Received Date : 21-Jul-2015

Revised Date : 17-Aug-2015

Accepted Date : 21-Sep-2015

Article type : Research Paper

Editor : D.Grantz

Metabolite Profiling Reveals Temperature Effects on the VOCs and Flavonoids of Different Plant Populations

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Short title: Temperature Effects on the VOCs and Flavonoids

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/plb.12403

Abbreviations: Analysis of variance (ANOVA), Fourier Transform Infrared (FTIR), GC with flame ionisation detector (GC-FID), gas chromatography-mass spectrometry (GC-MS), liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS), principal component analysis (PCA), partial least square discriminate analysis (PLS-DA), polydimethylsiloxane (PDMS), solid phase microextraction (SPME), volatile organic compounds (VOCs)

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Abstract

Temperature is one of the key factors in limiting the distribution of plants and controlling major metabolic processes. A series of simulated reciprocal transplant experiments were performed to investigate the effect of temperature on plant chemical composition. *Polygonum minus* from different lowland and highland origins were grown under controlled environment with different temperature regimes to study the effects on secondary metabolites. We applied gas chromatography-mass spectrometry and liquid chromatography time-of-flight mass spectrometry to identify the chemical compounds. A total of 37 volatile organic compounds and 85 flavonoids were detected, with the greatest response observed in the compositional changes of aldehydes and terpenes in highland plants under higher temperature treatment. Significantly less anthocyanidin compounds and greater amounts of flavonols were detected under higher temperature treatment. We also studied the natural variation in the different plant populations growing under the same environment and identified compounds unique to each population through metabolite fingerprinting. This study shows that the origin of different plant populations influences the effects of temperature on chemical composition.

Keywords: Aldehydes; flavonoids; GC-MS; LC-TOF-MS; metabolite fingerprinting; *Polygonum minus*; terpenes; volatile organic compounds

Introduction

Plant growth is greatly influenced by temperature. Under unfavourable temperature conditions, plants will adapt to the environmental stress by regulating physiological processes (Zobayed et al., 2005). For instance, temperature changes can induce cellular responses and lead to alterations in plant metabolism, which leads to the composition changes in plant metabolites (Chaves et al., 2011). This is often to counteract the oxidative stresses resulting from the formation of free radicals due to the overproduction of reactive oxygen species (ROS) (Mittler, 2002). ROS, such as superoxide radicals ($O2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) can cause damages to DNA, proteins, lipids and carbohydrates, which further exacerbate oxidative stress (Gill and Tuteja, 2010).

Plants produce many secondary metabolites with prominent functions in protection against abiotic stresses, which have also been utilised as natural products for human benefits (Edris, 2007). There are three major groups of secondary metabolites: phenolics, terpenes, and nitrogen and sulphur-containing compounds (Mazid et al., 2011). Generally, plants produce diverse secondary metabolites in response to the environment. Environmental metabolomics is an emerging field of comprehensive study on plant metabolism to answer environmental or ecological questions in relation to plant physiology (Brunetti et al., 2013; Viant and Sommer, 2013). This approach was taken to study biochemical variation between co-occurring genotypes of *Carex caryophyllea* (Field and Lake, 2011). However, comparative study of different plant populations using this approach is still lacking. In this study, we apply the metabolomics approach to investigate the effects of temperature on the

chemical composition of different plant populations growing under the same environment. We focus on volatile organic compounds (VOCs) and flavonoids due to their prevalent roles in plant responses to environment (Loreto et al., 2014; Mierziak et al., 2014).

Volatile organic compounds (VOCs) emitted by plants (plant volatiles) have important functions in plant communications, direct and indirect defences, as well as protection against abiotic stresses (Loreto and Schnitzler, 2010). Examples of VOCs include isoprene, terpenes, alkanes, alkenes, alcohols, esters, carbonyls and acids. Isoprene and monoterpenes are the most abundantly emitted VOCs and have been implied to confer protection against high temperatures possibly linked to photorespiration (Peñuelas and Llusià, 2003). Isoprene fumigation increased leaf thermotolerance by 3-10°C in a concentration-dependent manner in *Pueraria lobate* and *Quercus alba*, but not in non-isoprene-emitting *Phaseolus vulgaris* (Singsaas et al., 1997). The thermotolerance of monoterpene-emitting oak, *Quercus ilex* (Loreto et al., 1998) and non-isoprenoid-emitting *Q. suber* (Delfine et al., 2000) increased after leaves were fumigated with some (e.g., pinene and β-ocimene) but not all monoterpenes. Due to implication as atmospheric greenhouse gases, most studies focus on the emission of VOCs and regulatory factors (Peñuelas and Llusià, 2003; Peñuelas and Staudt, 2010), rather than composition of VOCs within the plant tissues. There is no study investigating the interpopulational variation and influence of temperature on plant composition of VOCs.

