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# Highlighting the effects of coumarin on adult plants of *Arabidopsis thaliana* (L.) Heynh. by an integrated -omic approach

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ABSTRACT

In this study, the effects of the allelochemical coumarin through a metabolomic, proteomic and morpho-physiological approach in *Arabidopsis* adult plants (25 days old) were investigated. Metabolomic analysis evidenced an increment of amino acids and a high accumulation of soluble sugars, after 6 days of coumarin treatment. This effect was accompanied by a strong decrease on plant fresh and dry weights, as well as on total protein content. On the contrary, coumarin did not affect leaf number but caused a reduction in leaf area. An alteration of water status was confirmed by a reduction of relative water content and an increase in leaf osmotic potential. Moreover, coumarin impaired plant bio-membranes through an increase of lipid peroxidation and $H_2O_2$ content suggesting that coumarin treatment might induce oxidative stress. Coumarin reduced the effective quantum yield of the photosystem II, the energy dissipation in the form of heat, the maximum PSII efficiency, the coefficient of the photochemical quenching and the estimated electron transport rate, while it significantly stimulated the fluorescence emission and the coefficient of the non photochemical quenching. Finally, the proteomic characterization of coumarin-treated plants revealed a down-regulation of the ROS detoxifying proteins, responsible of oxidative damage and consequently of physiological cascade effects.



## 1. Introduction

Allelopathy is a complex phenomenon defined as "any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influences growth and development of biological systems" (International Allelopathy Society, IAS, 1996). Due to this complexity, analytical techniques and bioassays are basic tools to describe the allelopathic phenomenon. In recent years there is a growing interest in allelopathy-related studies also due to rapid improvements gained by the use of -omics methodologies such as transcriptomics, proteomics and metabolomics (D'Abrosca et al., 2013; Duke et al., 2013; Scognamiglio et al., 2015; Weston et al., 2015). The integrated and simultaneous use of these techniques could enhance the probability of success to understand the ecological, evolutionary and functional role of allelochemicals, involved in plant-plant interactions (Duke et al., 2013). Omic-based approaches allow, in allelopathy, the identification and quantification of primary and secondary metabolites present in complex samples from living organisms (Scognamiglio et al., 2015; Weston et al., 2015) and the determination of the complex biosynthetic pathways of selected compounds, which provide indications on their mode of action and on new target sites (Duke et al., 2013).

Coumarins constitute a large class of allelochemicals derived from the *o*-hydroxycinnamic acid lactonization. They are widely distributed in the plant kingdom and synthesized by almost all higher plants (Araniti et al., 2014; Bourgaud et al., 2006) playing an important ecological role in both plant protection and bio-communication (Razavi, 2011) and modulating dynamical processes of species coexistence in plant communities (Esposito et al., 2008). Coumarin (1,2 benzopyrone), the simplest compound of this class, is well known for its high phytotoxic potential. It inhibits root growth in barley (Hu, 1985), durum wheat and maize (Abenavoli

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et al., 2004; Abenavoli et al., 2001). In particular, Lupini et al. (2010) demonstrated that the root zone within 20 mm from tip of maize primary root is the most sensitive to coumarin, suggesting that this effect could be mediated by auxin. Recently, the same authors suggested a functional interaction between coumarin and auxin polar transport in *Arabidopsis thaliana*, and particularly with AUX1 carrier, which determined rootward auxin transport (Lupini et al., 2014). Furthermore, coumarin decreases respiration and photosynthesis in plants (Moreland and Novitzky, 1987; Kupidlowska et al., 1994); it is involved in cell differentiation, showing an auxin-like behaviour (Abenavoli et al., 2001); it affects nitrate uptake and its translocation to the shoot, as well as respiration in *Triticum durum* (cv. Simeto) seedlings (Abenavoli et al., 2001). Coumarin inhibits durum wheat germination reducing initially (I phase of germination) water uptake, electrolyte retention capacity and later the activities of selected marker enzymes for metabolic resumption (Abenavoli et al., 2006). Later, Pergo et al. (2008) suggested that coumarin acts as a cytostatic agent, retarding germination and growth of *B. pilosa*. All such physiological and biochemical changes were implied, directly or indirectly, in plant growth inhibition.

Despite the large evidences regarding the biological activity of this natural compound, no clues are given regarding its mode of action and the cascading effects produced on adult plants. In this respect, in the present study, a metabolomic and proteomic approach associated with morpho-physiological analysis were applied to investigate coumarin effects on *Arabidopsis thaliana* adult plants.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

*Arabidopsis thaliana* Col-0 seeds were sterilized for 3 min in 50% EtOH and 0.5% NaClO with Triton X-100 at 0.01% and washed for three times in sterilized water. After sterilization, seeds were suspended in 0.1% agar solution at 4 °C for 72 h to synchronize the germination (Araniti et al., 2015; Araniti et al., 2013). Seedlings were then sowed in a hydroponic system containing a half strength Hoagland solution and grown in a growth chamber with a PAR of 120 μmol $m^{-2}$ $s^{-1}$, a photoperiod 8/16 h light/darkness, at 22 ±2 °C and 70% relative humidity, for 25 days. Successively plants (25 days old) were treated for 3, 6 and 9 days with 100 μM coumarin which was renewed every other day. Based on our experience (Abenavoli et al., 2008), this concentration was adopted in the experiments as the more effective and interesting dose driving modification on plant growth and development. Plant materials were collected during the lighted period, exactly after six hours of light exposition.

