Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 11, 2015

Original Article

COMPARATIVE CHEMOMETRIC PROFILES BETWEEN LEAF TISSUES OF *WITHANIA SOMNIFERA* CULTURED *IN VITRO* AND FIELD

PANKAJAVALLI THIRUGNANASAMBANTHAM[1], KALAISELVI SENTHIL[1*], TAEK-JOO OH[2], HYUNG-KYOON CHOI[2]

[1]Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam University for Women, Coimbatore 641043, India, [2]College of Pharmacy, Chung-Ang University, Seoul 156756, Republic of Korea
Email: aubio.ptc.kalai@gmail.com

Received: 22 Jul 2015 Revised and Accepted: 10 Sep 2015

ABSTRACT

Objective: Metabolomic profiling of herbal extracts is indispensable to standardize drugs and to inaugurate the scientific basis of their therapeutic properties. The present study was attempted with an objective to investigate a comparative GC-MS (Gas chromatography–Mass spectrometry) analysis of *in vitro* and field grown leaf tissues of "Indian ginseng".

Methods: GC-MS often serves the methods of option for screening and quantitative metabolite profiling. In the present study, metabolic profiling of methanolic extracts of field and *in vitro* cultured *Withania somnifera* (Ashwagandha) leaf tissues were carried out using GC–MS technique.

Results: A total number of 39 primary metabolites in leaf were identified. These include alcohols, organic acids, purine, pyrimidine, sugars and putrescine. Highly significant qualitative and quantitative differences were noticed between the leaf tissues cultured *in vitro* and from the field. Especially, significant elevation in the accumulation of GABA (γ amino butyric acid) and putrescine was recorded in *in vitro* cultured leaf samples.

Conclusion: We conclude that *in vitro* cultures offers an intrinsic advantage to produce therapeutically valuable compounds, relatively in a short span of time and this principle determine its use as an alternative to field grown sample.

Keywords: *Withania somnifera*, *In vitro* leaf, Field grown, GC-MS, GABA, Putrescine.

INTRODUCTION

Withania somnifera, commonly known as Ashwagandha, is widely known for its popularity as a traditional medicinal herb since time immemorial. It is used as herbal medicine in various forms (decoctions, infusions, ointments, powder and syrup) in different parts of the world [1-3] for all age groups of patients without any side effects even during pregnancy [4,5]. Various bioactive constituents of this plant have been reported to possess adaptogenic, anticancer, anticonvulsant, immunomodulatory, antioxidative and neurological effects [6].

The major biochemical constituents of this plant are C-28 steroidal lactones in a class of compounds named withanolides [7] built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring [8]. Among the withanolides, Withanolide A and Withaferin A were reported to be dominant metabolites [9,10] distributed among various tissues at varying concentrations [11] and were proven to posses many therapeutic properties. The annual production of this plant is not sufficient to meet the global requirement [12] as its therapeutic advantages continuously attract the attention of pharmacologists and researchers alike. Therefore, *in vitro* culture system could be an alternative to native field grown plants for the production of medicinal valuable compounds. *In vitro* cultures possess a tendency to produce secondary metabolites within a short period than the field-grown plant owing to active growth [13]. However, the metabolic constituents, particularly secondary metabolites differ within the tissue type and sometimes with growth conditions [14]. This causes difficulties in the compositional standardization of herbal formulations and the commercial exploitation of this plant. Thus, analyzing total metabolome of this plant is extremely important to understand the complex biochemical processes.

Noteworthy technical innovations in analytical systems like NMR, GC–MS and HPLC have opened up new avenues for plant metabolomics research because of rapid identification of a large array of metabolites both quantitatively and qualitatively. GC–MS is the best technique to identify the bioactive constituents of long chain hydrocarbons, alcohols, acids; ester, alkaloids, steroids, amino and nitro compound [15]. Gas chromatography (GC) is recognized as the most powerful technique to find the number and proportion of compounds present in a complex mixture of volatile compounds. When GC is coupled to mass spectrometry (GC-MS), additional information arises about each separated compound molecular mass, elemental composition, functional groups, and in certain cases, molecular geometry and spatial isomerism [16].

