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Physiology

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

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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

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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 ($\delta^{13}C$) 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;

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

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 $\delta^{13}C$ and $\delta^{15}N$ 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.

Table 1
Effects of rutin (0, 500, 750, 1000 μM) on leaf fresh (g) and dry weight (g) of *A. thaliana*.

Rutin (μM concentration)	Fresh weight (g)	Dry weight (g)	Fresh/dry weight ratio
0 (Control)	0.403 ± 0.09	0.10 ± 0.00	4.15 ± 0.87
500	0.220 ± 0.01*	0.06 ± 0.01	5.42 ± 2.70
750	0.295 ± 0.05*	0.06 ± 0.01	7.13 ± 3.14
1000	0.263 ± 0.03*	0.03 ± 0.00*	11.25 ± 3.42

After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by Duncan test for homoscedastic data, and by Dunnett test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.
* Asterisk shows the statistical significance as compared to control at $p < 0.005$.

The carbon isotope discrimination ($\Delta^{13}C$) is a measure of the carbon isotopic composition in plant material relative to the value of the same ratio in the air on which plants feed:

$$\Delta(\%) = \frac{\delta a - \delta p}{1 + \delta p} \times 1000 \quad (2)$$

where Δ represents carbon isotope discrimination, δa represents C isotope composition in the source air, and δp represents C isotope composition in the plant leaf tissue. Theory published by Farquhar et al. (1989) and Farquhar and Richards (1984) indicates that carbon isotope discrimination in plant leaves can be expressed in relationship to CO_2 concentrations inside and outside of leaves in its simplest form as:

$$\Delta = a + \frac{(b-a)ci}{ca}$$
$$\Delta = 4.4 + \frac{(27-4.4)ci}{ca} \quad (3)$$

where a is discrimination that occurs during diffusion of CO_2 through the stomata (4.4‰), b is discrimination by Rubisco (27‰), and ci/ca is the ratio of the leaf intercellular CO_2 concentrations to that in the atmosphere. Eq. (3) shows a direct and linear relationship between Δ and ci/ca. Stable isotope composition measurements were performed in CACATI (Centro de Apoio Cientifico Tecnologico a la Investigacion), University of Vigo, Spain.

Root-respiration measurement

Root oxidizability is an indirect estimation of tissue viability and was determined using 2,3,5-triphenyl tetrazolium chloride (TTC). The viable (respiring tissue) reduces TTC to red color triphenyl formazan by accepting electrons from mitochondrial electron transport chain (Comas et al., 2000). Thus, any decrease in root oxidizability refers to reduced respiration resulting from tissue damage and thus reduced viability. Briefly, root tissues (50 mg) were treated with 5 mL of 0.4% TTC solution (w/v) and 5 mL of 1/15 M phosphate buffer (pH 7.4). The mixture was incubated at 40 °C for 3 h followed by the addition of 2 mL 2.0 N H_2SO_4. The roots were ground in 10 mL of reagent grade ethyl acetate to extract red triphenyl formazan that was recorded at 485 nm and expressed as $A_{485}\, g^{-1}\, h^{-1}$.

Lipid peroxidation measurement

At harvest lipid peroxidation was determined by malonyldialdehyde (MDA) content measurement according to the procedure described by Hodges et al. (1999). Briefly, pre-frozen plant material (120 mg) was homogenized in 80% ethanol and centrifuged at 3000 × *g* for 10 min at 4 °C, the supernatant was incubated at 95 °C with 20% TCA containing 0.01% hydroxytoluenebutylate, with and without 0.5% thiobarbituric acid (TBA). Absorbance was measured at 440, 532 and 600 nm. The lipid peroxidation was calculated as malonyldialdehyde (MDA) (nmol mL^{-1}) equivalents according to this equation;

$$\mathrm{MDA(nmol/mL)} = \left(A - \frac{B}{157,000}\right) \times 10^6$$

where $A = (Abs_{532} - Abs_{600})_{TBA+} - (Abs_{532} - Abs_{600})_{TBA-}$ and $B = 0.0571 \times (Abs_{440} - Abs_{600})_{TBA+}$.