Flavonoids belong to a family of C6-C3-C6 polyphenol compounds and the major flavonoids subclasses include flavonol, flavones, flavanone, flavanol, anthocyanins and isoflavone. Flavonoids have been shown to be important for plants to adapt to various

environmental conditions (Lovdal et al., 2010). Several studies on flavonoids production at different temperatures have been reported. In *Hypericum brasiliense*, there was an increase in the levels of total soluble phenols and of all the phenolic compounds analysed in plants at low temperature (de Abreu and Mazzafera, 2005). Furthermore, low temperature treatment resulted in higher total phenolic compounds content than high temperature treatment in *Rehmannia glutinosa* under temperature stress, based on the quantification of 16 phenolics compounds (Chung et al., 2006). Nevertheless, study on the flavonoid composition of different plant populations is still scarce.

The genus *Polygonum* comes from a widespread and diverse Polygonaceae family, which are more commonly found in northern temperate regions than in the tropics (Burkill, 1966). Several studies have been conducted to study the phytochemical composition of *P. minus*. Flavonoids were found to be the most common compounds in *Polygonum* species (Datta et al., 2000; Lopez et al., 2006). Essential oil study using GC-MS, GCxGC-MS-TOF and GC-FID successfully detected about 42 compounds mainly comprised of aldehydes and terpenes (Baharum et al., 2010). Biological activity assays on the essential oils extracted from different tissues of *P. minus* showed potent antioxidant and antimicrobial activity against MRSA (Ahmad et al., 2014).

Previously, we applied Fourier Transform-Infrared (FTIR) analysis to study the effects of temperature on *P. minus* at the level of metabolite functional groups (Khairudin et al., 2014). However, FTIR is unable to discern specific metabolites which were influenced by temperature. In this study, we examined in details the chemical compositional changes due to the effects of temperature. Plant extracts were screened and profiled using hyphenated GC-

MS and LC-TOF-MS. These techniques were used as they provide high sensitivity of compounds detection (Zhou et al., 2009). The data obtained were analysed using principal component analysis (PCA) and partial least square discriminate analysis (PLS-DA) to identify compounds which contribute significantly to the differences observed in different plant populations under different temperature treatments. The results from this study show the interpopulational variations in response to different temperatures and demonstrate that higher temperature has a greater effect on the volatile compounds than flavonoids.

Materials and methods

Plant materials and sample preparation

Stem cuttings of *P. minus* were collected from three highland populations (altitude >1000 m): Cameron Highland (CH; 4° 29' 15.75" N, 101° 22' 11.02" E), Genting Highland (GH; 3° 25' 42.18" N, 101° 47' 21.45" E), Fraser Hill (FH; 3° 43' 05.24" N 101° 43' 57.13" E), and lowland populations (altitude <200 m): Ulu Yam (UY; 3° 16' 14.63" N, 101° 41' 11.32" E) and Lenggeng (LG; 2° 52' 55.40" N, 101° 55' 39.73" E). 9 stem cuttings from each population were propagated in M3 compost and grown in three controlled environment chambers (Conviron Controlled Environments Limited, Canada). Each growth chamber had an equal number of plants per population. Constant conditions of 12 h day: 12 h night cycle, 75% humidity and 180 μmol/m^{-2} s^{-1} light intensity were set for each chamber. The temperature of the first chamber was set to be 25°C day:18°C night (control), while the second and third chambers were 28°C day:20°C night (higher temperature) and 22°C day:15°C night (lower temperature) respectively. These were to simulate lowland and highland temperature according to the average Malaysia meteorological record from April to August 2008 (http://www.met.gov.my/). After six weeks, new leaves from the third to sixth

node of the stem were harvested, immediately immersed in liquid nitrogen and stored at -80°C until analysis.

