceeds the cells scavenging potential and the rate of repair processes fails to keep pace with the proceeding damage (Apel and Hirt, 2004). Persistent or acute stress results in irreversible damage and a subsequent loss in physiological competence. In the case of moderate stresses, ROS production and the ensuing alterations in oxidative signalling may induce defense mechanisms resulting in systemic acclimation. In the case of oxidative stress a common mechanism may lead to cell death, either by an uncontrollable production of ROS and necrosis or genetically programmed cell death (PCD) and to stress induced activation

Abbreviations: APCI, atmospheric pressure chemical ionisation; APX, ascorbate peroxidase; BSA, bovine serum albumin; CAT, catalase; DAB, diaminobenzidine; DAD, diode array detector; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; FLD, fluorescence detector; GLS, glucosinolates; HPLC, high performance liquid chromatography; MSD, mass detector; tricine, N-tri(hydroxymethyl)methylglycine; tris, hydroxymethylaminomethane; PAA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; PAR, photosynthetically active radiation; PCD, programmed cell death; ROS, reactive oxygen species; toc, tocopherol.

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of defense mechanisms and acclimation (Rao and Davis, 1999; Martindale and Holbrook, 2002; Fujita et al., 2006; Foyer et al., 2009; Mullineaux and Baker, 2010).

Ozone (O_3) is a model abiotic elicitor of ROS in plant cells. It enters the leaves mainly through open stomata and due to its reactivity immediately reacts with components of the apoplastic space generating various ROS (Ludwikow et al., 2004; Baier et al., 2005; Iriti and Faoro, 2008). The chain of events following O_3 exposition resembles hypersensitive response (HR) in plant–pathogen interactions (Lamb and Dixon, 1997). High concentrations of O_3 induce a secondary ROS burst in the chloroplast (Joo et al., 2005) and the cell wall (Torres and Dangl, 2005) and can activate defense mechanisms by impacting gene expression, accumulation of secondary metabolites and plant hormones: salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Overmyer et al., 2000; Tamaoki, 2008). According to Sandermann et al. (1998), moderate doses of O_3 may enhance the plant resistance by activating detoxification, antioxidant and other defense mechanisms.

A number of studies on O_3 induced oxidative stress in plants have been done, but most of them concern plant responses in severe conditions (over 100 ppb of O_3) resulting in foliar injury and losses in plant productivity. Mild oxidative stress or alterations in ROS homeostasis and the resulting changes in the plants physiology have received far less attention. Furthermore, the impact of stress on physiology and biochemistry of plants is usually examined in a short-term perspective i.e. after hours or days. Very few studies deal with aftereffects and long-term (after weeks or months) consequences of stress imposition. (Degl'Innocenti et al., 2002; Navakoudis et al., 2003; Guidi et al., 2005; Sasaki-Sekimoto et al., 2005; Gielen et al., 2006).

White cabbage (*Brassica oleracea* L. var. *capitata* f. *alba*), along with other crucifers, is one of the most willingly consumed vegetables in Europe and in the world (Kusznierewicz et al., 2007). It provides significant amounts of vitamins (A, C, E, K), and other phytochemicals, such as glucosinolates (GLS) and other sulfur containing compounds (Kurilich and Juvik, 1999; Kopsell et al., 2004; Nosek et al., 2011) which are beneficial for human health. Numerous epidemiological reports indicate a close relationship between the consumption of vegetables from the Brassica genus and protection against numerous chronic diseases, including several types of cancer (Byers and Perry, 1992), cardio- and cerebrovascular, ocular and many neurological diseases (Block et al., 1992; Podsędek, 2005).