Total protein content determination

Total protein contents were determined by Bradford's method as described by Pedrol and Ramos (2001). For each replicate, 100 mg of leaf material was homogenized in 0.8 mL of 0.05 M Tris buffer (pH 8.0) containing 0.05 g of the insoluble antioxidant polyvinyl polypyrrolidone, and the mixture was centrifuged at 2860 × *g* for 20 min. A 0.1 mL sample of the supernatant was mixed with Bradford's reagent and absorbance at 595 nm was measured and translated into protein content using a calibration line constructed with bovine serum albumin standards.

Statistical analysis

ANOVA was used to detect significant variation in sample means due to treatment effects. After Kolmogorov–Smirnov testing for non-normality and Levene'stesting for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by the Duncan test for homoscedastic data, and by the Dunnett test for heteroscedastic data to compare multiple means using SPSS 15.0 for Windows. Each value represents the mean (± S.E.) of four replicates.

Results

Fresh and dry weight of *A. thaliana* seedlings was measured and results are shown in Table 1. From the table it is clear that rutin significantly reduced leaf fresh biomass at all concentrations tested (500, 750, 1000 μM). However, the reduction in leaf fresh weight was not dose-dependent. Rutin reduced the leaf dry weight in *A. thaliana* at all concentrations while the maximum decrease was observed at 1000 μM rutin. The plants treated with 1000 μM

Table 2
Carbon (%) and nitrogen (%) and C/N ratio in leaves of *A. thaliana* following 1 week exposure to rutin at 0, 500, 750, 1000 μM concentration.

Rutin (μM concentration)	Carbon (%)	Nitrogen (%)	C/N ratio
0 (Control)	34.30 ± 0.12	7.52 ± 0.05	4.56 ± 0.02
500	35.14 ± 0.17*	7.03 ± 0.03	4.99 ± 0.04*
750	35.74 ± 0.10*	6.86 ± 0.01*	5.20 ± 0.02*
1000	35.82 ± 0.16*	6.48 ± 0.03*	5.52 ± 0.03*

After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by least significant difference tests for homoscedastic data, and by Tamhane's T2 test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.
* Asterisk shows the statistical significance as compared to control at $p < 0.005$.

Table 3
Leaf mineral contents, sodium, potassium, phosphate, hydrogen (mg g^{-1}), aluminum and copper (mg kg^{-1}) of *A. thaliana* following 1 week exposure to rutin at 0, 500, 750, 1000 μM concentration.

Rutin (μM concentration)	Sodium (mg g^{-1})	Potassium (mg g^{-1})	Phosphate (mg g^{-1})	Hydrogen (mg g^{-1})	Aluminum (mg kg^{-1})	Copper (mg kg^{-1})
0 (Control)	7758.00 ± 159.27	52,398 ± 1112.53	13,076.25 ± 541.79	5.20 ± 0.01	536.75 ± 74.93	16.58 ± 0.65
500	6616.75 ± 258.57*	50,056.5 ± 1805.76	11,688 ± 307.40	5.33 ± 0.01*	386.75 ± 29.76*	12.55 ± 0.76*
750	6779.75 ± 496.16*	51,879.75 ± 2858.22	12,249 ± 241.19	5.49 ± 0.09*	472.00 ± 30.80*	13.75 ± 0.69*
1000	6441.00 ± 126.04*	46,782.5 ± 896.37*	12,277 ± 195.58	5.35 ± 0.02*	315.75 ± 40.69*	15.55 ± 0.44*

After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by least significant difference tests for homoscedastic data, and by Tamhane's T2 test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.

