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Ruta graveolens Cultures as Screening Resources for Phyto-Pharmaceuticals: Bio-prospecting, Metabolic Phenotyping and Multivariate Analysis

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ABSTRACT

Ruta graveolens is a folklore plant, used by ancient Egyptians and Greeks and used in traditional medicine systems like Unani, Ayurveda and traditional Chinese medicine. The plant has potent anti-inflammatory, anti-cancer and anti-HIV activity. In this study, *R. graveolens* and six *in vitro* cultures (with varying degrees of differentiation) were screened for biologically active compounds by GC-MS analysis. Non-targeted comprehensive analysis, directed towards extracting broad spectrum biochemical information was used for bioprospecting. The relationship between metabolic components and lines was interpreted with the help of multivariate analysis (hierarchical cluster analysis and principal component analysis). The characteristic metabolic traits underlying clustering and separation of culture lines were also elucidated. Bio-prospecting based on GC-MS analysis indicated the presence of several metabolites with a wide range of bioactivities: photobilogical, anti-microbial, anti-viral, anti-oxidative, anti-proliferative, anti-inflammatory, anti-tumour, anti-platelet aggregation, anti-HIV, immunomodulatory, estrogenic activity, among others with potential economic importance. Multivariate analysis demonstrated that metabolic traits enabled the discrimination of genotypes that exhibited marked differences in pharmaceutically important metabolites. With the use of metabolic phenotyping, *in vitro* cultures can be used as novel screening resources for new or improved phyto-pharmacueticals.

Keywords: metabolic profiling, hierarchical cluster analysis, principal component analysis
Abbreviations: FC, furanocoumarin; **HCA**, hierarchical cluster analysis; **Ia3**, transformed clone; **PCA**, principal component analysis; **RC1**, dispersed cell line; **RS2**, shoot line; **RC3**, aggregated cell line; **RC6**, differentiated cell line

INTRODUCTION

Natural products have provided inspiration for most of the active ingredients in medicines: around 80% of medicinal products up to 1996 were either directly derived from naturally occurring compounds or were inspired by a natural product, and more recent analysis confirms the continuing importance of natural products for drug discovery (Harvey 2001, 2004, 2007). The search for active phytocompounds will be greatly advanced by the combination of various metabolomic approaches to differentiate between plant species, tissues, or phytopreparations, and to identify novel lead compounds for future development. Though the use of metabolomics in the development of active secondary metabolites from medicinal plants as novel or improved phyto-therapeutic agents is still limited, it can offer a comprehensive overview of the identity and quantity of metabolites in biological materials. Wang *et al.* (2005) pointed out that metabolomics could provide the needed links between the complex chemical mixtures used in traditional medicines and molecular pharmacology. In a complementary development, the use of metabolome-refined herbal extracts with other biochemical components in combination, rather than as isolated single compound(s), may prove to be very useful as broader and holistic therapeutic or pharmacological agents for a variety of human health care applications (Shyur and Yang 2008). Even though such approaches have been employed for traditional Chinese medicines (TCM), its applications for traditional Indian medicine are yet to be realized.

Ruta graveolens (Rutaceae), a folklore plant, is used as traditional medicine for various ailments. The name *Ruta* is derived from the Greek "reuo" (to set free), because this herb is so efficacious in various diseases. Ancient Egyptians and early Greeks used *Ruta* to improve eyesight. In Chinese medicine, it is used for its antifungal, antibacterial and anti-inflammatory properties. *Ruta* has been reported to be useful for the treatment of multiple sclerosis and also possesses hypotensive activity (Korengath *et al.* 2008). It has a long history of use in homeopathic, Ayurvedic and Unani preparations (Elia 2003). It has traditionally been used in treatment of leucoderma, vitiligo, psoriasis, multiple sclerosis, cutaneous lymphomas, and rheumatic arthritis. Recently its extracts were shown to have potent anti-inflammatory and anti-cancer activity (Pathak *et al.* 2003; Preethi *et al.* 2007; Diwan and Malpathak 2009). Plant parts contain more than 120 compounds of different classes of natural products such as acridone alkaloids, coumarins, essential oils, flavonoids, and furoquinolines (Feo *et al.* 2002; Oliva *et al.* 2003). Many of these compounds are physiologically active and therefore of immense pharmaceutical interest.

With the success achieved in increasing culture productivity, *in vitro* cultures are now being promoted as alternative source and screening resources (bio-prospecting) for new or improved phyto-pharmacueticals. Reports describing the successful use of *R. graveolens* culture for furanocoumarin (FC) production (Ekiert *et al.* 1998, 2001) have made them promising alternatives with potential for large-scale production (Gontier *et al.* 2005; Diwan and Malpathak 2008). Enhanced production of FCs was achieved by manipulations of media constituents (Massot *et al.* 2000) and culture conditions (Ekiert and Gomółka 1999; Ekiert *et al.* 2001). Effective elicitation by biotic and abiotic elicitors (Bolhmann *et al.* 1995; Orlita *et al.* 2007a, 2007b, 2008),

Received: 5 April, 2010. Accepted: 10 September, 2010.