Here, we performed a series of analysis of selected components of the antioxidant system, at different stages of growth and development of *B. oleracea* fumigated with a moderate dose of O_3 for 3 days in its juvenile stage. Changes in the antioxidant system were monitored immediately after and several weeks after fumigation. The results presented in this paper include the activity of antioxidant enzymes: catalase (CAT), ascorbate peroxidase (APX), total peroxidase activity and the content of low molecular antioxidants: tocopherols (α-toc, γ-toc), and sinigrin. The abundance and activity of antioxidants is indicative of the cells ROS homeostasis and metabolic processes associated with it (Foyer and Noctor, 2005; Potters et al., 2010). It cannot be ruled out that the response of *B. oleracea* plants to elevated O_3 concentration is prolonged over a period of time and that the character of induced changes is different during stress imposition and in the period after stress cessation. Thus, the dynamics of changes in the antioxidant system combined with a comparative study of plant growth parameters over the period of field vegetation after stress application allowed to speculate on the role of antioxidants and ROS homeostasis in plant development.

Materials and methods

Plant material

Brassica oleracea L. var. *capitata* f. *alba* cultivar Gregorian F_1 seeds were sown into multipots filled with peat substrate and cultivated in a greenhouse. During germination a uniform temperature of 20 °C was maintained, sprouts were cultivated in 16–18 °C. After 6 weeks seedlings were transferred to closed top plexiglass chambers for ozone fumigation (Skoczowski et al., 2000). The fumigation chambers were placed in a larger phytotron chamber equipped with an air conditioning and humidity control system. Ozone was supplied to the experimental chambers by the Aqua Medic Ozone 50 generator during the light period. A constant concentration of 70 ppb of O_3 was controlled by the 49C UV Photometric O_3 Analyser provided with a self-calibrating system (Thermo Environmental Instruments Inc. USA). Plants were exposed to O_3 for 3 days under an 11 h photoperiod at 400 μmol m^{-2} s^{-1} (PAR), with a constant day/night temperature of 17/13 °C and ca. 60% relative humidity. Control plants were placed in similar chambers not supplied with ozone. At the end of fumigation the first group of plants was collected for analysis (described as 0 weeks).

After fumigation *B. oleracea* seedlings were divided into four 40 plant groups and transferred into the experimental field of the Experimental Station of the University of Agriculture in Mydlniki, Krakow, Poland (50°5′5″ N, 19°51′8″ E). Standard horticultural practices were applied over the course of plant vegetation; *inter alia* they were watered and supplied with necessary nutrients required for optimal growth. Three, 7 and 10 weeks after transplanting, the plants were harvested for analysis. For a single analysis the second and third leaves from 3 plants were harvested. The last group of harvested plants consisted of fully developed heads and two, outermost leaves were used for measurements.

All analysis were performed in three independent experiments.

Biometric and yield measurements

During vegetaion in the field, plant rosette diameter and the number of leaves was measured 3 and 7 weeks after transplanting from the vegetation chambers. Cabbage heads were harvested twice (after 10 and 11 weeks in the field) after developing a typical for this variety size. Yielding was determined as the percentage of marketable yield per harvest.

Protein extraction and quantification

Frozen in liquid nitrogen, ground with a mortar and pestle plant leaves were homogenized in ice cold 100 mM Tricine-Tris buffer pH 8.0, containing 100 mM $MgSO_4$, 1 mM DTT and 3 mM EDTA (Miszalski et al., 1998). After extraction, probes were centrifuged for 5 min at 10,000 × *g* at 4 °C. Protein content was quantified according to Bradford (1976) using BSA as a standard.

Enzyme analysis

CAT activity was measured by native PAGE (polyacrylamide gel electrophoresis) and spectrophotometrically. For semiquantitive CAT activity determination 7 μg of proteins from crude plant extracts were separated in the noncontinues Laemmli buffer system (Laemmli, 1970), with a 3.5% stacking and 7.5% separating polyacrylamid (PAA) gel. CAT activity was visualized with $FeCl_3$ and $K_3Fe(CN)_6$ according to Woodbury et al. (1971).

The spectrophotometric measurement was performed according to the modified method described by Aebi (1984). Crude plant extract (5 μl) was added to 995 μl of phosphate buffer pH 7.0 containing 3 mM H_2O_2. CAT activity was determined from the decrease