Characteristic differences in metabolite profile in male and female plants of dioecious *Piper betle* L.

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Piper betle is a dioecious pan-Asiatic plant having cultural and medicinal uses. It belongs to the family Piperaceae and is a native of the tropics although it is also cultivated in subtropical areas. Flowering in *P. betle* occurs only in tropical regions. Due to lack of inductive floral cycles the plant remains in its vegetative state in the subtropics. Therefore, due to lack of flowering, gender distinction cannot be made the in the subtropics. Gender distinction in *P. betle* in vegetative state can be made using Direct Analysis in Real Time Mass Spectroscopy (DARTMS), a robust high-throughput method. DARTMS analysis of leaf samples of two male and six female plants showed characteristic differences in the spectra between male and female plants. Semi-quantitative differences in some of the identified peaks in male and female landraces showed gender-based differences in metabolites. Cluster analysis using the peaks at *m/z* 151, 193, 235 and 252 showed two distinct clusters of male and female landraces. It appears that male and female plants besides having flowers of different sexes also have characteristic differences in the metabolites representing two metabolic types.

[Bajpai V, Pandey R, Negi MPS, Bindu KH, Kumar N and Kumar B 2012 Characteristic differences in metabolite profile in male and female plants of dioecious *Piper betle* L. *J. Biosci.* **37** 1061–1066] **DOI** 10.1007/s12038-012-9269-4

1. Introduction

Piper betle L. is a pan Asiatic cultural and ethnomedicinal plant of antiquity. Globally its leaves are consumed by more than 600 million people and it ranks second to coffee and tea. The plant is a shade-loving perennial climber with dioecy where distinction between male and female plants in the vegetative state is not easy and gender distinction can be made only after flowering. In India more than 100 landraces are under cultivation besides its availability in the wild. In India the plant is being cultivated from native tropical regions to the subtropics. No flowering is observed in the subtropics due to the lack of inductive photoperiods. *P. betle* besides cultural and recreational uses is also known to have medicinal importance. Although gender-based differences in biological activities (Tripathi *et al*. 2006; Singh *et al*. 2009; Misra *et al*. 2009) in *P. betle* were demonstrated in recent years, there is lack of information on the chemical profile of male and female plants.

The recently developed Direct Analysis in Real Time Mass Spectroscopy (DARTMS) is a fast, reliable high-throughput technique which is emerging as an accepted metabolic profiling tool. Some of the recent examples of applications of are direct analysis of curcumin, caffeine, olive oil, etc. (Cody *et al.* 2005; Kim and Jang 2009; Vaclavik *et al.* 2009). Using this technique it was possible to distinguish between species and

Keywords. DARTMS; dioecy; landraces; metabolites; *Piper betle*

Supplementary materials pertaining to this article are available on the *Journal of Biosciences* Website at *http://www.ias.ac.in/jbiosci/dec2012/supp/Bajpai.pdf*

Published online: 30 October 2012

varieties (Vicent *et al*. 2009; Bajpai *et al*. 2010; Kim *et al*. 2011). DARTMS was also used for chemical fingerprints of *P. betle* landraces (Bajpai *et al*. 2010). In this communication we report profiling of male and female landraces of *P. betle* using DARTMS and show that its possible use in gender discrimination in dioecious plants where dioecy is cryptic and becomes evident only after flowering.

2. Material and methods

Fully grown mature leaves from flowering *Piper betle* (L) vines were obtained from the research station of Indian Institute of Horticultural Research, Bangalore. The plants with their accession number and vernacular name are listed in table 1.