Original Research Paper

for enhanced FC production was recently reported. Besides coumarins, *Ruta* cultures have several biologically active metabolites present like protocatechuic, vanillic, syringic and *p*-coumaric acid (Ekiert *et al.* 2009), triglycerides (Asilbekova 2001), lipids (Asilbekova 2000), furacridone alkaloids (Nahrstedt *et al.* 1985), volatile oils (Kuzovkina *et al*. 2009), acridone alkaloids (Sidwa-Gorycka *et al.* 2009), and arbutin (Piekoszewska *et al.* 2010).

Therefore *R. graveolens* and selected *in vitro* cultures were screened for biologically active compounds by GC-MS analysis. In this study, six *in vitro* cultures with varying degrees of differentiation were screened for biologically active compounds by GC-MS analysis. Shoot line RS2 was selected for its rapid growth rate and high FC productivity (Diwan and Malpathak 2008). *R. graveolens* cell cultures that displayed a wide range of cellular differentiation were selected. Such a range of organization is rarely observed, making it a model system for examining the relation between cellular differentiation and secondary metabolite production.

This non-targeted comprehensive analysis was directed towards extracting a broad spectrum of biochemical information of the metabolome of selected culture lines. The relationship between culture lines and metabolic components was interpreted with the help of multivariate analysis. The characteristic metabolic traits underlying the clustering and separation phenomenon in culture lines were elucidated using high factor scores of principal component analysis (PCA).

MATERIALS AND METHODS

Plant material

R. graveolens plants were established from Pune, Kolhapur and Kerela then screened for furanocoumarin productivity. Plants having furanocoumarin content higher than 8 mg g^{-1} DW were selected as elite plants and established in the Botanical Garden, University of Pune, India (Diwan and Malpathak 2009). Nodes, internodes and leaves of 6-month-old plants were used as explants for establishing *in vitro* cultures.

In vitro cultures

Cell suspension cultures with varying degrees of differentiation were established from leaves of elite plants. Cell lines with varying degrees of differentiation, dispersed cell line RC1 (devoid of organization and aggregation), aggregated cell line RC3 (compact cell aggregates) and differentiated cell line RC6 (development of shoot and root primordia), were established as described in Diwan and Malpathak (2009) and were selected for metabolite analysis. Several shoot lines were established from nodes and internodes of *R. graveolens* plants. Shoot line RS2 was selected due to its low doubling time and high productivity, as described by Diwan and Malpathak (2008).

Transformation

Agrobacterium tumefaciens strain LBA 4404 harboring binary vector pCAMBIA 2301 (Hoekema *et al.* 1983) with Kan resistance (KANR) and GUS as selectable marker was used for transformation experiments (unpublished data). Broadly, leaves and internodes were infected without preculture by injuring and inoculating 10 µl of bacterial solution, followed by 2 days of co-culture and then transferring the explants to selective regenerating medium containing 50 mg/l kanamycin. Stable transgene integration was confirmed by growth on selection medium for KANR by PCR and GUS activity. Several transformed clones of *R. graveolens* were obtained and Clone Ia3 was selected due to its rapid growth and enhanced productivity.

Preparation of extracts

For profiling, methanolic extracts of *in vivo* and *in vitro* materials were prepared. *In vivo* stems along with their leaves were harvested, dried at 40°C and pulverized. Roots were also dried and powdered as described above. *In vitro* plant material was harvested on the 21st day after culture initiation, dried at 40°C and powdered. Extraction of *in vivo* and *in vitro* material was carried out as follows. Finely pulverized *in vivo/in vitro* plant material (100 mg) was sonicated (33 Mhz) for 20 min at room temperature and cold-extracted in ultra-pure methanol overnight. The extract was centrifuged at 10,000 rpm for 20 min and the supernatant was filtered (using a 0.45 µM membrane filter; Laxbro, India). After filtration, the extract was evaporated to dryness at room temperature and dissolved in ethyl acetate (ultra-pure). The final concentration of all crude extracts was 1 mg ml^{-1}. Methanolic extracts of *in vivo* plant material (stems and roots) were used as reference during profiling.

Metabolic phenotyping

Plant extracts were subjected to GC-MS analysis.

1. GC-MS conditions

GC-MS analyses were performed using a gas chromatograph (Varian 3800 GC, USA) with a data handling system and FID coupled to ion trap detector, equipped with a DB-5 fused-silica column (30 m, 0.32 mm ID and 0.25 µM film thickness). Injection temperature was 300°C. Oven temperature was programmed from 50°C for 2 min then increased by 20°C/min until it reached 150°C, followed by 3°C/min until it reached 225°C and finally increased by 15°C/min until it reached 280°C, then held for 9 min with a total run time of 45 min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Injection was done in split mode (4:1) with a volume of 1.0 µl.

2. MS conditions

Analysis was performed on a Varian 4000 (USA), ion trap mass spectrophotometer. MS was scanned from 10 to 1000 m/z at a scan rate of 1 scan/sec.

3. Compound identification

The identity of components was established from their GC retention times by comparison of their MS spectra with those reported in the literature (Feo *et al.* 2002) and by computer matching with the NIST mass spectral library (http://www.nist.gov).