* Asterisk shows the statistical significance as compared to control at $p < 0.005$.

rutin were also darker and narrower and had furled margins that are typical signs of water deficit. Arabidopsis exhibited differential behaviors in carbon and nitrogen contents following 1 week rutin exposure. There was a tendency of stimulation in leaf carbon % that increased following a corresponding increase in the rutin concentrations (Table 2). Rutin significantly decreased the nitrogen % in *A. thaliana* leaves at 750 and 1000 μM while the C/N ratios were increased at all concentrations (Table 2). Reduction in sodium content (Na^+) was observed in *A. thaliana* leaves at all rutin concentrations compared to the control (Table 3). The potassium contents in *A. thaliana* leaves were significantly decreased at higher rutin concentrations (1000 μM) as compared to control. There was a tendency of stimulation in hydrogen content while aluminum content decreased at all rutin concentrations and maximum reduction was observed after treatment at 1000 μM. The copper contents were significantly less in Arabidopsis leaves than the control at all rutin concentrations (Table 3).

The results presented in Fig. 1 reveal that there was significant decrease in Ca^{2+} contents in *A. thaliana* leaves following treatment with rutin (500, 1000 μM). The rutin reduced the Zn^{2+} contents in Arabidopsis at 500 μM as compared to the control. As regards Mg^{2+} contents there was significant reduction at 1000 μM rutin

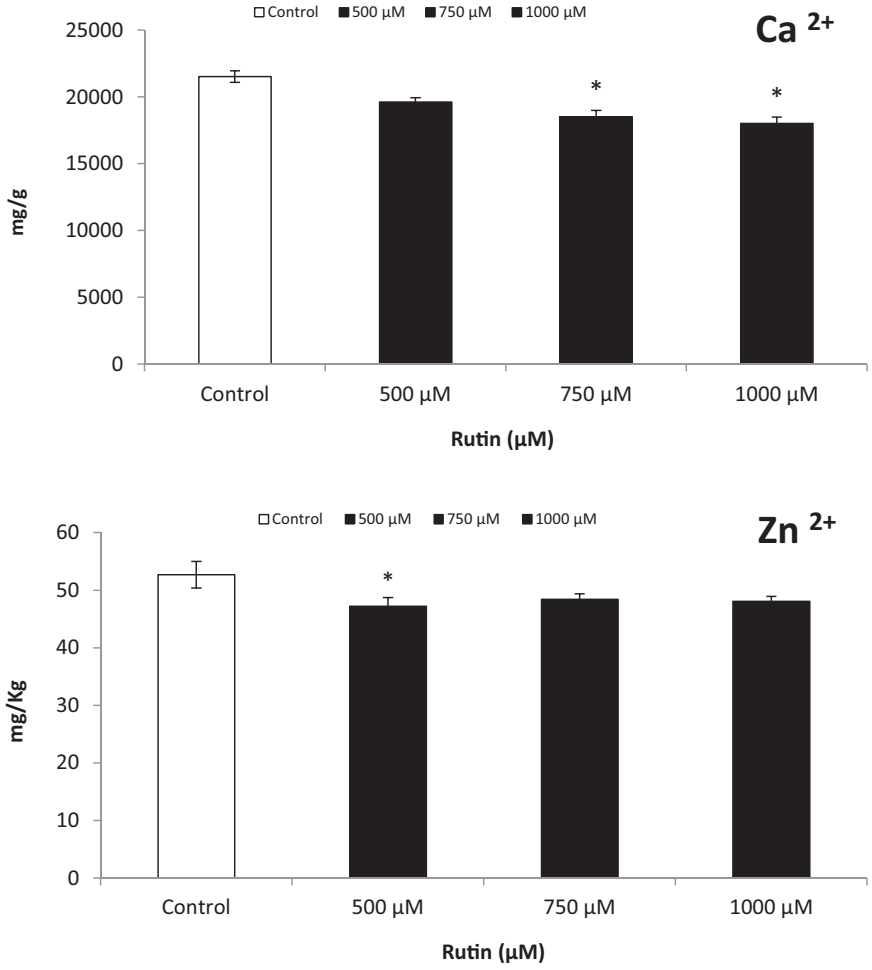

Fig. 1. Zinc and calcium contents (dry weight basis) of leaves of thale cress treated with different concentrations (500, 750, 1000 μM) rutin and in untreated control 1 week after treatment. *Asterisk shows the statistical significance as compared to control at $p < 0.05$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by Duncan test for homoscedastic data and by Dunnett test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.