DARTMS analyses were performed at Sophisticated Analytical Instrumentation Facility (SAIF) in Central Drug Research Institute (CDRI), Lucknow. The leaves were washed with deionized water and wiped dry before use. The mass spectrometer (JMS-T100LC; AccuTof, atmospheric pressure ionization time-of-flight mass spectrometer, Jeol, Tokyo, Japan) fitted with a DART ion source was used in the study. The mass spectrometer was operated in positive-ion mode with a resolving power of 6000 (full-width at half-maximum). For orifice 1 the potential was set to 28 V, resulting in minimal fragmentation. At the ring lens and orifice 2 the potentials were set to 13 and 5 V, respectively. Orifice 1 was set at 100°C. The RF ion guide potential was 300 V. The DART ion source was operated with helium gas flowing at approximately 4.0 L/min. The gas heater was set to 300°C. The potential on the discharge needle electrode of the DART source was set to 3000 V; electrode 1 was 100 V and the grid was at 250 V. Freshly cut pieces of betel leaf were positioned in the gap between the DART source and mass spectrometer for measurements. The constituents present in *P. betle* leaves are nonpolar to moderately polar and, therefore, were ionized by DATMS. Data acquisition was from *m/z* 10 to 1050. Exact mass calibration was accomplished by including a mass spectrum of neat polyethylene glycol (PEG) as well as 1:1 mixture PEG 200 and PEG 600 in the data file. m-Nitrobenzyl alcohol was also used for calibration. The mass calibration was accurate to within±0.002 Da. Using the Mass Center software, the elemental composition was determined on selected peaks.

Table 1. *Piper betle* genotypes with their accession numbers and vernacular names maintained at the Indian Institute of Horticultural Research (IIHR) Bangalore, India

Accession number	Vernacular name
IIHRBV4 ♂	*Tellaku Chinthalapudi*
IIHRBV9 ♂	*Shirpurkata*
IIHRBV24 ♀	*Halisahar Sanchi*
IIHRBV28 ♀	*Gachi*
IIHRBV37 ♀	*Sirugamani*
IIHRBV40 ♀	*Malvi*
IIHRBV45 ♀	*Khasi*
IIHRBV46 ♀	*Calcutta Bangla*

3. Statistical analysis

Cluster analysis was done on the basis of four peaks at *m/z* 151, 193, 235 and 252 by k-means (standardized, non-hierarchical) clustering. All statistical analyses were performed on STATISTICA windows version 7.0 (StatSoft, Inc., USA).

4. Results and discussion

Most of the current therapeutic classes are derived from natural product prototypes, and indeed, more than 90% of current therapeutic classes are derived from natural product prototypes. As per estimates, approximately two-thirds to three-quarters of the world's population still relies on medicinal plants for its primary pharmaceutical care (World Health Organization 2002). Recent resurgence in the natural products as drugs or source of drugs has renewed the interest in studies on medicinal plants. Although most of the medicinal plants are monoecious, some are also dioecious, bearing male and female flowers on different plants. This is due to the fact that only about 7% of known taxa are dioecious (Renner and Ricklefs 1995) and dioecy in plants is more an exception than a rule. Unlike in the animal world, dioecy in plants is mostly cryptic and can only be ascertained at flowering by the sex of flowers. Although human awareness of this phenomenon is as old as the Babylonian times (ca 2300 BC), when the different sexes were known for date palms (Negbi 1995), its significance in terms of biological efficacy escaped attention in the past. Reports on gender-based differences in some physiological attributes and also at the metabolite level have emerged in the recent past (Stanley *et al.* 2005; Kleps *et al.* 2007). The naturally occurring chemical diversity of *Tanacetum vulgare* plants in an area smaller than 3 km^2 was extremely high, exhibiting 14 different chemotypes (Kleine and Muller 2011). In plants the bio-/chemo-diversity due to gender is over and above the diversity observed within a species due to location, seasons, developmental stages, etc. In nature it is possible to have a gender-associated qualitative or quantitative difference which may ultimately impact the levels of metabolites and hence drug yield/recovery and efficacy of that taxon. Thus, the gender-related bio-/chemo-diversity within a species is of considerable importance depending upon its uses.