4. Data processing

Baseline correction, peak deconvolution and alignment, and normalization to the total sum of the chromatogram for the GC-MS data were performed. All the measured metabolites were treated on an equal level with auto-scaling prior to multivariate analysis.

5. Multivariate analysis

Hierarchical cluster analysis (HCA) and PCA were performed using the statistical program Matlab (MathWorks, USA, www.mathworks.com). An HCA-generated dendrogram was represented by a similarity factor between 1 and 0 with 1 being the most similar. HCA used Euclidean distances to calculate the matrix of all samples. PCA was performed to reveal the general clustering, grouping, and trends without prior knowledge with the intention of searching for metabolic differences between the culture lines.

RESULTS AND DISCUSSION

In vitro cultures were screened for biologically active compounds by GC-MS analysis. More than 200 peaks were typic-ally resolved from each extract. Metabolites that were identified with a high degree of certainty were compared on the basis of their normalized peak area and metabolites forming major constituents of the metabolome were depicted. GC-MS analysis corroborated the accumulation of compounds belonging to alkaloids, coumarins, phenolics as major biologically active metabolites in the *in vitro* extracts.

Table 1 Bio-prospecting of selected *in vitro* cultures for pharmaceutically important metabolites.

Class	Compound	RC1	RC3	RC5	RS2	Ia3	stm	rt	Activity
Essential oils	a-Pinene	-	-	+	+	+	+	+	Allelochemicals, antibacterial, antiviral,
	Limonene	-	-	+	+	-	+	-	antifungal
	n-Octanol	-	-	-	-	-	+	-	
	Non-2-ene	-	-	+	+	-	+	-	
	Nonan-2-one	+	+	-	-	+	+	+	
	Nonan-2-ol	-	-	-	-	-	+	-	
	Octanoic acid	-	-	+	+	+	+	-	
	Methyl salicylate	-	-	+	+	+	+	-	
	Decan-2-one	-	-	-	+	+	+	-	
	Decan-2-ol	-	-	-	-	-	+	-	
	Octyl acetate	-	-	-	-	+	+	-	
	Undecan-2-one	-	-	-	-	+	+	-	
	Undecan-2-ol	-	-	-	-	-	+	-	
	Dodec-2-ene	-	-	-	-	-	+	-	
	Tridecane	+	-	-	-	-	+	-	
	Decyl-2-acetate	-	-	-	-	-	+	-	
	Dodecan-2-one	-	-	+	+	+	+	+	
	Tridecan-2-one	-	-	-	-	-	+	-	
Coumarins	*p*-coumaric acid	+	+	+	+	+	+	+	
	4-Hydroxycoumarin	+	+	+	+	+	+	+	Antioxidant, anticancer (Kim *et al.* 2007)
	7-Hydroxycoumarin (umbelliferone)	+	+	+	+	+	+	+	Antioxidant, anticancer (Kim *et al.* 2007)
	7-Hydroxy-6-methoxycoumarin (scopoletin)	-	-	+	+	+	+	-	Immunomodulatory (Manuelea *et al*. 2006) Hepatoprotective (Kang *et al.* 1998)
	5,7-Dimethoxy coumarin (limettin)	-	-	+	+	+	+	-	Photobiological activity (Ashwood-Smith *et al.* 1983), antiproliferative activity (Alesiani *et al.* 2008)
	5-Methoxypsoralen bergapten	-	+	+	+	+	+	-	PUVA, anti-neoplastic, anti-proliferative,
	8-Methoxypsoralen xanthotoxin	-	+	+	+	+	+	+	anti-HIV (Kim *et al.* 2007), Topo I inhibitor (Diwan and Malpathak 2009)
	7-Methoxypsoralen	-	-	-	+	+	+	-	Anti-HIV, anticancer (Kim *et al.* 2007)
	5,6-Dimethoxyangelicin (pimpinellin)	-	-	+	+	+	+	+	Anti-inflammatory, anti-HIV, anticancer (Kim *et al.* 2007)
	Chalepensin	-	-	+	+	+	-	+	
	Daphnoretin	-	-	+	-	-	-	+	
	Isoimperatopin	-	-	-	+	+	+	+	
	Isopimpinellin	-	-	-	+	+	+	+	
	Marmesine	-	-	+	-	-	-	+	
	Scopolein derivative	-	-	-	+	+	-	-	Bacteriostatic activity, regulate blood pressure, anti-inflammatory activity (Kang *et al.* 1998)
	Angelicin	-	+	-	+	+	+	+	Spasmolytic activity (Patnaik *et. al.* 1987), treatment of thalassemia, anti cancer (Ebermann *et al.* 1996)
	Benzofuran-3-one (Coumaranone)	+	+	+	+	+	+	+	
	Phthalide	+	+	+	-	-	-	-	
	Osthol	-	-	+	-	-	-	-	Inducing cell differentiation anticancer (Kuo *et al*. 2005)
	Psoralen	+	+	+	+	+	+	+	Anticancer (Kim *et al.* 2007), Topo I inhibitor (Diwan and Malpathak 2009)
Quoline alkaloids	2,5-Cylohexadiene-1,4-dione,2,6-bis(1,1-dimethyl) quinone derivative	+	+						Anti tumour (Aschea *et al*. 2005), anti-oxidant (Mora and Dangles 2005)
	1-Methyl-2-[3',4'-(methylenedioxy)phenyl]-4-quinolone (Gravoline)	-	-	+	-	-	+	+	
	2-*N*-Nonyl-4-quinolone	+	+	-	+	+	+	-	
	t-Fagarine	-	+	+	+	+	+	-	
	Kokusaginine	-	-	-	+	+	+	-	
	Ardorinine	-	-	-	+	+	+	-	
	Akimmianine		-	-	-	-	+	+	
	Dictamnine	-	-	-	+	+	+	+	Photoactivity (like psoralen), ovicidal, anti-platelet aggregation (Sackett 1996)
	Skimmianine	-	+	+	+	+	+	-	
	1-methyl-2-*N*-nonyl-4-quinolone	-	-	+	+	+	+	-	
	2-*N*-dodecyl-1,4-quinolone	-	-	+	+	+	-	+	
	2-*N*-tetradecyl-4-quinolone	-	-	-	+	+	-	+	
	3-Methyl-2-nonyl-1H-quinolin-4-one	-	-	+	+	+	+	-	
	2,3,8-trimethyl-1H, 9H pyrrolo[3,2H]-quinolin-6-one	-	-	+	-	-		-	
	3-methyl-2-undecyl-1H-quinolin-4-one	-	-	-	+	+	+	-	
	2,2,6-trimethyl-2H,5H-pyrano[3,2-c]quinolin-5-one	-	-	-	+	+	+	-	
	Graveliferone	-	-	+	-	-	-	+	

