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Metabolite profiling of mangosteen seed germination highlights metabolic changes related to carbon utilization and seed protection

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ABSTRACT

Mangosteen seed is categorized as recalcitrant, becoming inviable if exposed to desiccation and low temperature. However, the molecular mechanism of mangosteen seed germination has not been fully understood. This study profiled the metabolites that were present in germinating mangosteen seeds at different stages (zero, one, three, five, seven and nine days after sowing) using liquid chromatography-mass spectrometry (LC–MS) and gas chromatography-mass spectrometry (GC–MS). A total of 38 tentative metabolites were profiled and classified into amino acid, organic acid, sugar, polyol, alkaloid and others. Metabolites identified implied a timed regulation for germination. Sugars as well as amino acids exhibited a declining trend throughout the germination period as they are likely to be utilized for development and growth of seedling. Furthermore, secondary metabolites such as alkaloids, flavonoids, and xanthone presence displayed an increasing trend at the middle of germination period which may provide protection for mangosteen seed against herbivory and stress. In brief, carbon utilization and seed protection associated with the modulation of primary and secondary metabolites, respectively are important metabolic signatures of mangosteen seed germination.

1. Introduction

Mangosteen (*Garcinia mangostana* L.), a tropical fruit widely known for its sweet unique taste can be found in Southeast Asian countries such as Malaysia, Philippines and Thailand (Lim, 1984; Palapol et al., 2009). Additionally, mangosteen is well known in traditional medicine as some part of it, either from trees or fruit, has been used to treat wounds, diarrhea, dysentery, skin infections and more (Lu et al., 1998; Nakatani et al., 2002; Pedraza-Chaverri et al., 2008; Obolskiy et al., 2009). Mangosteen extracts have also been shown to possess various medicinal properties such as antifungal (Obolskiy et al., 2009), antioxidant (Yoshikawa et al., 1994), antibacterial (Iikubo et al., 2002), anti-HIV (Chen et al., 1996; Vlietinck et al., 1998) and anticancer agents (Matsumoto et al., 2003; Moongkarndi et al., 2004; Han et al., 2009; Shan et al., 2011). These health benefits are mainly contributed by xanthones, a class of polyphenolic compounds present in only a few plant species including mangosteen (Jinsart et al., 1992; Jung et al., 2006; Obolskiy et al., 2009). The two major xanthones that can be found in mangosteen include α-mangostin and γ-mangostin (Obolskiy et al., 2009).

Despite its medicinal potential, mangosteen tree growth is slow and only matures in about five to seven years before it starts bearing fruit (Osman and Milan, 2006). Mangosteen is mainly propagated through seeds which are categorized as recalcitrant. These seeds are sensitive to desiccation and low temperature (Normah et al., 1997) as they maintain active metabolism when shed (Berjak and Pammenter, 2013). Also, at later stages of development, mangosteen seed has an elevated level of soluble sugars and secondary metabolites (Mazlan et al., 2018). The seed will not be able to germinate if its moisture content drops to 30% or lower (Normah et al., 1997, 2016). It can only be stored for a short period before becoming inviable for germination (Normah et al., 1997), making it difficult for a long-term seed preservation and maintaining planting materials throughout the year. Mangosteen seed shows *Garcinia*-type germination where radicle protrusion is followed by plumule emergence on the opposite side (Lim, 1984; Normah et al., 2016).

Studies on mangosteen seed are still limited particularly during the seed germination. Seed germination is dependent upon multitude of endogenous and exogenous factors such as temperature and pH level (Kulkarni et al., 2014) and is strongly supported by various types of reserve compounds synthesized and stored during seed development such as carbohydrates and proteins (Pritchard et al., 2002; Galili et al., 2014). Germination are mainly divided into three phases – imbibition

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(Phase 1), lag interval (Phase 2) and radicle protrusion (Phase 3) (Nonogaki et al., 2010; Rajjou et al., 2012). Rapid water uptake by seeds occurs during Phase 1 followed by Phase 2 which is characterised by an active seed metabolism. Lastly, seeds germinate at Phase 3 with radicle protrusion. Seed germination has been investigated in many plant species such as in Arabidopsis (Rajjou et al., 2006), barley (Yang et al., 2011), tea (Chen et al., 2011), maize (Huang et al., 2012), rice (Kim et al., 2008) and soybean (Cheng et al., 2010). For instance, it was revealed that key compounds such as sugars and amino acids were temporally regulated during Arabidopsis seed germination (Fait et al., 2006). Still, investigation on recalcitrant seeds such as mangosteen are relatively scarce and hence demands further analysis.

Previous studies by Normah et al. (2016) and Lim (1984) have highlighted the physical properties and morphology of germinating mangosteen seed in effort to understand its biology. However, metabolic changes during the germination of this recalcitrant seed have not been reported. Hence, the aim of this study is to analyze mangosteen seed germination using metabolomics approach via liquid chromatography-mass spectrometry (LC–MS) and gas chromatography-mass spectrometry (GC–MS). Information gained would provide better comprehension on the metabolism of recalcitrant mangosteen seed germination.