The developed GC-MS method was therefore applied to the analysis of metabolites in young and mature leaf samples of both *in vitro* and field grown plants of *Withania somnifera*. To the best of our knowledge, we present here the first GC-MS metabolite profiles of *in vitro* cultured tissues in comparison with field grown tissue.

MATERIALS AND METHODS

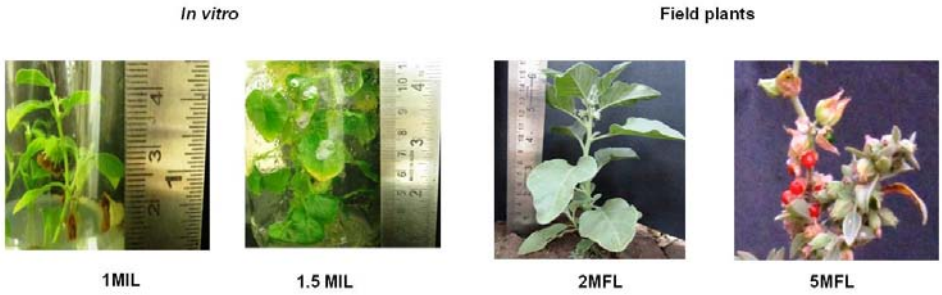
Solvents and chemicals

HPLC-grade methanol, water, pyridine and methoxyamine were purchased from Sigma (St. Louis, MO, USA). BSTFA [N,O-bis (trimethylsilyl) trifluoroacetamide containing 1% TMCS (trimethyl chlorosilane)] were obtained from Alfa Aesar (Ward Hill, MA, USA) and 2-Chloronaphtahalene as internal standard for GC-MS were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan).

Plant materials

Seeds of *W. somnifera* (L.) Dunal 'Jawahar' were obtained from Central Institute of Medicinal and Aromatic Plants (Lucknow), surface sterilized as per the procedure described by Murthy *et al.*[17] and inoculated in Murashige and Skoog solid basal medium containing 2% sucrose and incubated in the dark at 25 ˚C for germination. Shoots from *in vitro* germinated seedlings were cultured in basal MS media and were maintained at 25 ˚C for a 16 h photoperiod. *In vitro* leaf tissues after 30 d (1MIL) and 45 d (1.5MIL) of inoculation were collected (fig. 1). To obtain field grown root samples, the same seeds were sown in the field following standard cultivation practices. The seeds were sown in the month of August, which has been known as an ideal plantation time, in the field of Avinashilingam Institute, Coimbatore, Tamilnadu. The field lies

between 11 °1' North latitude and 76 °57' East longitude. The city receives an annual rainfall of around 700 mm from September to October with the north east and south west monsoons. The seeds are sown about 2 cm deep into the soil at a distance of about 50 cm. The method of sowing was preferred as it promotes the development of a healthy root system compared to being sown using the broad casting method. The temperature range was 28-30 °C. There was no application of fungicides or organic fertilizers. The field was irrigated twice in a week. Young and mature leaf samples were respectively collected after 60 d (2MFL) and 150 d (5MFL) after germination. The leaf samples grown *in vitro* and in the field were sampled for GC-MS analysis.



1MIL, 1 mo *in vitro* leaf; 1.5ML, 1.5 mo *in vitro* leaf; 2MFL, 2 mo field-grown leaf; 5MFL, 5 mo field-grown leaf;

Fig. 1: *In vitro* and field grown leaf tissues

Sample preparation for GC-MS analysis

20 mg of all dried samples was extracted with 1 ml of 70% methanol to analyze metabolites by GC-MS. The extracts were sonicated for 30 min, followed by centrifugation at 2,000 rpm for 5 min. The supernatant was filtered through a 0.45 µm filter (PTFE Sartorius Stedim Biotech, Göttingen, Germany). After extraction, 100 µl of each filtered sample was transferred into a GC vial for derivatization prior to GC-MS analysis.

