Environmental Pollution 212 (2016) 559–564

Contents lists available at ScienceDirect

Environmental Pollution

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

Short communication

Effects of different routes of application on ethylenediurea persistence in tobacco leaves☆

S. Pasqualini [a, *], E. Paoletti [b], G. Cruciani [a], R. Pellegrino [a], L. Ederli [a]

[a] Department of Chemistry, Biology and Biotechnology, University of Perugia, Borgo XX Giugno 74, I-06121 Perugia, Italy
[b] Institute of Sustainable Plant Protection, National Council of Research, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Firenze, Italy

ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form
4 March 2016
Accepted 4 March 2016
Available online xxx

Keywords:
Apoplast
Bel-W3
EDU
Nicotiana tabacum
Ozone
Quadrupole-time of fly liquid chromatography/massspectrometry (Q-TOF LC/MS)

ABSTRACT

Ethylenediurea (EDU) is a common research tool for investigating ozone impacts on vegetation, although the role of different application routes (foliar spray vs soil drench) on EDU persistence in the leaves is unknown. We quantified EDU concentrations in leaves of the O_3-sensitive Bel-W3 cultivar of tobacco treated with EDU as either foliar spray or soil drench. Foliar EDU concentrations were measured by Q-TOF LC/MS. When EDU was applied as foliar spray, 1 h was enough for reaching a measurable concentration within the leaf. EDU concentration increased over the 21-day period when the leaf was not washed after the application (treatment #1), while it decreased when the leaf was washed after the application (treatment #2). These results suggest that: a) dry deposition of EDU onto the leaf surface was gradually absorbed into the unwashed leaf, although the mechanisms of such uptake were unclear; b) concentration of EDU was decreased quickly (−35%) during the first 24 h from application and more slowly during the following three days (−20%) in the washed leaves. Degradation did not involve enzymatic reactions and was not affected by the presence of ROS. When EDU was applied as soil drench, foliar concentrations increased over time, likely due to adsorption onto soil organic matter and gradual re-solubilization by irrigation water. An analysis of EDU concentration in protoplast and intercellular washing fluid showed that EDU did not enter the cells, but was retained in the apoplast only. Possible implications of EDU in the apoplast and recommendations for EDU application are discussed.

1. Introduction

Ground-level or tropospheric ozone (O_3) is the most widespread phytotoxic air pollutant in the developed world and is a serious concern for natural and cultivated vegetation (Paoletti, 2007; Fuhrer, 2009). Over the past recent decades, control measures over precursors emission have reduced O_3 peaks, while background levels are stable or continue to rise (Sicard et al., 2013; Paoletti et al., 2014b). Assessing O_3 impacts on vegetation is challenging because O_3 does not accumulate in the tissues and plant responses are aspecific.

Ethylenediurea (N-[2-(2-(2-oxo-1-imidizolidinyl)-N'-phenylurea abbreviated as EDU) has been widely used as a versatile research tool (Carnahan et al., 1978; Manning et al., 2011; Agathokleous et al., 2015) for: (i) diagnosing the role of O_3 in occurrence of foliar injury in the field (Paoletti et al., 2009; Saitanis et al., 2015); (ii) determining the effects of ambient O_3 on growth and productivity of plants in the field (Hoshika et al., 2013; Carriero et al., 2015); (iii) screening plants for sensitivity to O_3 under ambient conditions (Pandey et al., 2014, 2015; Yuan et al., 2015); (iv) understanding the mode of action of O_3 (Paoletti et al., 2008, 2014a). In spite of much research, however, there are still knowledge gaps about EDU protection mechanisms and fate within the plant.

When used appropriately, EDU does not induce confounding or toxic effects of its own (Agathokleous et al., 2016). EDU may be applied as a foliar spray, as a drench to the soil, and by stem injection or gravitational infusion (Manning, 2000; Paoletti et al., 2007, 2011). Regner-Joosten et al. (1994) and Gatta et al. (1997) investigated uptake, partitioning and persistence of EDU in plants in short-term experiments (5-10-16 days), by using hydroponics or leaf incubation in EDU solution as treatments and HPLC with UV detector for EDU analysis. The results suggested that EDU is quickly (<2 h) uptaken and translocated to the leaf apoplast where it persists long (>8 d) showing a slow degradation (Gatta et al., 1997).

☆ This paper has been recommended for acceptance by B. Nowack.

* Corresponding author.
E-mail address: stefania.pasqualini@unipg.it (S. Pasqualini).

http://dx.doi.org/10.1016/j.envpol.2016.03.017
0269-7491/

However, the *in planta* degradation mechanisms of EDU are unknown. As EDU cannot move via phloem to newer untreated leaves, repeated applications are necessary to ensure continuous protection from O_3, with re-application ranging from 7 to 21 days, depending on species, environmental conditions and O_3 levels (Weidensaul, 1980; Paoletti et al., 2009).

Our purpose here was to quantify EDU persistence in leaf tissues of potted tobacco plants, after application of EDU as soil drench or foliar spray. We hypothesized that: (i) EDU is uptaken quickly independently on the application route; (ii) after application, foliar concentration decreases over time; (iii) EDU degradation within the leaf tissues is not driven by enzymatic reactions; (iv) EDU residues in the soil or onto the leaf can result in further uptake over time; (v) EDU accumulates in the leaf apoplast and does not enter the cells. To improve the analytical sensitivity and the quantification of EDU relative to previous studies, a Q-TOF LC/MS system was used. The innovative MetaSite software was finally applied to determine the three-dimensional structure of EDU and the potential metabolites generated during EDU degradation.