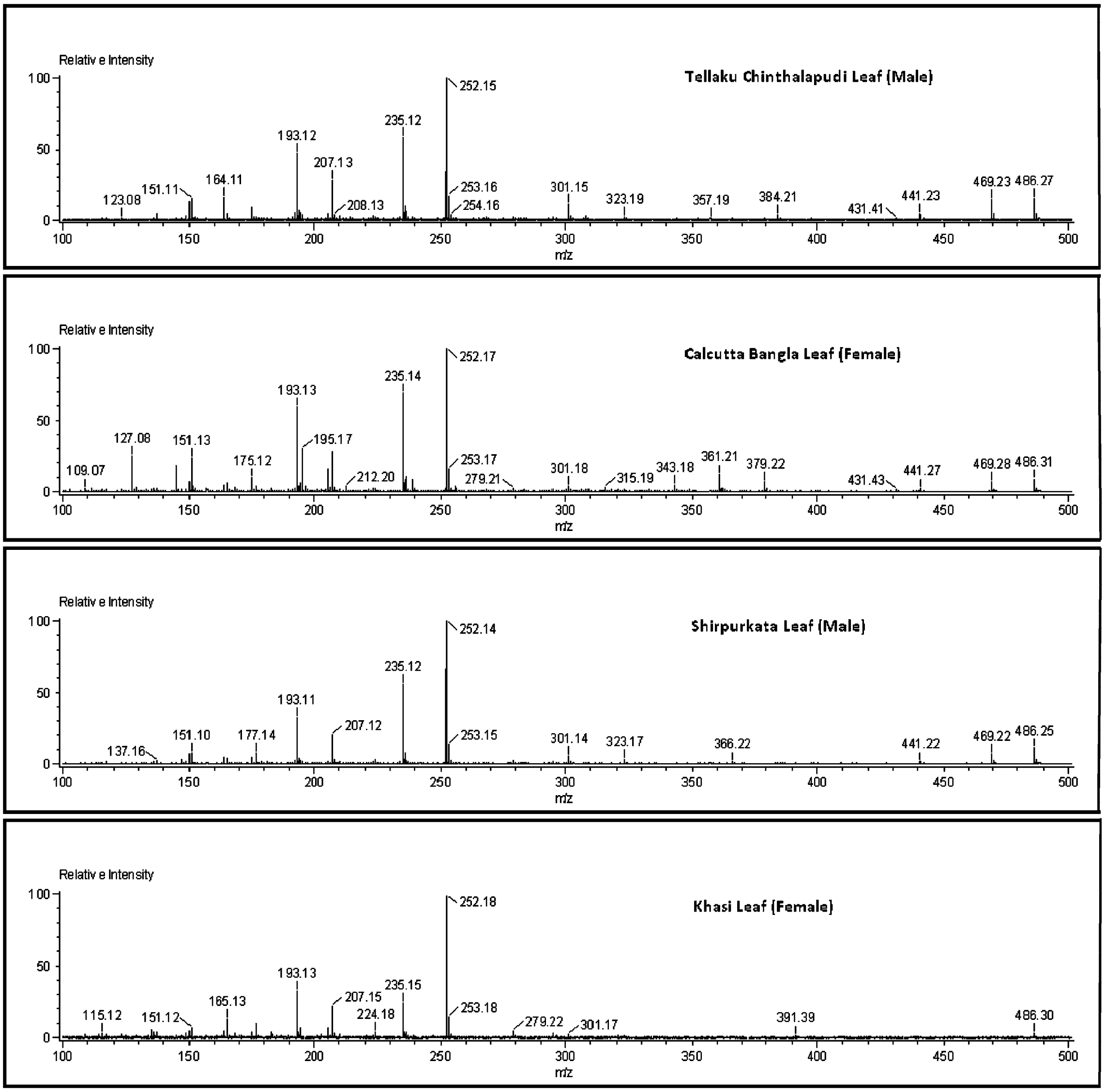

Figure 1. DART Mass Spectra of *Piper betle* landraces *Tellaku Chinthalapudi* (male), *Calcutta Bangla* (female), *Shirpurkata* (male) and *Khasi* (female) leaf.

Mass spectra of male and female landraces are shown in figure 1. It is evident from the spectra that there are differences in the peaks between male and female landraces. The peaks at *m/z* 150, 151, 164, 165, 175 193, 207, 235 and 252 are common to all landraces and hence constitutive for *P. betle*. Other major peaks seen in the spectra at *m/z* 136, 137, 149, 177, 189, 224, 236, 295, 300, 301, 308, 352, 366,4 31, 469 and/or 486 are specific to individual landraces. The DART mass spectra peaks were observed at *m/z* values corresponding to many of the reported phenols and their acetates in *P. betle* leaf (Kumar *et al.* 2010). Accordingly, the peaks at *m/z* 135, 151, 165, 177, 193, 207 and 235 were, respectively, identified as chavicol, allylpyrocatechol, chavibetol, chavicol acetate, allylpyrocatechol acetate, chavibetol acetate and allylpyrocatechol diacetate (table 2). It was not possible by DART mass spectra alone to differentiate between some of the phenols with same molecular weight. Thus, the peak at *m/z* 151 could be due to allylpyrocatechol or carvacrol. Due to their different molecular formulae, it was not possible to differentiate them based on their exact mass values; however, as reported in earlier studies (Kumar *et al.* 2010), it was considered as allyl pyrocatechol. The peak at *m/z* 165 could be due to eugenol or chavibetol. Since both the molecules have the same molecular formula a distinction could not be made. Based on peak percentage ionization semi-quantitative differences for peaks *m/z* 151, 193, 235 and 252 are presented in table 3. Peaks which showed differences based on

