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A targeted metabolomics approach toward understanding metabolic variations in rice under pesticide stress

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ABSTRACT

Diazinon insecticide is widely applied throughout rice (*Oryza sativa* L.) fields in Iran. However, concerns are now being raised about its potential adverse impacts on rice fields. In this study, a time-course metabolic change in rice plants was investigated after diazinon treatment using gas chromatography–mass spectrometry (GC–MS), and subsequently the statistical strategy of random forest (RF) was performed in order to find the stress-associated effects. According to the results, a wide range of metabolites were dynamically varied as a result of the plant response to diazinon such as biosynthesis and metabolism of sugars, amino acids, organic acids, and phenylpropanoids, all correlating with the exposure time. Plant response was involved in multiple metabolic pathways, most of which were correlated with the exposure time. In this study, RF was explored as a potential multivariate method for GC–MS analysis of metabolomics data of rice (*O. sativa* L.) plants under diazinon stress; more than 31 metabolites were quantitatively determined, and time-course metabolic response of the plant during different days after treatment was measured. Results demonstrated RF as a potential multivariate method for GC–MS analysis of changes in plant metabolome under insecticide stress.

Rice is considered as the most important food for populations of developing countries and is the main dish to half of the world's population [1]. It is an annual grass of the Gramineae family belonging to the *Oryza* genus [2]. *Oryza sativa* L. is grown all over the world and is cultivated in humid and temperate environments, making its production susceptible to fungi, insects, and mites. More than 70 insect species have been recorded as rice pests, so this would be considered as one of the major constraints on crop yields causing serious reduction in plant production or even blockage of it. To address this problem, several kinds of insecticides, fungicides, and herbicides are used in order to protect crops against pest damage. The application of pesticides in rice fields has become a popular approach to controlling pest damage during early periods of rice cultivation in Asia [3]. Diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl thiophosphate; Fig. 1) is an organophosphorus pesticide that was commercially introduced in 1952 [4]. Thanks to its inhibitory effects on acetyl cholinesterase enzyme in a vast majority of insects, diazinon is universally used in agricultural sectors for plant protection against a variety of sucking and leaf-eating insects [5]. Nevertheless, several reports have demonstrated that diazinon is immunotoxic [6], cytotoxic [7], and genotoxic [8]; hence, it exhibits toxic properties and potential risk to human health. Because rice is one of the most popular crops worldwide, it seems essential to probe the influence of diazinon on metabolite profiling of rice. To this end, metabolomics is one of the most powerful tools for providing an overview of metabolite changes under various abiotic stresses, namely pesticide stress [9].

So far, no report regarding the influence of diazinon on metabolite profiling of rice has been recorded. Therefore, the current study was undertaken to investigate the effect of diazinon on rice metabolite profiling under subtropical climatic conditions.

Recently, random forest (RF)[1] has been recognized as a powerful means for classification, especially in the fields of metabolomics [10]. High throughputs of analytical data acquired from mass spectrometry and chromatographic techniques have been extensively employed for classification using RF. RF analysis was developed as

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[1] *Abbreviations used:* RF, random forest; GC–MS, gas chromatography–mass spectrometry; MSTFA, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide; TMCS, trimethylchlorosilan; AMDIS, Automated Mass Spectral Deconvolution and Identification System; S/N, signal/noise; OOB, out-of-bag; ROC, receiver operating characteristic; AUC, area under the ROC curve; IS, internal standard; OP, organophosphorus; AChE, acetylcholinesterase enzyme; ROS, reactive oxygen species; TCA, tricarboxylic acid.

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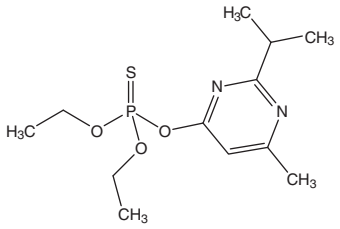

Fig.1. *O,O*-Diethyl *O*-2-isopropyl-6-methyl-pyrimidin-4-yl phosphorothioate (IUPAC).

an ensemble classification and regression approach by Breiman [11]. It is an ensemble classifier method that is an extension of the decision tree concept based on classification and regression tree. RF combines many trees to form a forest for analysis [12].

In this study, we have explored RF as a potential multivariate method for gas chromatography–mass spectrometry (GC–MS) analysis of metabolomics data of rice (*O. sativa* L.) plants under diazinon stress. More than 31 metabolites of sugars, amino acids, and organic acids were quantitatively determined, and time-course metabolic responses of the plant during different post-treatment days were investigated and measured.