Table 1 (Cont.)

Class	Compound	RC1	RC3	RC5	RS2	Ia3	stm	rt	Activity
Furo/Furano-quinones	4,6,7-Trimethoxyfuro[2,3-b]quinoline	-	-	-	-	+	-	-	
Acridone alkaloids	1,3-Dihydroxy-*N*-methylacridone	-	-	+	+	+	+	+	
	Rutacridone	-	-	+	-	-	+	+	
	1-Hydroxy-*N*-methylacridone	-	-	-	-	-	+	+	
	1-Hydroxy-3-methoxy-*N*-methylacridone	-	-	-	-	-	+	+	
	Furacridone	-	-	-	+	+	+	-	
Quinazoline alkaloids	Arborinin	-	-	-	+	+	+	+	
	Guaiacol	-	-	-	+	+	+	+	
	Indole	+	+	+	-	-	+	+	
	Benzoic acid	+	+	-	-	-	+	+	
	Diethyl phthalate	-	-	+	-	-	+	-	Estrogenic Activity (Harris *et al.* 1997)
	isobutyl phenol	-	-	+	+	+	+	-	
	4H-1-Benzopyran-4-one, 5 hydroxy-7-methoxy-2-methyl-6-(3-methyl-2-butenyl)	-	-	+	-	-	-	-	
Others	7-Chloro-1,3-dihydro-5-phenyl-1(trimethylsilyl)-2H-1,4-bezodiazepin-2-one	-					+		Sedative, anti-convulsants, photobiological (Cornelissen *et al.* 1980), anti-HIV (Breslin *et al.* 1999)
	Heptadecanoic acid, 16-methyl-,methyl ester	+						+	Antibacterial, antifungal (Agoramoorthy *et al.* 2007), anti tumour (Yu *et al.* 2005)
	1-Methyl ethyl ester 4-Oxazolecarboxylic acid, 4,5-dihydro-2-phenyl (oxazole derivative)	-					+		Anti neoplastic
	10-Methylnonadecane	-					+		
	Nonane, 3 methyl-5 propyl-	-					+		Preventing osteoporosis (Lewis *et al.* 2001)
	2-Nonanone,9-[tertahydro-2H-pyran-2-yl)oxy	-					+	+	Antifungal (Andersen *et al.* 1994)
	Hexadecane,1,1-bis(dodecyloxy) (alkane hydrocarbon)	-					+		DNA Polymerase inhibitory activity (Ogura *et al.* 1986)
	Benzophenone	-	-	+	+	+	-	-	anti-inflammatory (Muraria *et al*. 2004), anti-tumor activity (Matsumoto *et al*. 2003)
	Benzothizole	-	-	-	+	+	-	-	Anticancer (Chen *et al*. 2007)
	Syringol	-	-	-	+	+	+	+	
	Phthalic acid, isobutyl octyl ester	-	-	+	+	+	-	-	
	4H-Pyran-4-one, 2,3-dihrdro-3,5-dihydroxy-6-methyl-	-	-	-	-	+	+	-	Anti-proliferate and pro-apoptotic (Kim *et al*. 2004)
	Resorcinol	-	-	-	-	+	-	-	
	Pidolic acid 5-oxopyrrolidine-2-carboxylic acid	-	-	-	+	-	+	+	Arthritis (including osteoarthritis and rheumatoid arthritis), Alzheimer's disease, cancer, congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head trauma, spinal cord injury, neuro-degenerative disorders (acute and chronic), autoimmune disorders (Laird *et al*. 1999)
	Phenylalanine	+	+	-	-	-	+	+	
	Pyridine, 2-phenyl	-	+	-	-	-	-	-	Hyperuricemia, gout, inflammatory intestinal diseases, diabetic nephropathy, diabetic retinopathy (Miyata *et al*. 2007)
	Phenol, 2,5-bis(1,1 dimethylethyl)-	-		-	+	+	+	+	Inflammation, ischemia, muscular dystrophy, anti-inflammatory (Lee *et al*. 2007)
	Benzoic acid	+	+	-	-	-	-	-	
	Benzoic acid, 4-ethoxy-, ethyl ester	+	-	-	-	+	-	-	
	2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	-	-	+	-	-	+	-	
	Octyl butarate derivative	-	-	+	+	+	+	-	Correlated with high FC content (Cianfrogna *et al.* 2002)