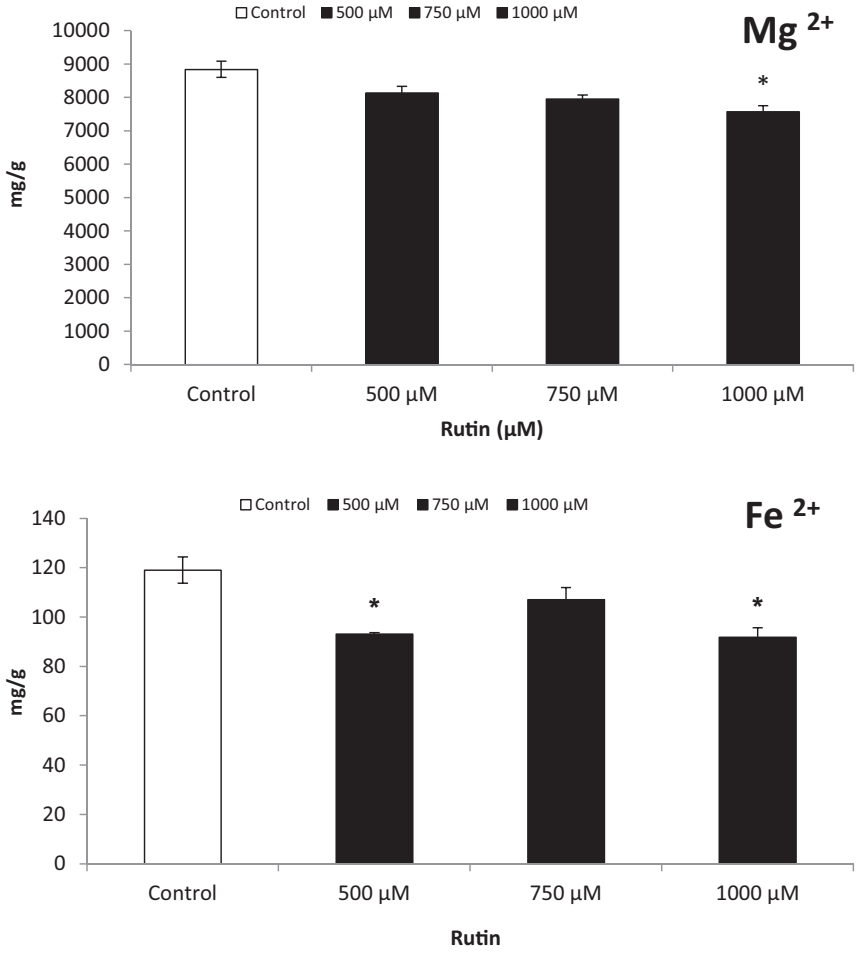

Fig. 2. Magnesium and iron contents (dry weight basis) of leaves of thale cress treated with different concentrations (500, 750, 1000 µM) of rutin and in untreated control 1 week after treatment. *Asterisk shows the statistical significance as compared to control at $p < 0.05$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by Duncan test for homoscedastic data and by Dunnett test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.

as compared to the control. The leaf Fe^{2+} contents were lowest at 500 and 1000 µM rutin concentrations as compared to the control (Fig. 2). Rutin treatments significantly affected N concentrations and nitrogen isotope composition ($\delta^{15}N$) (Fig. 3 and Table 2). Rutin reduced the $\delta^{15}N$ in *A. thaliana* leaves at all concentrations while the maximum reduction was observed at 1000 µM rutin. The lipid peroxidation values (measured as MDA contents) were higher following exposure to rutin at all concentrations compared to the control. Increase in the MDA contents was 25% more than the control at 1000 µM rutin treatment (Table 4). The root oxidizability

Table 4
Changes in lipid peroxidation (Malondialdehyde: nmol g^{-1} FW) and root oxidizability (TTC: mg g^{-1} FW) in leaves of *A. thaliana* following treatment with rutin (0, 500, 750, 1000 µM).

