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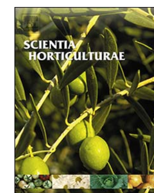

Metabolomics analysis of developing *Garcinia mangostana* seed reveals modulated levels of sugars, organic acids and phenylpropanoid compounds

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ABSTRACT

Mangosteen seeds are categorized as recalcitrant as they are sensitive to desiccation and low temperature. The seeds also do not go through the final developmental phase of maturation drying, hence, they are actively metabolic when they are shed. As such, metabolomics analysis could be useful to further understand mangosteen seed development. In this study, we used liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry approaches to analyze the profiles of polar compounds of mangosteen seeds harvested at four different stages of development (i.e. six, eight, 10 and 12 weeks after anthesis) and in mature seeds at 14 weeks. Principal component analysis (PCA) suggested temporal regulation of these metabolites during mangosteen seed development. Sugar levels such as glucose and fructose gradually increased throughout development, suggesting active metabolism until the mature stage. Organic acids such as citrate and malate as well as glycerol transiently increased during the seed growth phase which may imply their active energy and storage reserve production, respectively. High level of growth-related metabolites such as γ-aminobutyric acid found at discrete developmental stages infer their roles in the seed development. Furthermore, accumulation of secondary metabolites was active between 10 and 12 weeks after anthesis particularly metabolites related to phenylpropanoid pathway. The elevated levels of gambiriin A1, rutin, chlorogenic acid, 3-*O*-feruloylquinic acid, thymol-β-D-glucopyranoside and guaicol-β-D-glucopyranoside may enhance seed protection and defense. In conclusion, mangosteen seed displays recalcitrant seed characteristics, with metabolism geared to be germination-ready in terms of resources and seed defense.

1. Introduction

Mangosteen (*Garcinia mangostana* L.) is an evergreen tropical tree species and belongs to the Clusiaceae family. While it is widely known for its delectable fruit, mangosteen is also seen to have promising pharmaceutical properties. For instance, xanthones and anthocyanin found in fruit pericarp exhibit strong antioxidant and anti-inflammatory activities (Fang, 2015). Due to its high prospective value in commercial and medicine, there are many mangosteen-related studies on planting and mass propagation (Downton et al., 1990; de Carvalho, 2014). Even so, seed preservation and hence large plantation of mangosteen are not feasible. This is due to the recalcitrant properties of mangosteen seeds that do not tolerate desiccation and cold temperature, thereby preventing their long-term storage (Normah et al., 1997; Normah et al., 2016). At shedding the seed has a moisture content of above 50% (Normah et al., 1997; Normah et al., 2011; Normah et al., 2016) and dies when moisture content falls below 30% (Normah et al., 1997). Recalcitrant seeds are metabolically active when they are shed, as they do not go through the maturation-drying phase during their development (Berjak and Pammenter, 2013).

According to Yapwattanaphun et al. (2014), there is no embryo in the mangosteen seed during early development. In the young developing ovule, the endosperm is liquid and seed is derived from the outgrowth of integument cells (Yapwattanaphun et al., 2014). Seed coat (or testa) develops from the outer integuments embedded with tannin and it can go up to 0.25 mm in thickness (Lim, 1984; Normah et al., 2016). A fully ripened mangosteen fruit may contain only approximately two well-developed seeds (Osman and Milan, 2006; Te-chato, 2007). Mangosteen seed has a relatively high amount of starch compared to other *Garcinia* species (Normah et al., 2016). The seed oil content is between 21 to 28% of dry weight (Ajayi et al., 2007) whereas protein, lignin and tannin are found in trace quantity (Normah et al., 2016).

Metabolites such as sugars, amino acids and flavonoids are known

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to be involved in seed development in other plant species such as Arabidopsis, soybean and tomato (Fait et al., 2006; Mounet et al., 2007; Collakova et al., 2013; Doughty et al., 2014; Lastdrager et al., 2014). Using metabolomics analysis, the profile of those metabolites can be obtained for better understanding of their roles in the metabolism of seed development. Thus, the aim of this study was to describe changes in metabolite profiles of polar compounds of various classes such as sugars, phenolics and organic acids during mangosteen seed development using liquid chromatography-mass spectrometry (LC–MS) and gas chromatography-mass spectrometry (GC–MS). The information obtained would give insights to the metabolism during the development of this recalcitrant mangosteen seed.

2. Materials and methods

2.1. Fruit sampling

Mangosteen fruit were obtained from Universiti Kebangsaan Malaysia (UKM) mangosteen plots (Bangi, Selangor, Malaysia) (GPS coordinate: 2.922662°E, 101.786690°N). The trees were grown in tropical climate (24–32 °C) with high humidity and sunny all year round. The age of the tree is approximately 15–20 years. Each fruit was first tagged at anthesis and its development was monitored. Seeds were pooled from at least six biological replicates (independent fruit) at six (W6), eight (W8), ten (W10), twelve (W12) and fourteen (W14) weeks after anthesis and stored at −80 °C until required. The integument cells of the mangosteen seed ovule starts to grow a protuberance at W6 (Yapwattanaphun et al., 2014). It continues to grow and form a cell mass two weeks later at W8 (Yapwattanaphun et al., 2014; Yonemori et al., 2014). Mature seeds at W14 were determined by their ability to germinate, seed size and mass based on Normah et al. (2016) and Osman and Milan (2006).