Solid Phase Microextraction (SPME)

For the analysis of volatile compounds, a 100 µm of polydimethylsiloxane (PDMS) fiber was first conditioned by leaving it in the GC injection port at 250°C for at least 30 minutes before each extraction to remove any impurities. The fiber and the manual SPME holder were purchased from Supelco (Bellefonte, PA, USA). About 300 mg of fresh leaves were ground with liquid nitrogen and placed in a 20 mL vial. About 700 µL of distilled water was added to the ground leaves and the vial was covered tightly using a cap with septum to ensure no volatile could escape during the extraction. The fibre was then exposed to the sample headspace by inserting the fibre through the septum and the vial with the exposed fibre was heated at 45°C for 20 minutes. After standing for 20 min, the GC-MS analysis was conducted and the trapped analyte in the fibre was thermally desorbed by inserting the fibre into GC injector at 250°C for 10 min. Three biological and three technical replicates were used for each analysis.

Extraction of flavonoids

The extraction of flavonoids was carried out based on Gomez-Romero et al. (2010) with slight modifications. About 500 mg of freeze-dried samples were ground to a fine powder and extracted using 5 mL of methanol. The extracts were filtered after standing for one hour and the procedure was repeated twice. Three biological and three technical replicates were used for each analysis.

GC-MS analysis of volatile compounds

GC-MS analysis was conducted according to Baharum et al. (2010) using a gas chromatograph Clarus 600 T series with a Clarus 600 mass spectrometric detector (Perkin Elmer, USA) and a 30-m × 0.25-mm × 0.25-µm Elite-5MS column (Perkin Elmer, USA). The column temperature was increased from 40ºC to 220ºC at a rate of 4ºC/min; injector temperature, 250ºC; injection volume, 1 µL; transfer temperature, 280ºC. MS parameters were as follows: EI mode, with ionisation voltage 70 eV, ion source temperature, 180ºC; scan range, 50-600 Da. The volatile components were identified by matching their mass spectra with those of the Wiley 275 library (Wiley, New York) in the National Institute of Standards and Technology (NIST, version 2.0, USA) library and literature (Joulain and Konig, 1998; Joulain et al., 2001).

GC-FID analysis and n-Alkane standard solution

Retention indices of *P. minus* compounds were carried out according to standard method of Kováts Indices. The retention index of each compound was compare with literature to support the identification of the compounds (Adams, 1995). In order to perform Kováts indices, samples were analysed using a Hewlett Packard 5890 system GC-FID (Hewlett Packard, Palo Alto, CA, USA). The compounds were separated on 30 m × 0.25 mm × 0.25 µm DB-5HT column. The GC program was the same as those used for GC-MS analysis. *n*-alkane standard solutions C_8-C_{20} (mixture no. 04070) and C_{21}-C_{40} (mixture no. 04071) were purchased from Fluka Chemica.

LC-TOF-MS analysis of flavonoid extracts

Samples were analysed using LC-TOF-MS with a photodiode array (PDA) detector (micrOTOF-Q, Bruker, USA) equipped with a binary solvent delivery systems. Methanol extract was separated using a reversed phase C_{18} analytical column (250 × 2.0 mm, 5 micron). This separation used 0.1% formic acid as mobile phase A (15-50%) and acetonitrile as mobile phase B (50-85%) over 41 minutes of retention time, with 20 µL sample injection volume under 0.35 mL/min flow rate. Absorption spectra for the PDA were in the range of 190-950 nm. Parameters for the TOF detector in the positive-ion mode were as follows: nebulizer was set at 2 bars, the flow of dry gas was 7.0 L/min at a temperature of 190°C, end plate offset of -500 V and mass range of 50-1000 m/z. The analysis was done in triplicates for each methanol extract. Flavonoids were identified by comparing the exact value obtained from TOF system with the KNApSAcK database version 1.200.03 in the positive mode. The data was also cross checked with other databases such as Mass Bank and METLIN to putatively identify the compounds. The percentage area relative to the total percentage area of all detected compounds was calculated.