2. Materials and methods

2.1. Seed sample preparation

Mature mangosteen fruit were obtained from trees at mangosteen plots in Universiti Kebangsaan Malaysia (UKM), Selangor, Malaysia (GPS coordinate: 2.922662 °E, 101.786690 °N). Two batches of sample were made; one for metabolomics analysis and the other for representative pictures. Aril was removed from seeds to avoid fungal infection during germination. Clean seeds were planted in autoclaved sand and were grown in a greenhouse (24–26 °C) with daily watering. Mangosteen seed takes nine days of sowing before both radicle and plumule emerge (Osman and Milan, 2006; Normah et al., 2016). Hence, samples were taken every two days at one (D1), three (D3), five (D5), seven (D7) and nine (D9) day(s) after sowing with day zero (D0) as control. At each timepoint, three germinating seeds, acted as biological replicates, were cleaned, blotted dry and flash frozen in liquid nitrogen prior storage at −80 °C.

2.2. Metabolite extraction

Seeds were ground to fine powder and 0.1 g from each replicate was homogenized in 1.4 mL of methanol (Merck, Germany) and ribitol (50 µL, 2 mg mL^{-1}) as an internal standard. For 15 min at 70 °C, the mixtures were incubated for extraction. Extracts obtained were added with one volume of water, mixed briskly and subsequently centrifuged at 2200 × *g*. Supernatant acquired was vacuum-dried for four to six hours to eliminate the extraction solvent and stored in −80 °C until used.

2.3. Liquid chromatography-mass spectrometry (LC–MS) protocol

Methanol were used to resolubilised dried samples. This study follows Glauser et al. (2013) method with modifications (Mamat et al., 2018; Mazlan et al., 2018). In extract compound separation, liquid chromatography system (Ultimate 3000 UHPLC+, Thermo Scientific, USA) equipped with a C18 column was utilized. LC parameters were set for injection volume (1.0 µL), column temperature (60 °C) and flow rate (0.3 mL min^{-1}). The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Elution was performed with a 35-min gradient starting with increase from 0 to 5% B in the first 2 min, then further to 40% in the following 2 min and up to 95% B within the next 16 min. At 95% B, the mixture was held for 2 min before 0.1 min increase to 100% B. At 100% B, the mixture was held for 4 min prior drop to 5% B in 2 min. Finally, column reconditioning was performed for 7 min with the initial gradient.

Mass spectrometry analysis was using a MicroTOF-QIII (Bruker, Germany) system with electrospray ionization (ESI) source in a positive mode of ionization. The drying gas (nitrogen) was set at 45 psi with flow at 8 L min^{-1} and gas temperature of 200 °C. The voltage of ESI spray and the fragmentor were fixed at 4.5 kV and 200 V respectively. Range of the scan was between 50 and 1000 Da. Subsequently, the data were logged in a centroid mode.

2.4. LC–MS metabolite identification

The raw data for LC–MS were first analyzed using Compass Data Analysis (Bruker, Germany) to detect peaks and deconvolute the total ion current chromatogram. This was to generate the list of peaks for retention time to mass per charge ratio (Rt-*m/z*) that corresponds to putative compounds detected and intensity data sets. The data were then binned and tabulated. MetaboAnalyst (http://www.metaboanalyst.ca/) for data normalization (by sum) and transformation, scaling and statistical analysis. Rt-*m/z* values that were detected in all three biological replicates, in at least three stages of germination were selected for putative identification. The *m/z* values were compared with three online metabolite databases namely Massbank (http://www.massbank.jp), Metlin (https://metlin.scripps.edu) and Metfrag (http://msbi.ipb-halle.de/MetFrag/). For each *m/z* values, top three compounds that are found in plants were selected and narrowed down to one, based on thorough literature review. Calculation of relative intensity (in percentage, %) was done by using the Formula (1). Finalized identified metabolites were then tabulated and graphed.

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

2.5. Gas chromatography-mass spectrometry (GC–MS) protocol

The dried supernatant was dissolved and derivatized in 80 µL of methoxyamine hydrochloride (20 mg mL^{-1}) in pyridine at 30 °C for 90 min. Then, it was incubated for 30 min with temperature of 37 °C after the addition of 80 µL of *N*-Methyl-*N*-(trimethylsilyl)-trifluoracetamide (MSTFA). Both chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). GC–MS and its analysis was done as per Chen et al. (2014) with revision.

For each replicate, 1.0 µL of derivatized sample was injected into GC–MS with a split ratio of 25:1. The GC–MS system consisted of a GC Agilent 5975 gas chromatograph (Agilent, China), an autosampler and a single quadruple mass spectrometer (Agilent, USA). Using tris-(perfluorobutyl)-amine (CF43), adjustment to the mass spectrometer was made according to recommendations by the manufacturer. The AB-5MS 30 m column used has 0.25 mm for both inner diameter and film thickness (Abel Industries, Canada). The parameters were set for injection temperature (230 °C), interface (250 °C) and ion source (200 °C). Helium, the carrier gas used was set to flow at the rate of 1 mL min^{-1}. The temperature setup started with isothermal heating (70 °C, 5 min) followed by a 5 °C min^{-1} rising of oven temperature (up to 310 °C) and a final heating (310 °C, 1 min).

2.6. GC–MS metabolite identification

Metabolites were identified using NIST11 library. Then, metabolites with match score of below 700 were discarded. Compounds that were found to be redundant in the data were further trimmed. The metabolites that were detected in all three replicates for at least three stages were selected as the final list of metabolites. The data were imported into MetaboAnalyst where they were subjected to normalization using