Rutin (µM)	Malondialdehyde (nmol g^{-1} FW)	TTC (mg g^{-1} FW)
0 (Control)	51,499.91 ± 6763.87	0.863 ± 0.019
500	58,749.94 ± 4234.07*	0.831 ± 0.013
750	62,499.93 ± 11,694.02*	0.858 ± 0.012
1000	71,499.9 ± 20,052.01*	0.853 ± 0.008

After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by least significant difference tests for homoscedastic data, and by Tamhane's T2 test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.

* Asterisk shows the statistical significance difference as compared to control at $p < 0.005$.

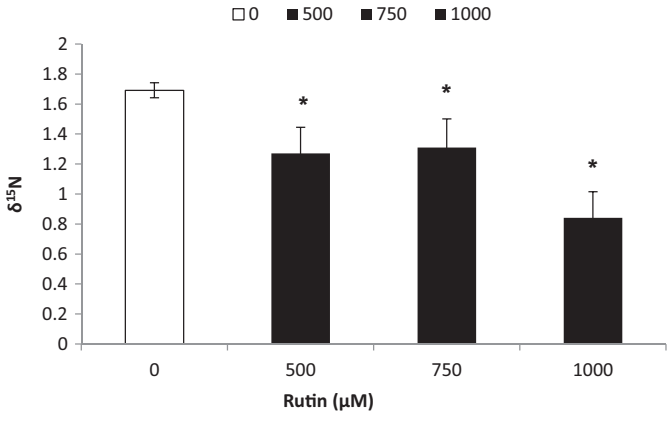

Fig. 3. Changes in nitrogen isotope composition ($\delta^{15}N$) in leaves of *A. thaliana* following exposure to 1 week of rutin at 500, 750 and 1000 µM concentrations and untreated 0 (Control). *Asterisk shows the statistical significance as compared to control at $p < 0.05$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by Duncan test for homoscedastic data and by Dunnett test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.

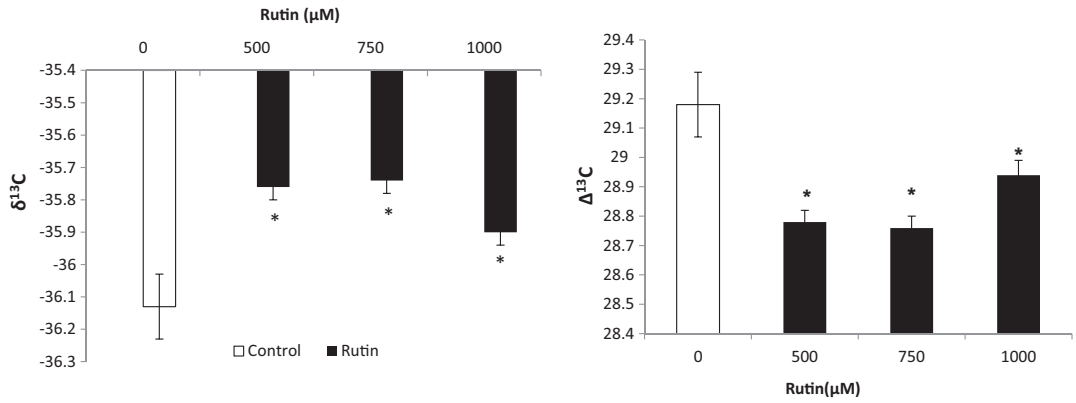

Fig. 4. Effect of different concentrations (500, 750, 1000 μM) of rutin on carbon isotope composition ($\delta^{13}C$) and carbon isotope discrimination ($\Delta^{13}C$) in leaves of *A. thaliana*. *Asterisk shows the statistical significance as compared to control at $p < 0.05$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by Duncan test for homoscedastic data and by Dunnett test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.

(RO) in Arabidopsis leaves were measured in terms of triphenyl tetrazolium chloride and rutin did not significantly affect the RO after 1 week treatment at all concentrations tested (Table 4).

