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Changes in the metabolome of lettuce leaves due to exposure to mancozeb pesticide

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ABSTRACT

This paper describes a proton high resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) metabolomic study of lettuce (*Lactuca sativa* L.) leaves to characterise metabolic adaptations during leaf growth and exposure to mancozeb. Metabolite variations were identified through multivariate analysis and checked through spectral integration. Lettuce growth was accompanied by activation of energetic metabolism, preferential glucose use and changes in amino acids, phospholipids, ascorbate, nucleotides and nicotinate/nicotinamide. Phenylalanine and polyphenolic variations suggested higher oxidative stress at later growth stages. Exposure to mancozeb induced changes in amino acids, fumarate and malate, suggesting Krebs cycle up-regulation. In tandem disturbances in sugar, phospholipid, nucleotide and nicotinate/nicotinamide metabolism were noted. Additional changes in phenylalanine, dehydroascorbate, tartrate and formate were consistent with a higher demand for anti-oxidant defence mechanisms. Overall, lettuce exposure to mancozeb was shown to have a significant impact on plant metabolism, with mature leaves tending to be more extensively affected than younger leaves.

1. Introduction

Mancozeb is an ethylenebisdithiocarbamate salt widely used as a dithiocarbamate fungicide to protect fruit, nut and field crops from a range of fungal diseases (Paro et al., 2012). Its toxicity to humans and to the environment is known to be mainly based on its primary metabolite, ethylenethiourea (ETU), which forms in the presence of water and oxygen and is highly mobile in soils due to its enhanced water solubility (Easton, Guven, & de Pomerai, 2001). Mancozeb toxicity relates to the broader issue of agricultural soil contamination (involving not only pesticides but also fertilizers and industrial contaminants), raising the question of how plant metabolism is disturbed (particularly in plants readily consumed as large scale foods i.e. fruits and vegetables) and how it impacts food compositional characteristics and nutritional quality. In this context, metabolic effects have, for instance, been investigated for tomatoes exposed to the insecticide thiamethoxan (Karmakar, Bhattacharya, & Kulshrestha, 2009) and cadmium (long-term) (Hédiji et al., 2010), and for mandarin oranges exposed to fertilisers and pesticides (Zhang et al., 2012). However, to our knowledge, little is known about the effects of mancozeb on plant metabolism, thus justifying the present paper on the metabolic effects caused by lettuce exposure to this pesticide.

The study of the impact of external perturbations on living systems' metabolism has increasingly entailed the use of metabolomics strategies, recognised to hold significant potential in crop and food quality and safety issues (Mannina, Sobolev, & Viel, 2012; Shepherd, Fraser, & Stewart, 2011). Usually based on nuclear magnetic resonance (NMR) and/or mass spectrometry (MS) analytical methodologies, in tandem with multivariate statistical analysis of the data, metabolomics has been increasingly used for fruit and plant metabolic studies tackling several food quality issues: transgenic modification (Kausch et al., 2012; Picone et al., 2011; Sobolev, Testone et al., 2010), geographical, environmental and genotype effects (Bernillon et al., 2013; Kim et al., 2013; Son et al., 2009; Spraul et al., 2009; Zhang, Breksa, Mishchuk, & Slupsky, 2011), resistance to insect attacks (Capitani et al., 2012) or drought (Silvente, Sobolev, & Lara, 2012), effect of biofortification agents (Blasco, Leyva, Romero, & Ruiz, 2013). In the context of environmental contaminants, metabolomics have been employed in tomato (Hédiji et al., 2010; Karmakar et al., 2009) and mandarin orange (Zhang et al., 2012) exposure studies, and in the study of lettuce exposed to arsenic in irrigation water (Beni et al., 2011). Additional metabolomics studies on lettuce have included the

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effects of transgenic modification (Sobolev, Testone et al., 2010) and iodine as a fortification agent (Blasco et al., 2013), in tandem with metabolome characterisation of lettuce extracts by NMR (Sobolev, Brosio, Gianferri, & Segre, 2005) and MS based methods (Abu-Reidah, Contreras, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2013).

This paper describes, for the first time to our knowledge, an NMR metabolomics study of lettuce exposed to the fungicide mancozeb, considering two different leaf growth stages in the plant: young and fully expanded leaves. Plant growth is known to lead to significant metabolomic changes and such information must thus be considered when evaluating the impact of exposure to the pesticides. The metabolite profiles of leaves exposed and non-exposed to mancozeb were analysed directly by high field (800 MHz) ^{1}H high resolution and magic angle spinning (HRMAS) NMR spectroscopy, building on the existing spectral assignment of whole lettuce tissue (Beni et al., 2011). Multivariate analysis and signal integration were employed to enable the identification of metabolite changes related to plant growth and to the concomitant effects of mancozeb exposure.