We identified 79 metabolites and some important metabolites are represented in **Table 1**. These were then screened for their reported biological activity. Thus commercially important (major) metabolites with a wide range of bioactivities such as photobiological, antimicrobial, antiviral, antioxidative, antiproliferative (Kim *et al.* 2007), antitumour (Matsumoto *et al.* 2003; Chen *et al.* 2007), anti-HIV, immunomodulatory (Manuelea *et al.* 2006), antiinflammatory, estrogenic activity (Harris *et al.* 1997). Topoisomerase I inhibitor (Diwan and Malpathak 2009), DNA Polymerase inhibitor (Ogura *et al.* 1986) and osteoporosis-preventing (Lewis *et al.* 2001) activities were also identified (**Table 2**).

Nonrandom distribution of the metabolite patterns in some metabolite groups was evident. Each tissue culture system had its characteristic set of metabolites. Dispersed cell culture line, RC1 accumulated large no of precursors, pathway intermediates and the derivatives of the general phenylpropanoid pathway. Aggregated cell culture, RC3 accumulated basic metabolites belonging essential oils, alkaloids, coumarins and quinones. Metabolic profile of differentiated cell line RC6, shoot line RS2 and transformed shoot clone Ia3, matched with the metabolic profile of *in vivo* stems. RC6 showed presence of shoot specific metabolites like few components of essential oils (α-pinene,

Table 2 Biologically active metabolites in the *in vitro* extracts of *Ruta graveolens*.

Activity	Compounds
Photobiological	Limettin, bergapten, xanthotoxin, psoralen, dictamnine, 7-chloro-1,3-dihydro-5-phenyl-1(trimethylsilyl)-2H-1,4-bezodiazepin-2-one
Antimicrobial	Scopolein, α-pinene, limonene, heptadecanoic acid, 16-methyl-methyl ester, 2-nonanone,9-[tertahydro-2H-pyran-2-yl)oxy
Antiviral	α-Pinene, limonene
Antioxidative	4-Hydroxycoumarin, 7-hydroxycoumarin, quinone derivative
Antiproliferative	Limettin, angelicin, 1-methyl ethyl ester 4-oxazolecarboxylic acid, 4,5-dihydro-2-phenyl
Antitumour	4-Hydroxycoumarin, 7-hydroxycoumarin, 7-methoxypsoralen, pimpinellin, osthol quinone derivative
Anti-HIV	7-methoxypsoralen, pimpinellin, 7-chloro-1,3-dihydro-5-phenyl-1(trimethylsilyl)-2H-1,4-bezodiazepin-2-one, Heptadecanoic acid, 16-methyl methyl ester
Immunomodulatory	Scopoletin
Antiinflammatory	Pimpinellin
Estrogenic activity	Diethyl phthalate
Topo I inhibitor	Bergapten, xanthotoxin, psoralen, angelicin
DNA Polymerase inhibitor	Hexadecane,1,1-bis(dodecyloxy) (alkane hydrocarbon), psoralen
Preventing osteoporosis	Nonane, 3 methyl-5 propyl-, pidolic acid 5-oxopyrrolidine-2-carboxylic acid

limonene, octanoic acid, methyl salicylate, dodecan-2-one), coumarins (scopoletin, limettin, bergapten, xanthotoxin, angelicin pimpinellin), qinoline alkaloids (dictamine, graveliferone) and some Rutaceae specific alkaloids (arborinin) (Feo *et al.* 2002; Oliva *et al.* 2003). It also showed presence of root specific metabolites like chalepensin, daphronetin, marmesin, graveliferone, quinoline alkaloids (2-*N*-dodecyl-1,4-quinolone, 2-*N*-tetradecyl-4-quinolone, gravoline), acridone alkaloids (1,3-dihydroxy-*N*-methylacridone, rutacridone 1-hydroxy-*N*-methylacridone, 1-hydroxy-3-methoxy-*N*-methylacridone) (Feo *et al.* 2002; Oliva *et al.* 2003). This may be due to unique organizational status of this cell line which has shoot and root primordia (Diwan and Malpathak 2009). Most of the essential oils, higher coumarin and alkaloid derivatives, were found to be restricted to shoot line and transformed shoot line as cellular organization is essential for their biosynthesis. A derivative of octyl butarate was found in culture lines with high furanocoumarin content. In this light, it is interesting to note that, *in planta*, octyl butarate was reported to be associated with tissues having high furanocoumarin content in parsnip (Cianfrogna *et al.* 2002). Such association *in vitro* has been observed for the first time. It was seen that there exists a specific and inheritable metabolic fingerprint, probably related to different types of cultures. Similarly, heritable changes in metabolic profiles of cucumber plants regenerated in different types of *in vitro* culture (leaf callus culture, cell suspension and meristematic clumps) were reported by Filipecki *et al.* (2005)