Plant photosynthesis discriminates against the stable carbon isotope when atmospheric CO_2 passes through stomata during carboxylation in RuBisCO. In this study, the carbon isotope composition ($\delta^{13}C$) values in *A. thaliana* leaves were less negative (−35.76, −35.74 and −35.9) after treatment with 500, 750 and 1000 μM rutin respectively, as compared to the control (−36.13) (Fig. 4). The carbon isotope discrimination ($\Delta^{13}C$) values decreased following rutin treatment as compared to the control. The maximum reduction in $\Delta^{13}C$ values (28.76) was observed at 750 μM rutin than control (29.18) (Fig. 4). The $\Delta^{13}C$ values indicate that limiting the diffusion of CO_2 through the stomatal aperture can result in less negative $\delta^{13}C$ values. Rutin had a dominant effect on the ratio of CO_2 from leaf to air (ci/ca) at all concentrations while higher reduction in this ratio (1.078) was observed following treatment with rutin at 750 μM compared to the control (1.100) (Fig. 5). The leaf protein contents were significantly decreased in *A. thaliana* leaves after 1 week rutin treatment while higher reduction (59.71 and 61.43) was obtained following exposure to 500 and 1000 μM respectively, compared to the control (65.68) (Fig. 6).

Discussion

The excessive use of herbicides can lead to the development of environmental pollution and an increase in the number of herbicide-resistant weed biotypes (Duke et al., 2002; Batish et al., 2007). Therefore, economic and environmental constraints of crop production systems have stimulated interest in alternative weed management strategies using natural products (Macías et al., 2007; Dayan et al., 2009). Duke et al. (2000) discussed that natural compounds have several benefits over synthetic compounds, e.g. having novel structure due to diversity of molecules and are mostly water-soluble and non-halogenated molecules. In the present study, a natural compound, rutin significantly reduced the fresh biomass of *A. thaliana*. Hong et al. (2005), concluded that rutin treatment showed slight stimulation in the growth of mung bean at concentrations lower than 40 μg mL^{-1} and inhibition at concentrations higher than 80 μg mL^{-1}. Moreover, Duke et al. (2000) floated the idea that higher concentrations of extract act as an herbicide, disturbing plant processes like nutrient uptake, membrane permeability and photosynthesis inhibition, resulting in less plant biomass.

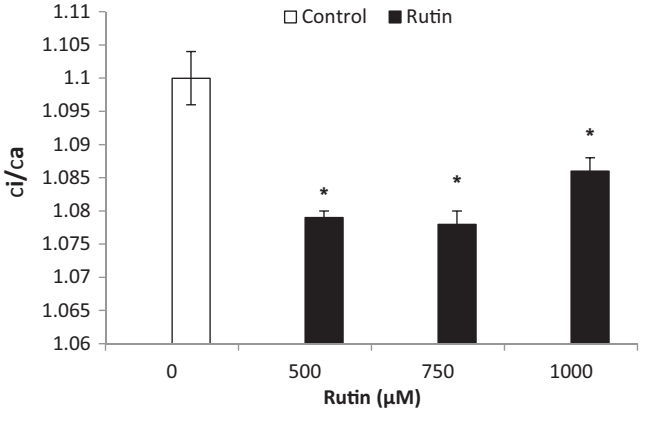

Fig. 5. Ratio of CO_2 concentrations from leaf to air (ci/ca) in *A. thaliana* following exposure to different concentrations of rutin (0, 500, 750, 1000 μM). *Asterisk shows the statistical significance as compared to control at $p < 0.05$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by Duncan test for homoscedastic data and by Dunnett test for heteroscedastic data. Each value represents the mean (±S.E.) of four replicates.

