Journal of Plant Physiology 171 (2014) 1325–1333

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Physiology

Higher peroxidase activity, leaf nutrient contents and carbon isotope composition changes in *Arabidopsis thaliana* are related to rutin stress

M. Iftikhar Hussain[a,b,*], Manuel J. Reigosa[a]

[a] *Department of Plant Biology and Soil Science, University of Vigo, Campus Lagoas-Marcosende, 36310- Vigo, Spain*
[b] *International Center for Biosaline Agriculture (ICBA), P.O. Box 14660, Dubai, UAE*

ARTICLE INFO

Article history:
Received 13 October 2013
Received in revised form 12 May 2014
Accepted 13 May 2014
Available online 16 June 2014

Keywords:
Arabidopsis thaliana
Mode of action
Physiological responses
Rutin, secondary metabolite

SUMMARY

Rutin, a plant secondary metabolite that is used in cosmetics and food additive and has known medicinal properties, protects plants from UV-B radiation and diseases. Rutin has been suggested to have potential in weed management, but its mode of action at physiological level is unknown. Here, we report the biochemical, physiological and oxidative response of *Arabidopsis thaliana* to rutin at micromolar concentrations. It was found that fresh weight; leaf mineral contents (nitrogen, sodium, potassium, copper and aluminum) were decreased following 1 week exposure to rutin. *Arabidopsis* roots generate significant amounts of reactive oxygen species after rutin treatment, consequently increasing membrane lipid peroxidation, decreasing leaf Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} contents and losing root viability. Carbon isotope composition in *A. thaliana* leaves was less negative after rutin application than the control. Carbon isotope discrimination values were decreased following rutin treatment, with the highest reduction compared to the control at 750 μM rutin. Rutin also inhibited the ratio of CO_2 from leaf to air (*ci*/*ca*) at all concentrations. Total protein contents in *A. thaliana* leaves were decreased following rutin treatment. It was concluded carbon isotope discrimination coincided with protein degradation, increase lipid peroxidation and a decrease in *ci*/*ca* values may be the primary action site of rutin. The present results suggest that rutin possesses allelopathic potential and could be used as a candidate to develop environment friendly natural herbicide.

Introduction

Allelopathy is an emerging branch of applied science that studies any process primarily involving secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of biological and agricultural systems, including positive and negative effects (IAS, 1996). These low molecular weight compounds include mediators of communication with other plants or insects and substances with essential roles in defense against pathogens and herbivores (Gershenzon, 2002; Unsicker et al., 2009). The important secondary metabolites identified as allelochemicals are phenolics, alkaloids, flavonoids, terpenoids, momilactone, hydroxamic acids, brassinosteroids, jasmonates, salicylates, glucosinolates, carbohydrates and amino acids (Bhowmik and Inderjit, 2003; Reigosa et al., 1999; Macías et al., 2007). The actions of these compounds are concentration-dependent, as they inhibit the plant growth at high concentrations and promote at low concentrations (Einhellig, 1986; Reigosa et al., 1999). Due to their multifunctional activity, they are considered as natural pesticides against pathogens, microorganisms, fungi, insects and weeds (Soltoft et al., 2008; Macías, 1995; Macías et al., 1999; Duke et al., 2000, 2002; Dayan et al., 2009).

Allelochemicals are released into the surrounding environment and alter the plant growth and development because there are hundreds of different structures and many of the compounds have several phytotoxic effects (Einhellig, 2002). It has been suggested that membrane perturbations are a common starting point for allelochemical effects but the current evidence does not allow narrowing to a primary site of action for most of them (Einhellig, 2002). Allelochemicals have been found to influence a number of physiological and biochemical reactions like transpiration, leaf water relations, photosystem II photochemistry, nutrient uptake, ATP synthesis, cell cycle, phytohormone metabolism, reactive oxygen species generation (ROS), carbon isotope discrimination and gene expression (Bertin et al., 2003; Dayan et al., 2000, 2009; Batish et al., 2006; Blum, 2005; Sánchez-Moreiras et al., 2010; Hussain and Reigosa, 2011; Hussain et al., 2011a; Soares et al., 2011). Moreover, some natural compounds with higher

---

* Corresponding author. Tel.: +34 986812616; fax: +34 986812556.
E-mail addresses: mih76@uvigo.es, mih786@gmail.com (M.I. Hussain).

http://dx.doi.org/10.1016/j.jplph.2014.05.009
0176-1617/

phytotoxic activity can induce programmed cell death in some plant species. Such is the case with cinnamic acid (Ding et al., 2007), naphthoquinones (Babula et al., 2009), hydroquinone (Keller et al., 2008) and chalcone (Díaz-Tielas et al., 2012). However, due to the specificity of many natural compounds, effects of some of the allelochemicals are expected to be more specific. In this context, different molecular biology techniques like mass spectrometer analysis (Bathellier et al., 2008), microarray techniques (Golisz et al., 2011) and metabolomics (D'Abrosca et al., 2013) can be used to study the mode of action of plant secondary metabolites.

Flavonoids are an extensive group of secondary metabolites derived from phenylalanine and malonyl-CoA pathway (Winkel-Shirley, 2001) with an enormous variety of physiological functions in plants (Peer et al., 2001). Rutin, quercetin and kaempferol have been suggested as possible allelopathic compounds released by plant roots (Uren, 2001). One of the crops with allelopathic potential, the common buckwheat (*Fagopyrum esculentum* Moench, family Polygonaceae), is rich in flavonoids, with rutin as the main one in green parts (Oomah and Mazza, 1996), seeds, sprouts (Bonafaccia et al., 2003; Zielińska et al., 2007) and root exudates (Kalinova et al., 2007). Because of its established phytotoxicity, rutin has been suggested as one of the possible allelopathic compounds in buckwheat (Golisz et al., 2007). However, the source of these allelochemicals is not only from the root exudates but also from crop residues, which could play a potential role in weed control programs through applications such as mulch, green manure, or incorporation as plant pellets into the soil. Therefore, rutin may be responsible for the inhibition of growth and biochemical and physiological traits of surrounding plant species, but it was subject to further studies.