Table 2. Exact mass of the identified constituents in the leaf of male and female landraces of *Piper betle*

m/z	Measured mass	Calculated mass	Molecular formula	Error (mmu)	Remarks
134	135.08128	135.08099	$C_9H_{11}O$	0.29	Chavicol
150	151.07640	151.07590	$C_9H_{10}O_2$	0.50	Allylpyrocatechol
164	165.09242	165.09155	$C_{10}H_{13}O_2$	0.87	Chavibetol
165	166.08864	166.08680	$C_9H_{12}NO_2$	1.84	Phenyl alanine
174	175.07764	175.07590	$C_{11}H_{11}O_2$	1.73	Unknown
176	177.09109	177.09155	$C_{11}H_{13}O_2$	0.47	Chavicol Acetate
192	193.08772	193.08647	$C_{11}H_{13}O_3$	1.25	Allylpyrocatechol Acetate
206	207.10140	207.10212	$C_{12}H_{15}O_3$	-0.72	Chavibetol Acetate
234	235.09649	235.09703	$C_{13}H_{15}O_4$	-0.55	Allylpyrocatechol diacetate
251	252.12287	252.12358	$C_{13}H_{18}NO_4$	-0.71	Unknown

percentage ionization between the two sexes were used for cluster analysis. Neighbor joining (NJ) tree shows two different clusters representing male and female landraces (figure 2). In *P. betle* molecular tools like randomly amplified polymorphic DNA (RAPD) were also used for biodiversity analysis. Cluster analysis based on RAPD data showed three major groups in *P. betle; Bangla*, *Kapoori* and others (Ranade *et al.* 2002; Verma *et al.* 2004). Thus, cluster analysis based on DARTMS is similar to the earlier reports. Inter- and intra-gender differences are marked out and need to be tested on a larger population of male and female plants for the assessment of bio-/chemo-diversity in *P. betle*.

Differences between male and female plants at the vegetative stage were recognized by the ecologists working on dioecious plants. Studies have shown herbivore preferences for gender (Hjalten 1992; Dormann and Skarpe 2002; Boecklen *et al*. 2004; Cornelissen and Stiling 2005; Uribe-Mu and Quesada 2006) which correlated with the levels of secondary metabolites. Higher amounts of phenols and antioxidants in females were the part of putative defense function against herbivore attack (Orians *et al.* 1996; Palumbo *et al.* 2007). In case of blue crabs (*Callinectes sapidus*) sex-specific differences in metabolites were also shown (Kleps *et al.* 2007). Thus, our findings on *P. betle* are in agreement with earlier reports. In recent years gender-related differences in biological activities were reported in *P. betle* (Tripathi *et al.* 2006; Misra *et al.* 2009; Singh *et al.* 2009) which could be due to differences in the metabolites as shown in this communication.

Recently, in a comprehensive study on gender-based differences in metabolites (Mittelstrass *et al.* 2011) it was inferred that male and females represent different metabolic types or metabotypes. They also showed sex-specific differences of cell regulatory processes that may play a role in differences in metabolites. A quantitative difference in acetylation of phenols was observed between male and females landraces. This observation is in agreement with that of Mittelstrass *et al.* (2011). Thus, DARTMS can be widely employed for population screening of economically important dioecious plants where only the vegetative parts are used as in the case of *P. betle*. More studies are required to analyse greater number of *P. betle* landraces and other important dioecious plants like *Tinospora cordifolia*, which are very widely used in indigenous systems of medicine. Thus, knowing the gender