2. Materials and methods

2.1. Samples

Seeds of *Lactuca sativa* L. (var. Queen of May), from Viveiros Litoral, Portugal, were germinated in water and transferred to plastic pots containing a turf and vermiculite (2:1) mixture. Cultures were maintained in a growth chamber at 20 ± 2 °C and exposed to a 16/8-h (day/light) photoperiod, using an average photosynthetic photon flux density of *ca.* 200 ± 20 μmol m^{-2} s^{-1}. Six 4-week old lettuces were sprayed with mancozeb pesticide (PESTANAL®, 2 mg/l, analytical standard, acquired from Sigma, USA) and a second set of six lettuces was used as a control. Seven days after pesticide exposure, the first bottom (expanded) leaves and first top (young) leaves were collected for both controls and exposed plants, snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Prior to analysis, samples were ground to a powder in a mortar (whilst frozen) and pooled for each 3 plants, to obtain *ca.* 500 mg/sample and partially average inter-plant variability (hence, control and exposed groups comprised 2 pools of young leaves and 2 pools of expanded leaves). Each pool was split into 2 aliquots (to account for experimental variability, potentially due to the constraints of rotor packing with a suspension rather than a solution), thus giving a total of 16 samples for analysis. Of each sample, 225 ± 20 mg were mixed with 250 μl of phosphate buffer (1 M, pH 6.50 in D_2O) containing 1 mM 3-(trimethylsilyl) propionate sodium salt (TSP)-d_4 for chemical shift referencing. 50 μl of each thawed sample were introduced into a spacer-containing 4 mm magic angle spinning (MAS) rotor.

2.2. NMR spectroscopy

NMR spectra were recorded on a Bruker Avance spectrometer, operating at 800 MHz for ^{1}H, at 277 K, using a 4 mm HRMAS probe, in which the rotor containing the sample was spun at the magic angle at 4 kHz. ^{1}H HRMAS spectra were acquired using the *noesypr1d* pulse sequence (Bruker pulse programme library) with water presaturation. A total of 256 transients were collected into 32,768 k (32 k) data points, with a spectral width of 9803 Hz, acquisition time of 1.67 s and relaxation delay of 4 s. Each free induction decay (FID) was zero-filled to 64 k points and multiplied by a 0.3 Hz exponential line-broadening function prior to Fourier transform (FT). All spectra were manually phased, baseline corrected and chemical shifts referenced internally to the TSP signal at δ 0.00. 2D homonuclear and heteronuclear spectra were registered for selected samples to aid spectral assignment. The total correlation (TOCSY) spectra were acquired using the pulse sequence *dipsi2phpr*, in the phase sensitive using States-TPPI mode, with water presaturation during relaxation delay. 4096 data points with 80 transients per increment and 280 increments were collected using a spectral width of 9804 Hz in both dimensions, a mixing time of 70 ms and a relaxation delay of 1.5 s. The ^{1}H–^{13}C phase sensitive (echo/antiecho) heteronuclear single quantum correlation (HSQC) spectra were recorded using the pulse programme *hsqcetgpsi*. 2048 data points with 80 scans per increment and 300 increments were acquired with spectral width of 11,161 Hz and 36,227 Hz in ^{1}H and ^{13}C dimensions, respectively. A relaxation delay of 1.5 s was used between pulses and a refocusing delay equal to 1/4 J_{C-H} (1.72 ms) was employed. For both TOCSY and HSQC spectra, zero filing to 1024 data points and forward linear prediction were used in f_1 and multiplication by a shifted sine-bell-squared apodization function was applied in both dimensions prior to FT and phasing. All peak assignments were carried out with basis on 2D NMR experiments and consultation of the Bruker Biorefcode spectral database, HMDB database (Wishart et al., 2013) and literature (Beni et al., 2011; Pérez, Iglesias, Ortiz, Pérez, & Galera, 2010; Sobolev et al., 2005).

2.3. Multivariate analysis

^{1}H NMR spectra were normalised to total spectral area and principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were applied using SIMCA-P 11.5 (Umetrics, Umeå, Sweden). Centered data and different spectral scaling methods were tested (unit variance and Pareto scaling), Pareto scaling having been selected for the final analysis. Multivariate analysis was applied to the whole 1D spectra (δ 0.4–9.23, water region δ 4.80–5.20 excluded) and to the δ 0.4–2.85 and δ 5.30–9.23 subregions (results not shown). PLS-DA loadings were back-transformed by multiplying all values by the square root of their standard deviation and the relevant peaks were integrated and normalised to total spectral area, using AMIX 3.9.5 (BrukerBioSpin, Rheinstetten, Germany). Integral variations were subjected to the student *t* or Wilcoxon tests (statistical relevance for $p < 0.05$). All statistical tests and boxplots were carried out using R-statistical software (R Core Team, 2012) (version 2.15.2), this having been also used, along with the Plotrix package (Lemon, 2006), to produce PLS-DA loadings plots colour-coded as a function of variable importance to the projection (VIP). In spite of the exploratory nature of PLS-DA results due to limited sample numbers, model validation was carried out by Monte Carlo cross-validation (MCCV) (7 blocks, 500 runs), with recovery of Q^2 values and confusion matrices of true and permuted classes and calculation of classification rates (CR), specificity and sensitivity.

3. Results

In terms of spectral reproducibility, aliquot spectra were found to be entirely superimposable, with biological variability (i.e. between different sample pools) resulting in negligible changes in the resonances arising from malate, succinate, fumarate and sucrose. Visual spectral comparison was, therefore, carried out based on the average spectra ($n = 4$ per condition) (Fig. 1). Fig. 1a and b shows the average ^{1}H HRMAS spectra obtained for control (non-exposed) young and expanded lettuce leaves and the arrows in Fig. 1b indicate several changes noted. Such changes comprise a prominent increase in malate (resonances 10), along with profile changes in the sugar and aromatic regions, including the loss of resonances 27 (assigned to polyphenolic species). Fig. 1c and d