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Short communication

Effect of arachidonic acid elicitation on lettuce resistance towards *Botrytis cinerea*

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ABSTRACT

Elicitation with arachidonic acid (AA) caused a significant increase in guaiacol peroxidase, polyphenol oxidase and protease activities in lettuce leaves and also resulted in elevated resistance of lettuce to the fungal pathogen (*Botrytis cinerea*). Research taken on this study indicates that AA induces mechanisms for the acquisition of resistance by lettuce. This observation explains why elicitation with this compound may provide an alternative to the conventional method for the protection of this vegetable against *Botrytis cinerea*.

1. Introduction

Lettuce (*Lactuca sativa* L.) is the most important vegetable in the group of leafy vegetables in the world and plays an important role in a healthy diet (Robak and Ostrowska, 2008; Mou, 2009). However, fungal and bacterial diseases threaten lettuce crops. Additionally, pesticide use carries risks for consumers because lettuce is a vegetable consumed mainly in salad mixtures and sandwiches (as a fresh vegetable) (Van Beneden et al., 2009). Grey mold, caused by the necrotrophic fungus *Botrytis cinerea* is the most common fungal disease of greenhouse and field-grown lettuce (Van Kan, 2005).

In recent years, research have looked for new, alternative methods of plant protection. Elicitation, being a method for the natural induction of plant resistance mechanisms, could be proposed as a new alternative, non-conventional and ecologically-friendly approach for plant protection. Plants possess a range of defense mechanisms that can be actively expressed in response to either pathogens and parasites or inoculation with elicitors.

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance in plants. Both ISR and SAR are effective against a wide range of pathogens. However, there are differences in the effectiveness of the signaling compounds involved against different types of pathogens. SAR is effective against pathogens that in noninduced plants are resisted through SA-dependent defenses, whereas ISR is effective against pathogens that are resisted through JA/ethylene-dependent defenses (Vallad and Goodman, 2004). SAR and ISR can be induced by elicitation.

The elicitors can be biotic or abiotic in nature (da Rocha and Hammerschmidt, 2005; Cao et al., 2013). Elicitation, in addition to induction of plant resistance, can also improve the nutritional and nutraceutical value of plants/sprouts (Świeca et al., 2014; Złotek et al., 2014).

Abiotic elicitors are the most interesting crop protectors, because of their high stability. Arachidonic acid (AA) is included to a group of chemical abiotic elicitors stimulating primarily induced systemic resistance of plants (Savchenko et al., 2010). Induction of immunity by AA treatment has been demonstrated in plants such as tobacco, millet and potato (Rozhnova et al., 2003; Rozhnova and Gerashchenkov, 2006; Amruthesh et al., 2005), but there have not been any studies on the effect of AA on the defensive reactions of lettuce.

The aim of this study was to examine the effect of elicitation with arachidonic acid on selected determinants of the resistance of lettuce and to test whether AA can protect lettuce (*Lactuca sativa)* from a necrotrophic fungal pathogen (*Botrytis cinerea*).

2. Materials and methods

2.1. Plant materials and growth conditions

Lettuce seeds (*Lactuca sativa* L. *var. capitata* cv. Justyna) were purchased from a commercial garden center. The seeds were sown

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into sowing boxes filled with universal soil for sowing seeds. Seven-day-old seedlings were transplanted to 600 mL pots containing universal garden soil (PNOS, Ożarów Mazowiecki, Poland) with sand (4:1 v/v) – two plant per pot. Plants were grown in a growth chamber (SANYO MLR-350H) at 25/18 °C, photoperiod 14/10 h day/night, with a photosynthetic photon flux density (PPFD) at a plant level of 500–700 μmol m^{-2} s^{-1} and a relative humidity of 70%. As recommended by the Regional Chemical-Agricultural Station in Lublin (Poland), the crop was fertilized at the following levels (in mg L^{-1}): N – 40, P – 445, K – 290, Mg – 100, Ca – 770, Cu – 50. The experiment was arranged in randomized block design with three replication. In each block (replication) twenty one-day-old plants (eight lettuce plants in each treatment, $n = 24$) were sprayed with a solution of the test elicitors in two concentration (1.5 mL per plant): 1 μM arachidonic acid (AA1) and 100 μM arachidonic acid (AA2) (arachidonic acid Sigma–Aldrich, purity ≥99%) prepared in deionized water (arachidonic acid was previously dissolved in a very small amount of ethanol). The control plants (C) were sprayed with deionized water. There were four pots with two lettuce plants per pot in each treatment. The concentrations of elicitors were selected based on previous screening experiments (data not published), so as not to induce negative effects on the health and growth of plants. Fifteen days after elicitation (29 days after transplantation, when plants were at early head development stage) the plants were harvested by cutting the plants from roots and leaves of lettuce were frozen. A composite sample of leaves from eight lettuce plants were used in the biochemical analyses.