Though interesting information can be revealed through point-by-point metabolite analyses, for greater in-depth understanding and for identifying culture specific metabolite pattern, multivariate analysis is necessary.

The data matrix was subjected to a multivariate analysis. First, HCA of the samples was performed using the Euclidean correlation. The organized (RC6, RS2, Ia3 lines as well as *in vivo* samples) and the unorganized cultures (RC1 and RC3) formed two separate groups (**Fig. 1A**). Within the organized cultures, culture lines RC6, RS2, Ia3 and *in vivo* shoots formed a distinct cluster, clearly separated from roots. Further, the organized cell line RC6 was clearly separated from the shoot lines and *in vivo* shoot samples.

These results indicate the existence of a specific metabolic phenotype reflecting the organisation status of the culture. We tested this by applying the PCA tool to our combined data set (**Fig. 1B**). PCA revealed two major types of metabolic differences within the cultures analyzed (represented by dotted circles). First, in accordance with HCA, PCA showed a clear difference between the selected culture lines, separating the organized cultures, on the one hand, from the unorganized cultures, on the other hand. In addition, PCA revealed a clear metabolite pattern within each metabolite cluster, separating the *in vitro* cultures lines from one another. The stems and roots (*in vivo*) samples were located in the middle of both vectors. This is logical, since these were the reference samples.

Elucidating individual metabolites

Having established that metabolic phenotyping coupled with HCA and PCA could be employed to distinguish the general metabolic component in different lines, the natural progression was to characterize the metabolic traits underlying the clustering and separation phenomena. For this, the component matrix of the PCA was screened for metabolites with high loadings in the factors of the specific principal components that produced clustering and separation in the scatter plots. The greater the loading, the more the variable is a pure measure of the factor (Tabachnick and Fidell 2001) and the more influence it has on the generation of the principal component. Therefore, high-loading variables are responsible for generating clusters and separation in principal component. It has been suggested that loadings in excess of 0.71 are 'excellent', 0.63 'very good', 0.55 'good', 0.45 'fair', and 0.32 'poor' (Comrey and Lee 1992). In this study metabolites with only excellent loadings were extracted from the component matrix for the first principal component from datasets of all culture lines (**Table 3**).

Scatter plots of PCA factor scores for dataset of selected culture lines are presented in **Fig. 2**. Plotted points represent individual samples, while arbitrary ellipses have been included to assist interpretation and simply border all metabolite patterns of individual lines.

Metabolites characterizing the clustering were assigned identities (based on mass spectrum and retention time) and classified as 'essential oils', 'alkaloids', 'coumarins', or 'other' (including derivatives, precursors, pathway intermediates) molecules. The principal components derived from factor-1 and factor-2 clustered and separated all culture lines. In factor-1, 36% of high-loading metabolites were alkaloids. In addition, there was evidence of essential oils (21%), others (21%) with a few examples coumarins (17%) (**Table 4, Fig. 2**). With these results it is appropriate to suggest that, the clustering and separation of all organized and unorganized culture lines with minimal overlap was heavily related to differences in essential oil components, alkaloids and coumarins. Within the organized cultures, lines separated on the basis of their alkaloid and coumarin composition. Similarly characteristic metabolite pattern, recognized using PCA and high loadings metabolites in matrix component of PCA, was successfully used as a selection tool for genotype discrimination in *Populus* (Robinson *et al.* 2005).

In this study all 79 monitored metabolites showed variation in the selected culture lines which differed in their organization level. Each tissue culture system had its characteristic set of changed compounds. This revealed two important conclusions: first that culture lines can be distinguished on the basis of their metabolic complement; and

