Scientia Horticulturae 193 (2015) 127–135

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Botrytis cinerea infection in three cultivars of chrysanthemum in 'Alchimist' and its mutants: Volatile induction of pathogen-infected plants

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ARTICLE INFO

Article history:
Received 16 February 2015
Received in revised form 22 June 2015
Accepted 24 June 2015
Available online 24 July 2015

Keywords:
Chrysanthemum
Odors
Volatile organic compounds
Pathogens
Botrytis cinerea

ABSTRACT

Three closely related chrysanthemum cultivars were examined in terms of the effect of *Botrytis cinerea* infection and leaves injury on the emission rates of fourteen volatile organic compounds (VOCs). Mechanical injury or pathogen infestation of vegetative tissues can induce VOCs production, which can provide defensive functions to plants. Infection with *B. cinerea* induces the plant defense system in chrysanthemum leaves and stimulates the emission of VOCs that may deter herbivores and attack natural enemies. The change in VOCs emission, as a result of injury or fungal infection in chrysanthemum, has not been studied yet.

Our results indicate that a pathogen can induce several VOCs in chrysanthemum plants. In the greenhouse, we measured VOCs from chrysanthemum 3 or 7 days following foliar inoculation. We confirmed that several green leaf volatiles ((GLVs); (Z)-3-hexenal, (E)-2-hexenal, (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol, (Z)-3-hexen-1-yl acetate), terpenes (β-pinene, β-myrcene, (Z)-ocimene, linalool, β-caryophyllene, (E)-β-farnesene), and shikimic acid pathway derivatives (benzyl acetate, methyl salicylate, indole) were positively induced from chrysanthemum plants infected with *B. cinerea* (Pietrowska et al., 2015). The quantities of the VOCs induced were higher 7 than 3 post infection days. Control plants released only very little but detectable amounts of VOCs. We also set up additional control plants (pierced plants) to facilitate pathogen infection. It was an additional stress and we collected odors also from those plants. In this case the quantities of the VOCs induced were higher 3 than 7 post-injury days. Also our paper suggests a large qualitative and quantitative overlap of VOCs induction from chrysanthemum plants with a mechanical injury when compared to both mechanical injury and the injury from fungal pathogens.

1. Introduction

Chrysanthemum (*Chrysanthemum* × *grandiflorum*/Ramat./Kitam; syn. *Chrysanthemum morifolium*) is the second (after rose) world most popular ornamental plant (Matsumura et al., 2010; McKellar et al., 2005).

The *in vivo* VOCs emission in chrysanthemum has not been studied yet, though several studies have focused on VOCs and other secondary metabolites extracted from various plants and organs. A vast review on secondary metabolites in the genus *Chrysanthemum* was elaborated by Kumar et al. (2005) and Chang and Kim (2013), focusing on the biological activity of the secondary compounds extracted from different plants tissues, and their medicinal as well as pesticide applications. The findings on the content of VOCs and other secondary metabolites in Chinese chrysanthemum flowers revealed 58 volatile compounds (Sun et al., 2010). Kim et al. (2014) analyzed the volatile compounds extracted from leaves from fifteen taxa of Korean *Chrysanthemum* species. With the chemotypes received, they were able to classify the taxa tested into three groups, different in terms of the quantity of particular secondary compounds.

Plants emit complex blends of volatile organic compounds (VOCs) into the surrounding atmosphere from many of their organs (terpenes, fatty acid derivatives, benzenoids, phenylpropanoids,

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http://dx.doi.org/10.1016/j.scienta.2015.06.040
0304-4238/

and amino-acid-derived metabolites), which is frequently associated with a range of biotic and abiotic stress factors (Boczek et al., 2013; Gantner and Najda, 2013; Holopainen and Gershenzon, 2010; Pichersky and Gershenzon, 2002; McCormick et al., 2012). The emission of VOCs from plants varies extensively depending on the species, organ, developmental stage and environmental conditions (Holopainen and Gershenzon, 2010) and the same compounds are released by most plant species, irrespective of their taxonomic affinities, *e.g.* the monoterpenes: (E)-β-ocimene and linalool, sesquiterpenes (E,E)-α-farnesene and (E)-β-caryophyllene, green leaf volatiles (GLVs): including (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate (Arimura et al., 2009; Danner et al., 2011; Haltishke et al., 2008; Kigathi et al., 2009; Schaub et al., 2010; Wu and Baldwin, 2009). Some of the VOCs emitted can serve as repellents to the attacking insect, as well as attractants to the natural enemies (Dicke and Baldwin, 2010; Hare, 2011; Kessler and Baldwin, 2001; Maffei, 2010; Piesik et al., 2013a,b,c; Schwachtje and Baldwin, 2008; Yi et al., 2009).

Plants have a tremendous capacity to counteract pathogens (De Vos and Jander, 2010; Dicke et al., 2009; Halitschke et al., 2008; Holopainen and Gershenzon, 2010; Leitner et al., 2008; Poelman et al., 2011; Vickers et al., 2009; Yi et al., 2009 Holopainen and Gershenzon, 2010; Leitner et al., 2008; Poelman et al., 2011; Vickers et al., 2009; Yi et al., 2009). Essential oils can be active against the fungal pathogens and significantly reduce fungal hyphal growth (Alvarez-Castellanos et al., 2001). Piesik et al. (2011a) found that several common GLVs and mono- and sesqui-terpenes were induced by the infection of maize with *Fusarium* spp., with a greater induction 7 than 3 post-foliar infection days.