2.2. Biochemical analysis

2.2.1. Guaiacol peroxidase (POD) assay

Soluble guaiacol peroxidase activity was determined spectrophotometrically according to the method described by Chance and Machly (1955). One unit (1 U) of peroxidase activity was defined as 0.001 ΔA_{470} in min per mg protein

2.2.2. Polyphenol oxidase (PPO) assay

Soluble polyphenol oxidase activity was measured spectrophotometrically, based on the procedure reported by Wisserman and Lee (1980). One unit (1 U) of PPO activity was defined as 0.001 ΔA_{410} in min per mg protein.

2.2.3. Proteases (POT) assay

Soluble proteases activity was measured according to Anson (1938). One unit (1 U) of POT activity was defined as 0.001 ΔA_{280} in min per mg protein.

2.3. Gravimetric analysis

Samples of fresh leaves from control and elicited cultivation were weighed, freeze-dried and weighed again to obtain the total dry weight. The results were expressed as g/plant (Tamagnone et al., 1998).

2.4. Scanning electron microscopy (SEM)

Scanning electron micrographs were made using the method of CRYO-SEM. The samples were frozen in liquid nitrogen, placed in cryochamber for 5 min at 95 °C where a process of sublimation of ice took place, and then coated with a thin layer of platinum (about 10 nm). The samples were then placed in a chamber of the electron microscope FESEM (Zeiss Ultra Plus) and were observed and photographed from cryo-mounted table. Microscopical images and measures were taken using a digital image processing system (AxioVision rel. 4.8, Carl Zeiss, Germany). Plants were kept in the light for 3 h before freezing the leaves.

2.5. Pathogen culture and inoculation of lettuce

In the experiments used two races of *Botrytis cinerea* – Osf 75/2011 obtained from oat seedlings and As 22/2010 obtained from *Tagetes tenuifolia.* Pathogen culture, plant inoculation and evaluation of the disease development on lettuce's leaves were performed according to Kułek and Floryszak-Wieczorek (2005). The pathogen was cultured on potato glucose agar medium in 28 °C. Conidial spore suspension (10^5 mL^{-1}) was prepared from seven-days-old fungus cultures in a solution of 0.1 M glucose and 0.05 M KH_2PO_4.

The leaves of thirty-six-days-old lettuce, when plants were at early head development stage (from control and previously elicited plants) were inoculated with *Botrytis cinerea* spore suspension by spraying (15 mL per plant).

In addition, comparison treatments were also used: absolute control (instead of fungal spores – only medium) – AC.

The plants after inoculation placed in growth chamber at 20 ± 2 °C, photoperiod 14/10 h day/night, with PPFD at plants level of 500–700 μmol m^{-2} s^{-1} and relative humidity of 95%. After seven days the development of the disease was evaluated by measurement of area of disease spots on leaves (Kułek and Floryszak-Wieczorek, 2005). Disease severity was also recorded by estimating the percentage of the brown leaf spot symptoms (disease index, DI [%]) according to Soleimani and Mohajer (2011).

2.6. Statistical analysis

All experiments were performed in triplicate (3 parallel experiments in the same condition) and after that each sample was measured in triplicate. Results were evaluated for statistical significance using univariate analysis of variance (ANOVA, Tukey's post hoc test). The factor was elicitor dose. p values <0.05 were regarded as a significant.

3. Results and discussion

3.1. Enzymes activities

Enzyme assay (POD, PPO, POT) experiments showed that treatment of lettuce seedlings with AA increased POD, PPO and POT activities (Fig. 1). The POD activity value was 0.025 U mg $protein^{-1}$ in AA1-treated lettuce, 0.036 U mg $protein^{-1}$ in AA2-elicited lettuce and only 0.017 U mg $protein^{-1}$ in untreated plants. The influence of AA on POD activity stimulation had been studied by Sood and Sohal (2012) who observed induction of POD activity in *Brassica juncea* L. after AA elicitation.

AA used at 100 μM concentration caused about 48% higher PPO activity in elicited lettuce plants than in the control plants and 1 μM AA concentration caused about 40% higher activity (Fig. 1). The data we obtained may indicate the participation of PPO in lettuce resistance. Raj et al. (2006), Thipyapong et al. (2004) and Wang and Constabel (2004) had previously suggested that PPO participated in plant defense. Additionally, Mouekouba et al. (2013) and Wang et al. (2011) found a positive correlation between enhanced peroxidase and polyphenol oxidase activities and protection against *Botrytis cinerea* in tomatoes.

3.2. Ultra structure of leaves

Scanning electron micrographs of the lower surface of the lettuce leaves showed the epidermal cells significantly smaller in the AA-treated leaf samples (Figs. 2A and 3). Furthermore, compared with the control, the stomata from AA1 and AA2-treated leaf