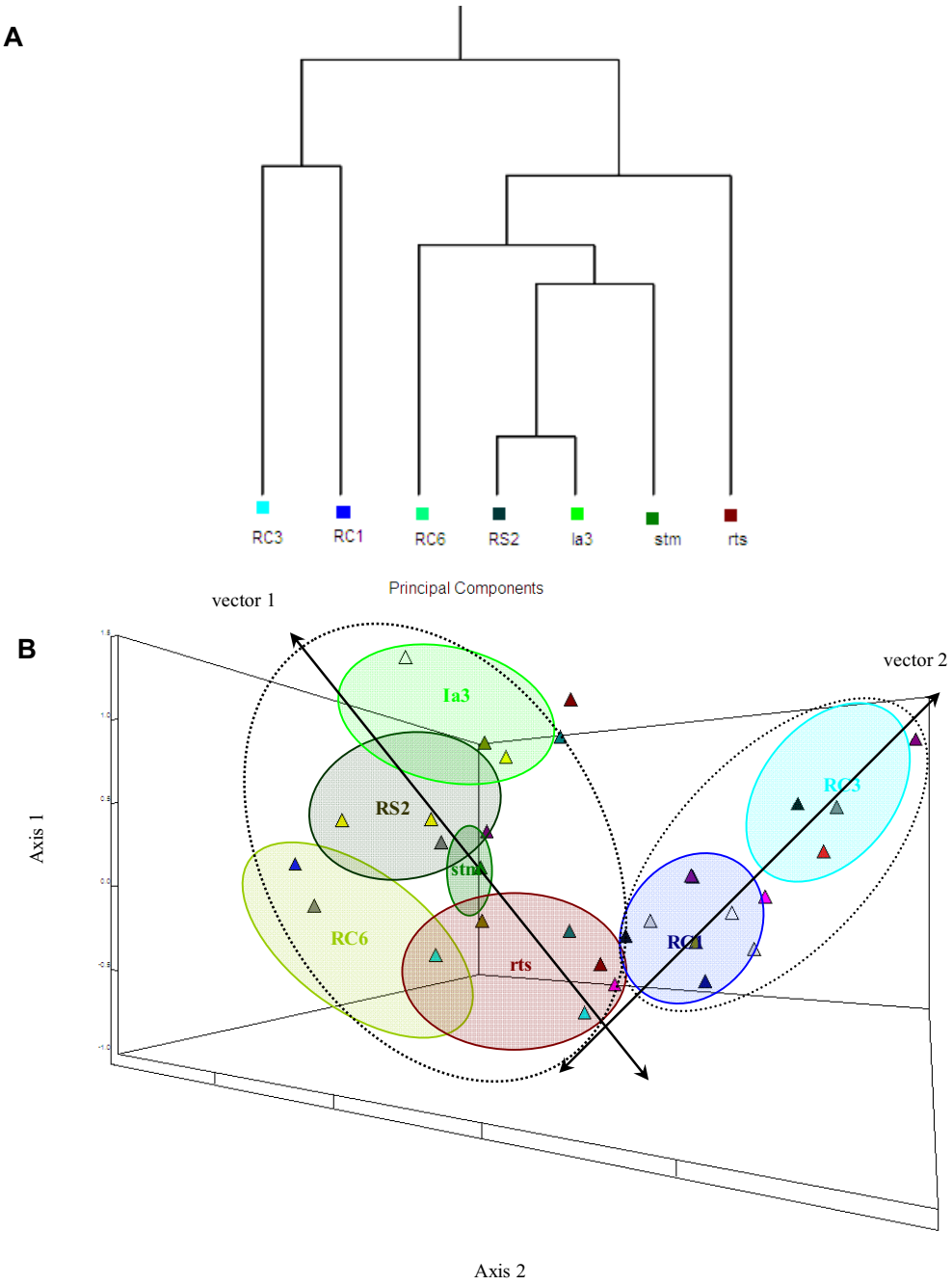

Fig. 1 Multivariate analyses of selected culture lines. (**A**) HCA of the selected culture lines. Hierarchical tree of the selected culture lines based on 79 individual metabolites. (**B**) PCA of the selected culture lines. PCA plot shows two major types of differences between the cultures: organized (vector 1) and unorganized (vector 2).

second metabolic pattern is under strict control of organization level among the systems tested. Similarly with the GC-MS aided biochemical phenotyping it was possible to analyze the patterns of metabolites within the metabolite groups in *Cucumis sativus* plants of a different tissue culture origin (Filipecki *et. al.* 2005).

CONCLUDING REMARKS

Bio-prospecting based GC-MS analysis of the culture lines indicated presence of several (major) metabolites with wide range of bioactivities such as photobiological, anti-microbial, anti-viral, anti-oxidative, anti-proliferative, anti-inflammatory, anti-tumour, anti-platelet aggregation, anti-HIV, immunomodulatory, estrogenic activity etc. with potential economic importance. This non-targeted screening of metabolites was advantageous as it permitted analysis of certain natural compounds that are difficult to isolate, purify, and synthesize. Multivariate analysis demonstrated that metabolic traits can be dissected reliably and accurately by metabolomic analyses, enabling the discrimination of individual genotypes of the same plant that exhibit marked differences in pharmaceutically important metabolites. With the use of metabolic phenotyping, *in vitro* cultures can be used as novel screening resources for new or improved phyto-pharmacueticals.

ACKNOWLEDGEMENTS

The financial assistance provided by University Grants Commission (UGC), New Delhi and UGC-DRS-SAP, New Delhi, is duly acknowledged.

Table 3 High loadings factors of the principal components that produced clustering and separation in the scatter plots - PC-1.