Chemical data analysis

A peak table of volatile compounds was generated using the Turbomass software (Perkin Elmer, USA), while for flavonoids analysis, the peak table was generated using Bruker Daltonics DataAnalysis 3.4 (Bruker, Germany). All peaks from each sample were combined into a single peak table and were then transferred into Microsoft Excel 2013. The retention time data was binned into region of 0.01 min to minimise the effect of peak shifts. The peak integral within each 0.01 min region was computed and normalised with respect total integral of the chromatogram.

Statistical analysis

Analysis of variance (ANOVA) and Tukey's multiple comparison were used to test for statistically significant differences in metabolite composition between temperatures treatments using SPSS v12.0.1 (Chicago, IL, USA). Changes in metabolite content with $P \leq 0.05$ were considered to be significant. Two-way ANOVA analysis was used to study the effect of different populations with temperature, population and temperature x population interaction as independent factors. For multivariate analysis, table with normalised percentage peak area of each identified compound was imported to SIMCA-P+ 12.0 software (Umetrics, Sweden), and multivariate analysis was performed using Pareto scaling (Par). The separation of the samples was observed in a scatter plot. Score contribution plot shows the contribution of each compound in sample separation. Partial least square discriminate analysis (PLS-DA) was performed based on temperature treatments when the samples cannot be separated well in principal component analysis (PCA). Heatmaps and hierarchical clustering were generated in R using heatmap.2 function with scaled data of percentage peak area. Venn diagrams were generated using a webtool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Results and Discussion

Temperature influence on the composition of VOCs in different plant populations

To study the effects of different growing temperature regimes on the chemical compositions of different plant populations, we conducted a reciprocal transplant common garden experiment under a controlled environment. Stems cuttings from three highland populations and two lowland populations were sampled and propagated inside plant growth chambers with temperature settings simulating the natural highland (22°C day:15°C night) and lowland (28°C day:20°C night) average growing temperatures. The mean of the two temperature

regimes were used as a control setting. There were no apparent differences in plant growth of different populations after six weeks. New leaves were harvested from the third node of stem for analysis.

For the analysis of volatile organic compounds, solid phase microextraction (SPME) was performed followed by GC-MS analysis. 27 out of 37 putatively identified volatile compounds were verified based on the retention indices by GC-GID using *n*-alkane standards (Supp Table 1). These volatile compounds were grouped according to different chemical groups. In all populations, aldehydes were the major compound (>80%), followed by terpenes (5-20%), while alcohols and alkanes were only present in trace amount (0.1-1.2%) (Figure 1). Aldehydes were mainly comprised of decanal and dodecanal. The effect of temperature in different populations varied as evidenced by the variation in the significant changes between individual compounds. The decanal and dodecanal contents were significantly influenced by temperature treatments in populations CH and FH but not in populations GH and LG, whereas only dodecanal was significantly decreased at lower temperature in population UY. Significant increase in other minor aldehydes was observed in populations FH, GH and LG at higher temperature. Overall, higher temperature treatment reduced the relative amount of aldehydes. This could be due to the increase in terpenes and alkane. Alcohol content was not influenced by the temperature but showed significant interpopulational variations (two-way ANOVA, $P<0.05$).

Notably, we discover an interesting inverse relationship between the composition of decanal and dodecanal after normalisation against total aldehyde content. The percentage of dodecanal was almost twice that of decanal at higher temperature treatment, whilst the converse is true at lower temperature treatment (Figure 2). At baseline temperature treatment,

the amount of decanal and dodecanal was similar. These decanal to dodecanal ratios were significantly different between the different treatments in all populations. This suggests that decanal and dodecanal composition in *P. minus* is interrelated and is very sensitive to growing temperatures as we can detect significant effect within the 6^{o}C difference consistently across all different populations.

Higher temperature treatment significantly increased the level of terpenes, especially terpene compounds other than caryophyllene and farnesene. These other minor terpenes which contributed to the significant increase during higher temperature treatment include bornyl acetate, bergamotene, geranylacetone, valencene, nerolidol, zingiberene, elemene, germacrene D and ocimene (Figure 3).