The few studies of its interactions with other plants have observed inhibition of seed germination and root growth of *Arabidopsis thaliana* (Hussain and Reigosa, 2014). The goal of the present work was to evaluate the mode of action of rutin on the growth and development of adult plants of *A. thaliana*. Respiration in Arabidopsis roots was studied to identify the effects of rutin on plant root metabolism. Spectroscopic determination of protein, minerals and peroxidation of lipids in Arabidopsis leaves were analyzed in order to elucidate the mechanism of action of this flavonoid on *Arabidopsis* cell biochemistry. Mass spectroscopic quantification of stable carbon and nitrogen isotopes were also analyzed because it is a useful tool to better understand rutin's biochemical and physiological impact on Arabidopsis.

Materials and methods

Plant material and growth condition

In the present study, *Arabidopsis thaliana* was selected because of its uniform germination, rapid growth and it represents an excellent model for the study of plant responses to allelochemicals and other environmental stresses (Baerson et al., 2005). Seeds of *A. thaliana* L. (Heyn.) ecotype Columbia (Col-0) were sterilized for 3 min in two consecutive aqueous solutions of EtOH (50%) and NaClO (0.5%), both with Triton X-100 (0.01%), washed in autoclaved water three times, vernalized for 48 h at 4 °C in 0.1% agar to favor synchronized germination, and transferred to Petri dishes containing agar with Murashige–Skoog nutrients (from Sigma–Aldrich) and sucrose at a concentrations of 1%. The Petri dishes were kept for 15 days under 60 μmol m^{-2} s^{-1} of light in a growth chamber at 22 ± 2 °C. The plantlets were transferred to individual pots (5 cm in diameter and 6 cm high) containing inert perlite moistened with 50% Hoagland nutrient solution and placed in a growth chamber having temperature of 22 ± 2 °C, a photocycle of 8 h of light (120 μmol m^{-2} s^{-1}) and 16 h of darkness, and a relative humidity of 55%. The plantlets were watered twice a week with 50% Hoagland nutrient solution during the first 2 weeks and then watered every other day until the age of 5 weeks (three more weeks in pots). At this stage, when plants had nine fully developed leaves (2 weeks in agar gel + and 3 weeks in perlite), the rutin treatments were imposed.

Rutin treatment and experimental design

Stock solution of rutin was prepared in a solvent based on the compound's solubility. Rutin (rutin hydrate) was dissolved in dimethyl sulfoxide (DMSO) and distilled water {(distilled water + tween 20) (1 L/0.1 mL)} was added equal to the volume of DMSO to prepare the stock solution. Distilled water + tween 20 was added to the stock solution to prepare different concentrations (500, 750, 1000 μM) of rutin. The procedure was repeated to prepare a control without rutin. The pH of all these chemical solutions, including control, was adjusted to 6.0 with KOH (Martin et al., 2002). After the 5th week of *A. thaliana* growth, each seedling was treated with 15 mL of rutin (500, 750, 1000 μM) or control (distilled water + DMSO + tween 20). The Hoagland solution (50%) was applied on alternate days during treatment period (day 0, 2, 4, 6). To better understand the effects of rutin and its interference with *A. thaliana* seedlings growth, the changes in fresh/dry biomass, elemental analysis, lipid peroxidation, carbon (δ^{13}C) and nitrogen isotope composition analysis and root oxidizability were measured. The experiment was arranged in Randomized Complete Block Design (RCBD) with four replications.

A. thaliana biomass determination and elemental analysis

At harvest, the C, H, and N contents of dried leaves (3 mg) were determined in a Fisons Instruments EA1108 apparatus with a detection limit of 10 ppm. The 50 mg dry leaves were used to measure Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+}, Fe^{2+}, Na^{+}, K^{+} and PO_4^{3-} contents by inductively coupled plasma optical emission spectrometry in a Perkin Elmer Optima 4300DV.

Carbon and nitrogen isotope composition analysis

Collected plant leaf samples were immediately dried in a forced-air oven at 70 °C (Gallenkamp oven, Loughborough, Leicestershire, UK) to constant weight and ground in Ball Mills (Retsch MM 2000, Haan, Germany). Dry ground plant material was weighed (1700–2100 μg) with a weighing meter (Metler Toledo GmbH: Greifensee Switzerland), filled in tin capsules (5 × 3.5 mm, Elemental Microanalysis Limited, UK). Each capsule was entered automatically in combustion oven at 1600–1800 °C in the presence of oxygen and subsequently isotope ratios were determined in an Isotopic Ratio Mass Spectrometer (Finnegan: Thermo Fisher Scientific, model MAT-253, Swerte Germany) coupled with an Elemental Analyzer (Flash EA-1112, Swerte Germany). The Isotopic Ratio Mass Spectrometer has an analytical precision better than 0.05‰ for ^{15}N and 0.3‰ for ^{13}C.

Carbon and nitrogen isotope compositions were calculated as;

$$\delta(\%) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000 \quad (1)$$

where R_{sample} is the ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$, and R_{standard} were the standards used. Atmospheric N_2 was the standard for nitrogen while Vienna PeeDee Belemnite (VPDB) was the standard for carbon. The accuracy and reproducibility of the measurements of δ^{13}C and δ^{15}N were checked with an internal reference material (NBS 18 and IAEA-C6 for C), and (IAEA-310A and IAEA-N1 for N), and acetanilide for C/N % ratios, respectively.