	Class	Metabolite	Loading value	
			PC1	PC2
1	Essential oils	α-Pinene	1.9699	-0.39357
2		Limonene	1.1697	-0.31531
3		Non-2-ene	1.1507	-0.33531
4		Nonan-2-one	1.5398	0.97095
5		Octanoic acid	1.73026	-0.65118
6		Methyl salicylate	1.71026	-0.60118
7		Decan-2-one	1.30027	-0.8007
8		Octyl acetate	0.75597	-0.46611
9		Undecan-2-one	0.79597	-0.46611
10	Coumarins	7-Methoxypsoralen	1.31027	-0.8707
11		Pimpinellin	1.9699	-0.36357
12		Chalepensin	1.70549	-0.26333
13		Isoimperatopin	1.6299	-0.56309
14		Isopimpinellin	1.6299	-0.46309
15		scopolein derivative	1.13086	-0.65046
16		Angelicin	1.83409	0.05983
17	Alkaloids	Gravoline	1.11904	0.86342
18		2-*N*-Nonyl-4-quinolone	1.57445	-0.22778
19		ϒ-Fagarine	2.19446	0.49828
20		Kokusaginine	1.39027	-0.8007
21		Ardorinine	1.40027	-0.8807
22		Dictamnin	2.17409	0.20935
23		Skimmianine plant	1.91445	-0.07825
24		1-Methyl-2-*N*-nonyl-4-quinolone	1.63026	-0.65118
25		2-*N*-dodecyl-1,4-quinolone	1.73049	-0.21333
26		2-*N*-tetradecyl-4-quinolone	1.3905	-0.36286
27		3-Methyl-2-nonyl-1H-quinolin-4-one	1.73026	-0.65118
28		3-Methyl-2 undecyl-1H-quinolin-4-one	1.65026	-0.68118
29		2,2,6-Trimethyl-2H,5H-pyrano[3,2-c]quinolin-5-one	1.37027	-0.8007
30		1,3-Dihydroxy-*N*-methylacridone	1.9699	-0.30357
31		Furacridone plant	1.37027	-0.8607
32		Arborinin	1.6499	-0.51309
33	Others	Guaiacol	1.6299	-0.51309
34		Indole	1.04323	0.85982
35		Diethylphthalate	1.95505	0.69851
36		Isobutyl phenol	1.71026	-0.65118
37		Benzophenone	1.13986	-0.60046
38		Benzothizole	1.12086	-0.69046
39		Syringol	1.90991	0.06343
40		Phthalic acid, isobutyl octyl ester	1.47086	-0.50094
41		Phthalide	0.82419	1.29898

Only metabolites loading >0.75 in the component matrix are shown. The loading of each peak is shown and metabolites are identified.

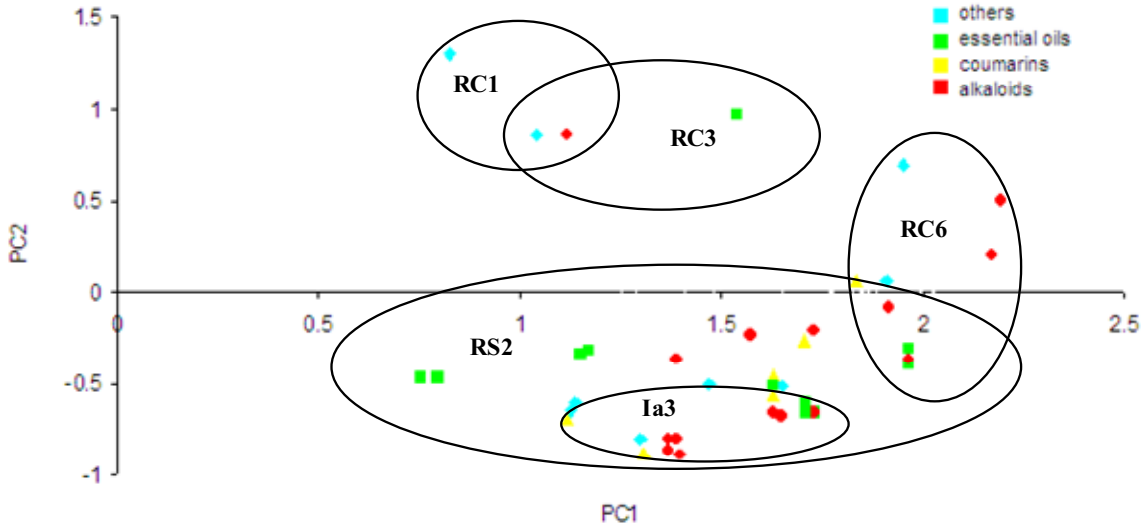

Fig. 2 Scatter plot of high loading metabolites of PC1. Only metabolites loading >0.75 in the component matrix are shown. The loading of each peak is shown and metabolites are identified.

Table 4 Molecule classification of the metabolites loading highly in factors of PCA component matrices for the principal component

Molecule type	RC1	RC3	RC6	RS2	Ia3
Essential oils	-	1	2	7	2
Alkaloids	1	1	4	5	7
Coumarins	2	-	-	5	1
Others	-	1	1	6	1
Total	3	3	7	23	11

Numbers represent the number of molecules from the stated class that load high in first principal component.

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