Materials and methods

Materials

Seeds of Shiroodi variety (*O. sativa* ssp. *indica*) were obtained from the Rice Research Institute of Iran and were subsequently cultivated in the same open field to avoid the influence of growing location. Diazinon was employed to prevent the plant from insect damage, whereas its applied concentration was based on the recommended permitted dosage from the Plant Protection Organization. Plants were subjected to 10% granular formulation of diazinon (15 kg hectare^{-1}) during heading and flowering time. Untreated plants were planted under the same experimental conditions. Rice leaves were taken from control and treated plants at 24, 48, 72, 96, and 120 h after treatment. Seven replicates at each time point were collected from different plants and immediately chilled in liquid nitrogen. Frozen leaves were manually ground in a mortar using liquid nitrogen in order to keep samples at cryogenic temperature, and eventually all samples were stored at −80 °C until metabolite analysis [13].

Chemicals

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared by a Milli-Q system (Millipore, Billerica, MA, USA). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), methoxyamine hydrochloride, sorbitol, trimethylchlorosilan (TMCS), and pyridine were obtained from Sigma–Aldrich (Steinheim, Germany).

Sample preparation

The extraction procedure and derivatization of the metabolites were carried out using a modified method described by Roessner and coworkers [14]. Approximately 50 mg of powdered rice leaves was homogenized in 1400 µl of 100% methanol (4 °C), and 50 µl of internal standard (2 mg sorbitol ml^{-1} water) was added. The mixture was then vortexed for 15 min at 70 °C. The extract was vigorously mixed with 1400 µl of water (4 °C) and subsequently centrifuged for 10 min at 2200*g*. The polar metabolite fraction containing aliquots of the methanol/water (1000 µl) was transferred to an Eppendorf tube and dried in vacuum for 6 to 16 h. A combination of oxidation and silylation reactions was conducted as the derivatization procedure. First, dried residue was dissolved and derivatized via adding 80 µl of methoxyamine (20 mg ml^{-1} in pyridine) solution and incubated in a 30 °C water bath for 90 min. Then, 80 µl of MSTFA and 0.8 µl of TMCS (1% MSTFA) were added for trimethylsilylation (37 °C for 30 min).

GC–MS analysis

A Varian ion trap MS 4000 and electron impact ionization detection was coupled with a Varian cp-3800 gas chromatograph. MS transfer line and ion trap temperatures were set at 270 and 200 °C, respectively. Here, 1 µl of derivatized sample was injected into the gas chromatograph, and metabolites were separated on a DB-5 MS column (30 m × 0.25 mm × 0.25 µm). The injection temperature was set at 250 °C, and the ion source temperature was adjusted to 200 °C. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. Analysis was performed under the following temperature program: 5 min of isothermal heating at 70 °C, followed by a 5 °C min^{-1} ramp for oven temperature to 310 °C and a final 10-min heating at 310 °C. The ion trap was operated in full scan mode at a mass range of 50 to 650 *m/z*. Both chromatograms and mass spectra were evaluated using the GC–MS Postrun Analysis (Varian), Samples were injected randomized into GC/MS.

Data processing

Mean-centering was applied in order to remove the overall offset. Moreover, auto-scaling avoids the possibility that a few high-intensity variables will dominate the final solution [15]. All acquired raw data were subjected to mean-centering and normalization before multivariate analysis by the following equations:

$$N_i = \frac{A_i}{A(\text{sorbitol})} \text{ then } R_i = \frac{N_i}{\text{mean}(n)},$$

where N_i, A_i, A(sorbitol), R_i, and mean(n) stand for normalized area, peak area, area of sorbitol as internal standard, mean-centering data, and the average of replicates.

RF was performed using the random forest package [16]; peak identification was achieved in AMDIS (Automated Mass Spectral Deconvolution and Identification System) software via searching the mass spectra against the standard mass spectral library (NIST and Wiley).

Results and discussion

The aim of the current study was to classify and follow the treated group and the control group, which would detect possible main variations in metabolite profiling of rice under diazinon stress, based on the data acquired from mass spectroscopy as well as retention times of the peaks obtained from MS workstation software. Peak identification was performed by integrating peaks at signal/noise (S/N) = 10 by the use of AMDIS.

Due to the observation of various intensities in GC–MS data, especially for following metabolites with a wide range of concentrations, it would be necessary to calibrate and normalize the peaks. Thus, GC–MS data set related to the treatment of rice with diazinon was split into training and test sets using random division. To well illustrate the performance of the proposed approach for two metabolomics datasets (treated and control), our models should be validated by predicting the classes of the test set that was not used in training set. The model based on random selection was constructed on the training set, which contained approximately two-thirds of the samples (7 replicates × 5 time points = 35