Multivariate analysis of combined data on VOCs provides an overview of the compounds which contributed to the separation of samples due to different temperature treatments. Principal component analysis (PCA) of the volatile data was not able to distinctively discriminate samples between temperature treatments (Supp Figure 1). Thus the PLS-DA was applied to optimise the separation between the three classes of treatments (Figure 4). The compounds responsible for the separation between samples along the first axis to explain 44.76% variation were among the abundant compounds mentioned above. The increase in dodecanal under higher temperature treatment, compared with decanal and farnesene which were more abundant at lower temperature treatment. Overall, the plot indicates that temperature strongly influenced the production of specific aldehydes and terpenes, notably a greater effect of higher temperature treatment.

The accumulation of these compounds under the higher temperature treatment varied between populations, such that the highland populations (CH, FH, GH) accumulated more of aldehyde and terpene compounds than the lowland populations (LG, UY). This is in accordance with the results from our previous FTIR analysis which showed increases of functional groups related to both compounds in highland populations under higher temperature treatment (Khairudin et al., 2014). This result suggests that plants originated from the highland population, which are normally growing under lower temperatures, might be more stressed when exposed to higher temperature.

Most studies focus on the effect of temperature on the emission of VOCs as compared to the content of VOCs in the tissue in current study. The amount of terpene compounds increased during the higher temperature treatment in this study. Terpenes serve a variety of functions in plants, which include deterring herbivores and attracting pollinators. However, the function of terpenes as a protective mechanism against temperature changes is still poorly understood. At high temperature, the increase in thylakoid membrane ion permeability causes proton leakage and reduces the production of ATP during photosynthesis. According to Singsaas (2000), terpene could act as a membrane stabiliser at high temperature and prevent proton leakage. This was supported by Velikova and Loreto (2005), in which the study showed more efficient photosynthesis in plants with greater amount of isoprene under heat stress. Conversely, terpene compounds could act as antioxidants (Loreto and Velikova, 2001). Double bond of terpene compounds could scavenge and regulate the activated oxygen species from abiotic stresses to protect the plant from oxidative damage. This is in agreement with Rosenfeld et al. (2002) who also reported an increase in caryophyllene with increasing temperature.

Conversely, farnesene production was increased at lower temperature treatment. According to Rupasinghe et al. (1998), farnesene was found to be increased by 6-fold in 'Anjou' pears (*Pyrus communis* L.) stored for 3 days at 0°C compared with those stored at 10°C. This suggests a possibility that farnesene is a low temperature-induced secondary metabolite. However, the role of farnesene in response to lower temperature is still unclear and further studies will be required to understand its mechanism.

Temperature influence on the composition of flavonoids in different plant populations

To compare the flavonoid constituents under different temperature treatments, LC-TOF-MS analysis was performed on methanol extracts. The putative identification (M+H) of flavonoids was done by comparing the exact value obtained from TOF system with the KNApSAcK database version 1.200.03 and cross-checked with Mass Bank and METLIN. A total of 85 flavonoid compounds were detected from all of the samples and categorised according to different classes (Supp Table 2). The effect of temperature treatments on different classes of flavonoids is showed in Figure 5. The interpopulational variation in the flavonoid composition was more apparent than that of VOCs. For example, flavone and flavanone were almost absent in population FH which was abundant in anthocyanidin compared with other populations. Such distinct observation was not found in the case of VOCs. Despite the interpopulational variations, the overall flavonol and flavanol content were significantly lower at the lower temperature treatment compared to the increase in anthocyanidin. Flavone was the only flavonoid significantly increased by the higher temperature treatment. Temperature treatment posed no significant effect on the amount of coumarin and flavanone, except for population CH and LG respectively.

Multivariate analysis was performed based on the individual putatively identified flavonoid compounds. PCA analysis showed tendency of sample clustering according to different populations instead of temperature treatments (Supp Figure 2). Samples were clustered separately in PLS-DA scatter plot based on different temperature treatments (Figure 6). From the loading plot, (+)-gallocatechin and 7,4-dihydroflavone were key in separating samples from between higher and lower temperature treatments respectively (Figure 6). (+)-Gallocatechin was present in all treatments, with the highest amount under higher temperature treatment (Supp Figure 3).