### 2.2. Metabolomic analysis

#### 2.2.1. Extraction procedure for metabolomic analysis

After collection, the aerial parts of *Arabidopsis* were immediately freeze-dried and liophylized at −40 °C. Plant materials (50 mg) were then transferred to a 2 mL microtube. NMR samples were prepared in a mixture of phosphate buffer (Fluka Chemika; 90 mM; pH 6.0) in $D_2O$ (Sigma-Aldrich) containing 0.1% w/w trimethylsilylpropionic-2,2,3,3-$d_4$ acid sodium salt (TMSP, Sigma-Aldrich), used as internal standard, and methanol -$d_4$ (Sigma-Aldrich). A volume of 1.5 mL of phosphate buffer in $D_2O$ and methanol -$d_4$ (1:1) was added to the plant samples. The mixture was vortexed at room temperature for 1 min, ultrasonicated (Elma® Transonic Digitals) for 40 min, and centrifuged (Beckman Allegra™ 64R) at 13,000 rpm for 10 min. An aliquot of 0.6 mL was transferred to a NMR tube and analyzed by NMR (Kim et al., 2010).

#### 2.2.2. NMR experiments

NMR spectra were recorded at 25 °C on a 300.03 MHz for $^1H$ and 75.45 MHz for $^{13}C$ on a Varian Mercury Plus 300 Fourier transform NMR. $CD_3OD$ was used as the internal lock.

Each $^1H$ NMR spectrum consisted of 256 scans with the following parameters: 0.16 Hz/point, acquisition time (AQ) = 1.0 s, relaxation delay (RD) = 1.5 s, 90° pulse width (PW) = 13.8 μs. A presaturation sequence was used to suppress the residual $H_2O$ signal. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline-corrected and calibrated to TMSP at 0.0 ppm.

$^1H$-$^1H$ correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra were recorded. COSY spectra were acquired with a 1.0 s relaxation delay and 2514 Hz spectral width in both dimensions. The window function for COSY spectra was sinebell (SSB = 0). HSQC and HMBC spectra were obtained with a 1.0 s relaxation delay and 3140 Hz spectral width in f2 and 18116 Hz in f1. Qsine (SSB = 2.0) was used for the window function of the HMBC. The optimized coupling constants were 140 Hz for HSQC and 8 Hz for HMBC. FIDs were Fourier transformed and the resulting spectra were manually phased and baseline-corrected and calibrated to TMSP at 0.0 ppm, using $^1H$ NMR processor (ACDLABS 12.0).

To quantitate metabolites, specific signals were manually integrated and scaled to the internal standard signal (TMSP). The amount of each metabolite was calculated and expressed as percentage from control.

### 2.3. Morpho-physiological analysis

Based on metabolomic results, all the morpho-physiological analysis, excepting photosynthetic parameters, were conducted after six days of coumarin-treatment. Indeed, three days of treatment were not enough to appreciate changes in metabolites content, whereas nine days severely damaged *Arabidopsis* plants.

#### 2.3.1. Dry weight (DW), fresh weight (FW) and DW/FW ratio

Four complete rosettes treated for 6 days were collected, weighed (FW) and oven-dried at 70 °C for 72 h. Successively, samples were newly weighed and DW and DW/FW ratio were evaluated.

#### 2.3.2. Leaf number, leaf area and leaf relative water content (RWC)

Leaves from four complete rosettes treated for 6 days were counted. Successively, leaves, at the same development stage, were collected, photographed and the leaf area was measured using the software Image Pro Plus v.6.0 (Media Cybernetics Inc., Bethesda, MD, USA).

Leaf relative water content (RWC) estimation was carried out as previously described by Araniti et al. (2017a). Leaf discs, cut from leaves at the same developmental stage from four different plants treated for 6 days, were incubated in 100 mL of distilled water for 24 h (Araniti et al., 2017a). Successively, the turgid weight of leaf samples was taken. The leaf samples were then oven-dried at 70 °C for 72 h. Dry weight was recorded and the raw data were elaborated through the following equation:

$$RWC(\%) = [(FW - DW)/(TurgidWeight - DW)] * 100$$

#### 2.3.3. Leaf osmotic potential [Ψ (π)], leaf membrane stability index [MSI (%)]

Leaf Ψπ was measured on four leaves treated for 6 days as previously reported by Araniti et al. (2017a). Once harvested, leaves were inserted into an empty syringe and frozen at −20 °C. After 24 h,