2.2. Metabolite extraction

Pooled whole seeds were finely ground and three replicates (0.1 g fresh weight each) were used for metabolite extraction of each developmental stage. Samples were homogenized in 1400 µL of 100% methanol and 50 µL internal standard (2 mg ribitol/mL) and incubated for 15 min at 70 °C. The extract was mixed vigorously with 1 vol of water and centrifuged at 2200× *g*. The supernatant was transferred into a sterile 1.5 mL Eppendorf tube and vacuum dried for four to six hours to eliminate the solvent. The dried extract was then stored at −80 °C until required for LC–MS and GC–MS analyses.

2.3. LC–MS protocol

LC–MS analysis was carried out using Dionex liquid chromatography platform (Ultimate 3000 UHPLC+, Thermo Scientific) coupled with a MicroTOF-QIII (Bruker) MS system. The LC parameters were as follows; 1.0 µL injection volume, column temperature was set at 60 °C and 0.3 mL/min flow rate. The LC mobile phase consisted of (A) 0.1% formic acid in water and mobile phase B (100% acetonitrile). The elution was carried out for 35 min using the following gradient: 0–5% B in 2 min, 5–40% B in 2 min and 40–95% B in 16 min. The mixture was then held at 95% B for 2 min before increasing to 100% B in 0.1 min. The mixture was held at 100% B for 4 min and was decreased to 5% B in 2 min. The column was then reconditioned with initial gradient for seven minutes.

Mass spectrometry (MS) analysis was performed using MicroTOF-Q hybrid quadrupole time of flight mass spectrometer (Bruker Daltonics), equipped with an electrospray ion source (ESI) with the following settings; nebulizer pressure: 1.2 bar, drying gas: 8 L/min at 200 °C, capillary voltage: 4500 V, end plate off set: −500 V, funnel 1 RF: 200 Vpp, funnel 2 RF: 200 Vpp. The samples were analysed from three replicates in positive ionization mode. The range of scan spectra was set from 50 to 1000 *m/z* range. All spectra were recorded in centroid mode.

2.4. Metabolite identification for LC–MS

Raw data obtained from the LC–MS were analyzed using ProfileAnalysis (Bruker) by bucketing and binning prior to statistics and multivariate analysis (MVA). Bucketing was carried out using the Find Molecular Features (FMF) and time alignment function to create compounds and mass lists for each compound. The MS spectra type was put as line spectra and the bucketing option was employed by normalizing to the sum intensity of obtained Rt-*m/z* pairs. The data were subjected to MVA, then transformed into log value and scaled using pareto to highlight relevant variations in the metabolite profiles. The *m/z* values were selected for identification if they were detected in all three replicates for at least two stages of seed development. For metabolite identification, searches using the chosen *m/z* values were scrutinized using three interrelated methods. The first method was a search in the following online databases Metlin (https://metlin.scripps.edu), Massbank (http://www.massbank.jp) and Metfrag (http://msbi.ipb-halle.de/MetFrag/). The second method used SmartFormula (from ProfileAnalysis) to determine the new *m/z* values for further search using the three previously stated databases. The last method was using fragment search which determines the putative metabolite based on the new *m/z* values. Then, the data was subjected to statistical analysis. Calculation of relative intensity, reI (in percentage, %) was done by using the formula below (1). Finalized identified metabolites were then tabulated and graphed.

$$\text{Relative intensity, reI} = \frac{\text{Intensity}}{\text{Total intensity in all metabolite across all stages}} \times 100 \quad (1)$$

2.5. GC–MS protocol

Dried supernatant was dissolved in 80 µL of 20 mg/mL methoxyamine hydrochloride and derivatized for 90 min at 30 °C in pyridine. Then 80 µL N-Methyl-N-(trimethylsilyl)-trifluoracetamide (MSTFA) was added to the mixture and incubated for 30 min at 37 °C. GC–MS and data analysis were performed as described by Chen et al. (2014) with modification.

The derivatized sample (1.0 µL) was injected into GC-MS instrument (Agilent, USA) with 25:1 split ratio. The analytical platform consisted of an autosampler, a GC Agilent 5975 gas chromatograph and a single quadrupole mass spectrometer (Agilent, USA). The mass spectrometer was tuned to manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43) while AB-5MS 30 m column with 0.25 mm inner diameter and 0.25 µm film thickness (Abel Industries, Canada) was used to perform the chromatography separation. Setting for injection temperature was 230 °C, the interface 250 °C and the ion source 200 °C. Helium was used as a carrier gas, set with constant flow rate of 1.0 mL/min. Additionally the temperature was set for 5 min isothermal heating at 70 °C, followed by a 5 °C/min oven temperature ramp up to 310 °C and a final 1.0 min heating at 310 °C.

2.6. GC–MS metabolite determination

Raw data obtained from GC–MS were analyzed manually, tabulating data for compound name, retention time and peak areas. National Institute of Standards and Technology (NIST 2008) mass spectral library was used for compound identification at a match score of 700 and above. Compounds were trimmed based on the presence in all three replicates for at least two stages of seed development. The data were further analyzed and subjected to normalization using the internal standard ribitol, log transformation as well as statistical analysis and MVA. Following that, compounds that were found to be redundant in the data were further trimmed. The semi-quantitative concentration of

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