The higher production of flavonol compounds at higher temperature treatments was supported by Wahid (2007), showing that the production of soluble phenolics were higher at higher temperature treatment. Our previous study showed that higher temperature treatment gave higher FTIR absorbance at wavenumbers corresponding to phenolic compounds (Khairudin et al., 2014). It was shown that plants growing at low or high temperature exhibit high phenylalanine ammonia lyase (PAL) activity, which lead to the accumulation of flavonoids (Janas et al., 2000). The study of Wang and Zheng (2001) showed that high temperature conditions promote the accumulation of phenolic compounds in cultivated strawberry plants.

In this study, plants under higher temperature treatment produced more flavonols and flavanols than that of lower temperature treatment with relatively more abundant anthocyanidins. Anthocyanidins were found to be more abundant under lower temperature treatment in all populations. This is supported by the increased of anthocyanin content under low temperature in apples, pear and *Arabidopsis* (Leyva et al., 1995; Steyn et al., 2005; Steyn et al., 2009; Ubi et al., 2006). Gene expression of PAL, chalcone isomerase, and enzymes

related to anthocyanin biosynthesis increased under lower temperature (Gazula et al. (2005). Mori et al. (2007) suggested the loss of anthocyanin in grape skin under high temperature involved chemical degradation, enzymatic degradation and also inhibition of anthocyanin biosynthesis. Enzymatic degradation is due to the accumulation of polyphenol oxidase and peroxidase enzyme induced by high temperature which later inhibit the production of anthocyanin (Vaknin et al., 2005),.

Natural variations in chemical composition among different populations of *P. minus*

To discern the influence of interpopulational variation in response to different temperature treatments, we performed further multivariate analyses on the combined datasets of VOCs and flavonoids based on different populations. From the PCA analysis of combined data, clustering of samples according to temperature treatments and different populations was not distinct at principal component 1 and 2 (Supp Figure 4). There was a stronger tendency for the samples to cluster according to population than temperature treatments. This indicates variations of different populations in response to different temperatures, i.e., temperature effect was not the same across all populations.

PLS-DA analysis was performed to investigate the interpopulational variations (Supp Figure 5). Samples under higher temperature were distinctly separated from that of lower and baseline treatments which clustered together. Population LG was found to be the most distinct compared with other populations. The top 10 contributors to the separations between samples according to different populations were all flavonoid compounds (Supp Figure 5c). This indicates interpopulational variation was largely due to flavonoid composition.

Compounds which were uniquely identified in different populations can serve as metabolite fingerprints to differentiate between different samples (Supp Table 3). There were significantly greater number of total compounds detected in population LG (39) than FH (31); and at higher temperature treatment (39) compared to baseline (33) and lower temperature (35) treatments (ANOVA, Tukey's test, $P < 0.05$). Overall, more unique compounds were found in samples from lowland populations (LG and UY) (Figure 7).

Conclusions

In summary, the results obtained from this study showed the variations in plant metabolism at different temperatures, with distinct accumulation of flavonoid and terpene compounds in response to different temperature treatments, which also depends on the origin of the different plant populations. Metabolites that were associated with higher temperature treatment were identified from PLS-DA loading plots, majority of which were assigned to aldehydes and terpenes. The metabolite fingerprinting approach detects shifts in the metabolome of *P. minus* in response to different temperature treatments. The content of flavonols and flavanols at higher temperature treatment were greater compared to lower temperature, perhaps to counteract a greater oxidative stress under suboptimal higher temperature. The difference observed when comparing the highland and lowland populations might reflect the different adaptive abilities of different populations to their natural growing temperatures. Unique compounds identified from each population can serve as metabolite fingerprints. This study demonstrated that temperature had greater effects on the composition of VOCs than flavonoids, which was varied according to different populations. This knowledge is useful to inform cultivation conditions and choice of *P. minus* population to acquire plants producing desired flavonoid compounds. Furthermore, this study also implies that the effect of climate

change on natural plant populations will be significantly varied and modelling of plant responses should include biogeographical distribution.