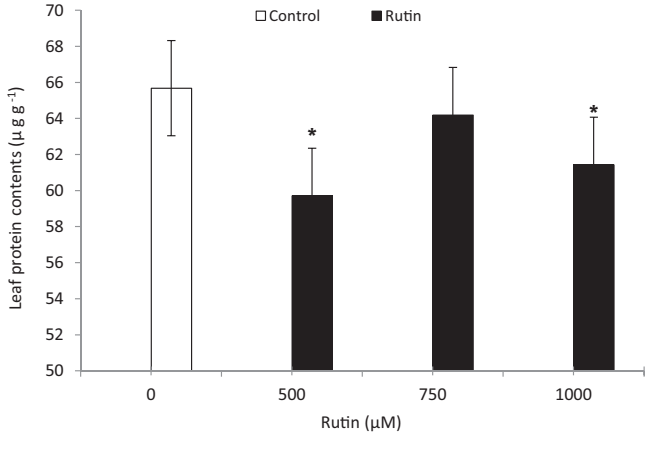

Fig. 6. Effect of rutin (0, 500, 750, 1000 μM) on leaf protein contents (μg g^{-1} dry weight) in *A. thaliana* measured 1 week after treatment. Every column in each bar represents the mean (±S.E.) of four replicates. *Asterisk shows the statistical significance as compared to control at $p < 0.05$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by Duncan test for homoscedastic data and by Dunnett test for heteroscedastic data.

Natural products have relatively short half-life and are therefore considered safe on environmental toxicology standpoint (Duke et al., 2002). Our results reported that rutin decreased the leaf nitrogen, calcium, magnesium, iron and zinc contents. Reduction in leaf N content is a first sign of leaf senescence, leading to reduced synthesis of RuBisco (ribulose-1,5-bisphosphate carboxylase oxygenase), that contributes 15–37% of leaf N content (Imai et al., 2008). While other allelochemicals also have shown to inhibit the uptake of phosphorus, K^+, (Glass, 1974; Harper and Balke, 1981), NO^{3-} and NH^{4+} (Bergmark et al., 1992), as well as the accumulation of macro- and micronutrients in the tissues (Baziramakenga et al., 1994). Similarly, cinnamic acid and ferulic acid were reported to reduce the availability of P, Fe and other nutrients. This reduction was attributed to the altered permeability of cell membranes (Franche et al., 2009). We report here that the Ca^{2+} and Zn^{2+} contents decreased following the rutin treatment. It is concluded that the calcium deficiency can reduce the root growth and induce early senescence (Hepler, 2005; Himelblau and Amasino, 2001) and Ca^{2+} may act as a messenger in the transduction of auxin signals, although its exact role is still controversial. On the other hand, Zn^{2+} is required for the synthesis of auxin from tryptophan and its deficiency can reduce auxin-mediated processes (Singh, 2005; Yang et al., 2005). Several researchers have reported that allelochemical contact with root cell membranes leads to depolarization, efflux of ions, reduction of hydraulic conductivity, and water and nutrient uptake (Baziramakenga et al., 1995; Lehman and Blum, 1999). Subsequent resulting changes in plant water relations and mineral nutrition lead to a cascade of secondary effects and it is difficult to separate the primary from the secondary effects (Duke et al., 2000; Sánchez-Moreiras et al., 2010; Ding et al., 2007; Dayan et al., 2009; Hussain et al., 2011b; Díaz-Tielas et al., 2012).

To date, the primary mechanisms of allelopathy have remained elusive. Several action modes have been suggested, including direct inhibition of PSII components and ion uptake, interruption of dark respiration, and ATP synthesis and ROS-mediated allelopathic mechanisms (Inderjit and Duke, 2003). Compared with interruption or inhibition of photosynthesis, allelochemical-induced peroxidation of root cell membranes by ROS is more likely, since the root is the first organ to be exposed to allelochemicals in the rhizosphere. In the present study, rutin induced an accumulation of ROS in Arabidopsis roots (Table 4). This ROS generation could be an important factor that regulates the occurrence of phytotoxicity in Arabidopsis roots. However, little information is available about the mechanisms by which allelochemicals induce ROS formation. Rutin treatment enhanced the MDA contents in Arabidopsis indicating that the accumulation of lipid peroxidation resulted in a significant decrease in cell membrane stability. It was reported that an increase in MDA content may reduce integrity of the membrane which can produce irreversible damage to the plant cell physiology (Halliwell and Gutteridge, 2007). Allelochemicals are reported to depolarize the cell membrane, inducing lipid peroxidation and causing a generalized cellular disruption that ultimately leads to cell death (Zeng et al., 2001). The leaf protein contents showed a remarkable reduction in Arabidopsis after 1 week rutin treatment. Similarly, Baziramakenga et al. (1997) reported that many phenolic acids reduced the incorporation of certain amino acid into proteins and thus reduced the rate of protein synthesis. The increase in lipid per oxidation was correlated with a decrease in protein content (particularly for 1000 μM rutin-treated plants). Early reduction in protein content (Fig. 6) becomes especially interesting because these are the first effects observed in senescent leaves and in particular of thylakoids membrane proteins which are responsible for the stability of the antenna complex (Liu et al., 2008). Change in protein levels is a well-established senescence marker, in particular the decrease of the ribosome's, the reduction of Rubisco, the breakdown of chlorophyll-binding proteins, or the proteolysis of photophosphorylation-related proteins during senescence that result in lower levels of proteins (Lim et al., 2007). The enhanced ROS production causes oxidative damage, leads to lipid peroxidation and damages macromolecules such as pigments, proteins, nucleic acids and lipids (Apel and Hirt, 2004; Blokhina et al., 2003).