Table 3. Peak percentage ionization of some of the important compounds in the leaf of male and female *Piper betle* landraces (mean±SD, *n*=5)

	Peak position and % ionization				
Piper betle landraces	*m/z* 151	*m/z* 193	*m/z* 235	*m/z* 252	Total
Calcutta Bangla (F)	5.60±0.89	14.00±1.58	15.80±1.64	27.00±4.24	62.40±3.78
Gachi (F)	7.20±1.64	15.20±2.17	15.80±1.48	24.80±1.92	63.00±3.08
Halisahar Sanchi (F)	6.20±1.92	16.40±2.41	16.80±1.10	27.20±2.77	66.60±3.21
Sirugamani (F)	19.72±2.51	22.20±0.55	12.16±2.11	14.02±1.99	68.10±1.62
Khasi (F)	5.20±1.30	13.60±2.41	15.60±1.52	28.80±2.77	63.20±4.15
Malvi (F)	5.40±1.52	16.40±1.82	18.80±1.48	24.80±2.68	65.40±1.95
Shirpurkata (M)	3.20±1.48	10.40±2.30	12.80±2.17	36.00±2.24	62.40±4.16
Tellaku Chinthalapudi (M)	3.80±0.84	9.60±1.52	11.40±1.14	41.60±2.51	66.40±1.14

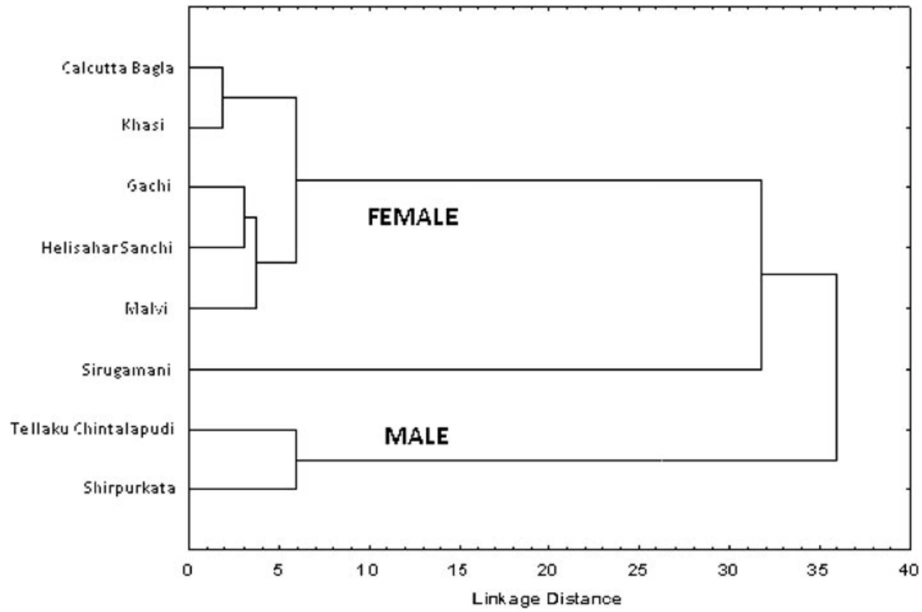

Figure 2. Cluster analysis (k-means) of male and female landraces of *Piper betle* based on four discriminating ions (*m/z* 151, *m/z* 193, *m/z* 235 and *m/z* 252).

of dioecious plant at the vegetative stage may be important as it will help in reducing the overall cost by selective cultivation of landraces that may have greater use value. It may be also important in the case of dioecious medicinal plants where only vegetative parts are used. Metabolomic studies in dioecious plants will help in understanding phenomenon of dioecy, which is largely considered as an adaptation for out-breeding and also prove useful in judicious bio-resource utilization.

Acknowledgements

Grateful acknowledgement is made to SAIF, CDRI (CSIR), Lucknow, India where the mass spectrometric studies were carried out. Authors confirm that there is no conflict of interest and everybody contributed equally. NK mentored the study and the paper was written by NK and BK. VB and RP did the experimental work. The plants used in the study were maintained by HB in betel vine breeding programme. Statistical analysis was done by MSN. CDRI Communication no. 8339.

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MS received 05 June 2012; accepted 30 August 2012

Corresponding editor: MAN MOHAN JOHRI