The aim of our study has been to examine chrysanthemum plants in terms of VOCs emission after mechanical injury and fungal infection. Here, we studied VOCs induction following *B. cinerea* infection in three chrysanthemum cultivars. The degree of VOCs induction was examined 3 and 7 days after mechanical injury and foliar inoculation. VOCs emitted by plants were collected, identified and the concentrations of fourteen particular VOCs were estimated. We examined whether the plants infected always have a greater VOCs induction than uninfected plants (control plants).

2. Material and methods

2.1. Plants cultivation

Three chrysanthemum cultivars (*Chrysanthemum* × *grandiflorum* /Ramat./ Kitam.) were used in the experiment; 'Alchimist' and its two radiomutants 'Alchimist Golden Beet' and 'Alchimist Tubular' (Zalewska et al., 2011). To ensure a good health of experimental plants obtained from *in vitro* tissue culture. Following the acclimatization, chrysanthemum plants were grown in the greenhouse at the Plant Growth Center at the UTP University of Science and Technology in Bydgoszcz, Poland.

The plants were cultivated in 9 cm diameter pots, one plant per pot. Plants grew vegetatively from April to June, supplementary light was provided in April and May to extend a natural daylight up to 16 h. The plants were cultivated under ambient relative humidity of 70–85%, day temperature of $22 \pm 1\,^{\circ}$C, night temperature of $18 \pm 1\,^{\circ}$C. The plants were watered 2–4 times weekly, and fertilized twice per week with a Peters Professional General Purpose 20-20-20 NPK fertilizer (Scotts, USA). Aligned, single shoot plants 20–24 cm in height and 18–22 leaves were selected for leaf treatments.

2.2. Leaf inoculation

B. cinerea was grown on PDA plates for 14 days and conidia suspensions were prepared by adding 20 ml of sterile water to the plates containing the fungus and gently scraping the conidia from the agar surface using a spreader. The conidia suspensions were filtered with sterile gauze into a 500 ml beaker and the concentration was determined with the Neubauer haemacytometer. All the leaves of chrysanthemum healthy plants were injured (pierced with a sterile needle) and inoculated with conidia suspension of the density of 10^6 cfu ml^{-1} by spraying (250 ml H_2O; 0.01% Triton-X-100) either 3 or 7 days prior to the VOCs collection. The control or injured (no inoculated) plants were sprayed with water without suspended *B. cinerea* 3 or 7 days prior to the VOCs collection.

2.3. Disease and rating

The severity of gray mold caused by *B. cinerea* was assessed on chrysanthemum leaves. The first suspension of the density of the assessment of plants' reaction to *B. cinerea* was done 3 days after inoculation. The second examination was carried out 7 days after inoculation. There was estimated the percentage of the leaf surface area with necrotic lesions. The macroscopic estimation was accompanied by the mycological analysis for the leaves with disease symptoms. Pathogens were isolated from tissue on the PDA medium for the confirmation of the identity of *B. cinerea*. The material for the analysis was randomly sampled from the roots and the stem base with disease symptoms, regardless of the experiment combination. 5 mm sections from diseased leaves were prepared. The leaf pieces were rinsed for 45 min in tap water, disinfected for 15 s in 1% $AgNO_3$ solution and then rinsed three times in sterile distilled water and placed onto potato dextrose agar (PDA) with 50 mg of streptomycin per 1 L, in Petri dishes. Representative cultures were identified by their morphology on PDA and synthetic nutrient agar (SNA medium) using available literature. The frequency (defined as the percentage of isolates of an individual species in the total number of fungal isolates) of *B. cinerea* in each fungal community was determined.

2.4. Volatile collection system

Volatiles were collected from experimental chrysanthemum plants enclosed within Nalophan (polyethylene terephtalate), odor and taste-free cooking bags made of plastic film (Charles Frères-Saint Etienne-France). The collection apparatus allowed for the collection of VOCs odors from 8 plants simultaneously, and lasted 2 h. A volatile collector trap (6.35 mm OD (outside diameter), 76 mm long glass tube; Analytical Research Systems, Inc., Gainesville, Florida, USA) containing 30 mg of Super-Q adsorbent (Alltech Associates, Inc., Deerfield, Illinois, USA) was inserted into each of 8 Tygon tubes (the connection between airflow meter and collector trap). Purified humidified air was supplied at the rate of 1.0 L min^{-1} over the plants, and a vacuum pump sucked 20% less (0.8 L min^{-1}) to avoid collecting odors from any gap of the system. Additionally, four blanks (odors gathered from empty Nalophan bags) were collected and verified the lack of background non-chrysanthemum VOCs based on the lack of detectable peaks in chromatograms.

2.5. Analytical methods

Volatiles were eluted from the Super-Q in each volatile collection trap with 225 μL of hexane, followed by adding 7 ng of decane as an internal standard. The previous experiments showed that this quantity of hexane was sufficient to extract all the trapped volatiles (Piesik et al., 2010, 2011b,c). Individual samples (1 μL) were