Acknowledgements

This research was supported by the Genomics and Molecular Biology Initiative of the Malaysia Genome Institute (07-05-MGI-GMB 004), Research University Grant under Arus Perdana (UKM-AP-BPB-14-2009) and Fundamental Research Grant Scheme (UKM-RB-06-FRGS0102-2009). We also acknowledge Centre for Research and Instrumentation Management, Universiti Kebangsaan Malaysia for Research Instrumentation Development Grant 2010 (PIP-CRIM).The authors would like to thank Prof. Dr. Michael Burrell from the University of Sheffield for his guidance and two anonymous reviewers for their constructive comments.

Compliance with Ethical Standards

The authors declare no conflict of interest that could have direct or potential influence or impart bias on current work. All funding sources have been stated in the acknowledgement. All authors had read and approved the final manuscript. This manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere. We also confirmed that the samples used did not involve endangered or protected species.

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Figure legends

Figure 1. Composition of volatile organic compounds in *P. minus* grown under baseline, lower temperature and higher temperature treatments. Data are mean of percentage peak areas (±SE) from 3 biological replicates. Some error bars show only –SE for clarity. CH: Cameron Highland, FH: Fraser Hill, GH: Genting Highland, LG: Lenggeng, UY: Ulu Yam; L: lower temperature treatment, H: higher temperature treatment, B: baseline temperature treatment. Different letters indicate statistically significant differences (ANOVA corrected for multiple comparisons, $P<0.05$) between temperature treatments for individual compounds in each population (small letters), between temperature treatments for all populations (capital letters).

Figure 2. Ratio of decanal and dodecanal composition in *P. minus* grown under baseline, lower temperature and higher temperature treatments. Data are means (±SE) of normalised percentage peak areas against total aldehyde peak areas of 3 biological replicates. Different letters indicate statistically significant differences (one-way ANOVA, $P<0.05$) between temperature treatments.

Figure 3. Heatmap of relative abundance of different terpenes in *P. minus* grown under baseline, lower and higher temperature treatments. Data are means from 3 biological replicates. Colour scale is relative to the abundance of each compound. CH: Cameron Highland, FH: Fraser Hill, GH: Genting Highland, LG: Lenggeng, UY: Ulu Yam; L: lower temperature treatment, H, higher temperature treatment, B, baseline temperature. * indicates significant difference at $P<0.05$, one-way ANOVA.

Figure 4. PLS-DA model obtained from the classification of *P. minus* samples based on volatile compounds according to the temperature treatments. The percent of the variation of the data explained by each component is provided in the plot. (a) Score plot. Box, circle, triangle, diamond and inverted triangle symbols indicated FH, GH, CH, LG and UY populations respectively. Baseline, lower and higher temperature treatments are represented respectively by grey, white and black symbols. Each point represents sample with three biological replicates. (b) Loading plot of volatile compounds of *P. minus.*

Figure 5. Composition of different flavonoid classes in *P. minus* grown under baseline, lower and higher temperature treatments. Data are mean of percentage peak areas (±SE) from 3 biological replicates. CH: Cameron Highland, FH: Fraser Hill, GH: Genting Highland, LG: Lenggeng, UY: Ulu Yam; L: lower temperature treatment, H: higher temperature treatment, B: baseline temperature treatment. Different letters indicate statistically significant differences (ANOVA corrected for multiple comparisons, $P<0.05$) between temperature treatments for individual compounds in each population (small letters), between temperature treatments for all populations (capital letters).

Figure 6. PLS-DA model obtained from the classification of *P. minus* samples based on flavonoids according to temperature treatments. (a) Score plot. Box, circle, triangle, diamond and inverted triangle symbols indicated FH, GH, CH, LG and UY populations respectively. Baseline, lower and higher temperature treatments are represented respectively by grey, white and black symbols. Each point represents sample with three biological replicates. (b) Loading plot of flavonoid compounds of *P. minus.*

Figure 7. Venn diagram on the number of compounds found according to (a) temperature treatment or (b) different populations. Data inclusive of all 3 biological replicates from each population of each treatment. L: lower temperature treatment, H: higher temperature treatment, B: baseline temperature treatment; CH: Cameron Highland, FH: Fraser Hill, GH: Genting Highland, LG: Lenggeng, UY: Ulu Yam.