2. Materials and methods

2.1. Plant material

Seeds of *Nicotiana tabacum* L. cv. Bel-W3 were kindly provided by Ted Woodlief of North Carolina State University (Raleigh, NC, USA). The seeds were germinated in plastic pots (0.3 L volume, 10 cm upper diameter) filled with soil (modular tray substrate with 85% organic matter as dry weight - Klasmann Deilmann, Sedelsberg, Germany) and agriperlite mixture in the ratio 3:1 (v/v), after vernalization for 2 d at 5 °C, and then transferred to a growth chamber with 12 h photoperiod, photosynthetic photon fluence density (PPFD) of 200–250 μmol m^{-2} s^{-1}, day/night air temperature of 28/26 °C, and relative humidity 60%–75%. Plants were water irrigated every day and fertilized weekly with half-strength Hoagland's solution. The third leaf from the base of 4-week-old plants was used in all experiments.

2.2. EDU application

N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea (EDU) was applied either as foliar spray or as soil drench. EDU, kindly provided by Prof. William J. Manning of University of Massachusetts, (Amherst, MA, USA) was 100% active ingredient wettable powder. The solution was prepared immediately before the application by dissolving 300 mg EDU in 1 L (300 ppm) of warm deionised water with and without the wetting agent Triton X-100 (0.1% v/v) for foliar spray and soil drench, respectively. Such EDU concentration was selected according to previous studies with tobacco (Batini et al., 1995). When the solution was back to ambient temperature, the third leaf was marked with a small plastic tie and sprayed to drip point with either 0 or 300 ppm EDU solution by an atomizer nozzle to both the upper and lower surfaces until run off (about 5 mL/leaf). During EDU application, all the other leaves and the pot were protected with plastic wrap. To remove EDU eventually adsorbed on leaf surface (dry deposition), we allowed EDU solution over the leaf to evaporate (1 h), then the attached leaf was either not washed (treatment #1) or gently washed under running water on both sides for at least 3 min by tilting the pot so that the water did not go into the soil, and finally dried with paper (treatment #2). The latter treatment simulated a shower occurring after EDU application under ambient conditions. When EDU was applied as soil drench (treatment #3), 4-week-old plants were irrigated with either 0 or 300 ppm EDU solution (100 mL/plant). The solution that was not retained into the pot was removed from the tray at 24 h from EDU application and subsequently the plants were water irrigated every day.

2.3. Leaf sampling and EDU quantification

EDU concentration was measured in EDU-sprayed leaves over a period of 7 (treatment #2) or 21 days (treatment #1). The longest time point was set as 21 days, since that was the longest period used between two EDU applications, as reviewed in Paoletti et al. (2009). The first sampling was carried out when the solution over the EDU-sprayed leaf was completely evaporated (day 0, 1 h from EDU spraying) and after 1, 2, 3, 7, 10, 18 and 21 days from the application. When EDU was applied as soil drench (treatment #3), the third leaf was collected from the plants after 1, 2, 3, 7, 10, 18 and 21 days from EDU application. In order to remove the solution of EDU possibly deposited on the leaf surface before the analysis, the leaf sampled from treatment #1 was immersed in distilled water for 2 min under gentle stirring and then washed under running water for at least 3 min and dried with paper. The leaves from all treatments were frozen and finely pulverized in liquid nitrogen, dried in lyophilizator (Heto Power Dry LL3000 freeze dryer - Thermo Fisher Scientific, Somerset, USA) and stored at −80 °C until the analysis. Ten mg of lyophilized tissue, corresponding to approximately 100 mg of FW, was extracted with 1 ml of methanol under agitation for 30 min at room temperature. After centrifugation (20,000 × *g* for 5 min), 100 μL of supernatant was diluted 1:10 with methanol. Analytical determination of EDU was performed using an Agilent 6550 UHD Accurate-Mass Q-TOF LC/MS system equipped with a dual Jet-Stream source (Agilent Technologies, Palo Alto, CA) operating in positive ion mode. The chromatographic separation of EDU was achieved with a Supelco C8 2.7 μm 2.1 × 50 mm column (Sigma-Aldrich, St. Louis, USA) at 35 °C and a flow rate of 0.5 mL/min with a binary linear gradient of solvent A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid) from 0.5% B to 95% B in 3.5 min, and a total run time of 5 min. EDU was detected as $[M+H]^+$ and $[M+Na]^+$ adducts and quantified with five external calibrating solutions in the concentration range of 100–1000 ppb (R^2 = 0.99937). A detection limit of 10 ppb was obtained.

In order to investigate if EDU reacted with O_3-derived ROS, we set up an *in vitro* experiment in which EDU concentration was measured in aqueous solution containing 300 ppm EDU and different reactive oxygen species (ROS), namely 1 mM H_2O_2, O_2^- or $^{\bullet}OH$. The xantine (300 μM)/xanthine oxidase (0.075 U/mL) system and Fenton reaction (100 μM H_2O_2 100 μM $FeSO_4$ and 300 μM EDTA) were used to generate O_2^- and $^{\bullet}OH$, respectively (Haber and Weiss, 1934; Fridovich, 1970). EDU concentration was measured in control (without ROS) and in a ROS-generating system during 30 min of incubation. The experiment was replicated three times.

2.4. EDU metabolites calculation

In order to search for potential EDU metabolites, a mixed approach (*in silico–in vitro*) was used. In the first step, potential EDU metabolite structures were first generated *in silico* by MetaSite software. MetaSite is a computational procedure that predicts metabolic transformations related to phase I and phase II metabolism (Cruciani et al., 2013, 2014). The MetaSite algorithm considers both enzyme-substrate recognition, which is a thermodynamic factor, and the chemical transformations induced by the metabolic enzymes, which is a kinetic factor. Improved by experimental information from the Human CYP Consortium Initiative, a joint venture between pharmaceutical companies, MetaSite gives automatically back of fragments and metabolites generated in different biomatrices. The potential metabolites that