Analysis of the natural abundance of stable C isotopes in plant dry matter provides useful information on the long-term water use efficiency of plants (Farquhar et al., 1989; Farquhar and Cernusak, 2005). Present results confirm that the stress effect on $\delta^{13}C$ was higher in Arabidopsis as demonstrated by a reduction in $\delta^{13}C$ values that was correlated with *ci*/*ca*. The $\delta^{13}C$ values indicate the limitation in the diffusion of CO_2 through stomatal apertures which results in less negative $\delta^{13}C$ values in the rutin treated plants compared to the control. Barkosky and Einhellig (2003) reported that $\delta^{13}C$ in soybean leaf tissue was significantly less negative after treatment with 0.75 mM *p*-hydroxybenzoic acid. In the present work, the ratio of CO_2 from leaf intercellular spaces to the atmosphere was reduced. This stomatal closure at least in part contributed to the declines in leaf fresh and dry biomass. The interference with the plant–water balance appears to be one of the mechanisms of action of phenolic compounds causing a reduction in plant growth (Barkosky and Einhellig, 2003). Moreover, Cernusak et al. (2008) reported that $\delta^{13}C$ data may be used to study plant WUE, respiration and secondary fractionation processes, and to partition net ecosystem CO_2 fluxes between photosynthesis and respiration. Nitrogen is the element that often limits plant growth and development in many terrestrial ecosystems. In this study, rutin significantly decreased the leaf nitrogen contents and $\delta^{15}N$ values. Anthropogenic activity has altered the amount and relative abundance of the forms of nitrogen (NH^{4+}, NO^{3-} and amino acids) that are available for plant absorption (Evans and Belnap, 1999). Robinson et al. (2000) conducted a study to integrate the stress responses (drought and nitrogen) of 30 genotypes of wild barley (*Hordeum spontaneum* C. Koch.). They concluded that, root $\delta^{15}N$ was correlated negatively with total dry weight, whereas shoot and whole-plant $\delta^{15}N$ were not correlated with dry weight. The difference in $\delta^{15}N$ between shoot and root varied with stress in all genotypes.

Conclusions

Rutin is a well known allelopathic compound from buckwheat but its phytotoxic potential on adult plants remains unclear. The present study demonstrates that rutin exerted several phytotoxic and inhibitory effects in Arabidopsis, including the decline in fresh biomass, leaf mineral content, protein content and increase in lipid peroxidation, and are reminiscent of its biochemical, physiological and cellular stress responses. It seems to be either inducing water deficiency stress (probably through action in the roots) or directly triggering anti-drought defenses. Reduction in carbon isotope discrimination coincided with the reduced CO_2 ratios (from leaf intercellular spaces to air), increased lipid peroxidation and reduced leaf protein contents that probably lead to the appearance of necrosis and early senescence in the Arabidopsis leaves. Our results confirm that the use of rutin caused early senescence induction in Arabidopsis that may be the primary site of action of rutin.

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