Samples in GC vials were dried under the stream of nitrogen for 5 min, followed by the addition of 30 µl of 20,000 µg/ml methoxylamine hydrochloride in pyridine, 50 µl of BSTFA (N,O-Bis (trimethylsilyl) trifluoroacetamide; Alfa Aesar, Ward Hill, MA, USA) containing 1% TMCS (trimethyl chlorosilane), and 10 µl of 2-chloronaphthalene (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) 200 µg/ml in pyridine as an internal standard) to the dried vials, respectively. After derivatization, the samples were incubated for 60 min at 60 °C and then subjected to GC-MS analysis.

GC-MS analysis

The GC-MS analysis was performed using 7890A Agilent GC system (Agilent Technologies, CA, USA) equipped with a 5975C MSD detector (Agilent Technologies) and automatic sampler (7683 B series, Agilent Technologies). Electro impact ionization mode with ionization energy of 70 eV was used for GC-MS detection. Analytes were separated on a fused silica capillary column of 5% phenyl methylpolysiloxane phase (DB-5, Agilent Technologies) with dimensions of 30 mm × 0.25 mm i.d. × 0.25 µm film thickness. Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. the inlet temperature was set to 250 °C with an injection volume of 1.0 µl and a split ratio of 1:10. The mass range was 50-700 Da and data were obtained in MSD with a full scan mode. The oven temperature was programmed initially at 70 °C and followed by a rise to 150 °C (at 5 °C/min) and then to 250 °C (at 3 °C/min; hold 2 min) and finally to 320 °C (at 10 °C/min; hold 3 min).

Data processing

To quantitatively compare global metabolic profile among all samples, the raw datasets by GC-MS analysis was processed as described previously [18]. The mass spectra deconvolution was performed using the AMDIS (Automated Mass Spectral Deconvolution and Identification System, http://chemdata. nist. gov/mass-spc/amdis/) and then created the ELU files which were analyzed with an online peak-filtering algorithm (SpectConnect, http://spectconnect. mit. edu). The identification of the metabolites was achieved by comparing mass spectra with those of the NIST-Wiley Mass Spectra Library. After peak area normalization with an internal standard, multivariate statistical analysis was performed. In order to analyse statistically, the relative intensities and significant differences of the datasets obtained by GC-MS analysis of each sample was subjected to analysis of variance (ANOVA) using PASW Statistics 19 software (IBM, Somers, NY), followed by Turkey's significant-difference test with a 95% confidence level. In order to reduce the dimensionality of the data set, PCA (Principal Component Analysis) was mainly applied and used as the first step of data analysis to identify patterns and outliers. PCA transforms original valuables into new uncorrelated variables by means of the principal components that account for the variability in original datasets as much as possible. The loading plots derived by PCA indicated the importance of each variable for the model and were used to identify metabolites responsible for the separation and to interpret the relationship among variables and clusters on the PCA score plot [19].

RESULTS

GC-MS analysis of *W. somnifera* leaf extracts

The results pertaining to GC-MS analysis led to the identification of the number of compounds from the GC fractions of the 70% methanolic extract of *W. somnifera* leaves grown in field or *in vitro* conditions 39 metabolites were identified. As shown in table 1, the following 39 metabolites were identified: alcohols (glycerol, myo-inositol, and xylitol), amino acids (asparagines, aspartic acid, glutamic acid, glutamine, glycine, histidine, homoserine, isoleucine, lysine, proline, serine, threonine, and tryptophan), organic acids (citric acid, fumaric acid, galactonic acid, glucaric acid, gluconic acid, glucuronic acid, glyceric acid, malic acid, malonic acid, pyruvic acid, rythronic acid, succinic acid, α-aminoadipic acid, and γ-aminobutyric acid), purine (xanthine), pyrimidine (uracil), sugars (fructose, galactose, glucose, mannose, melibiose, and xylose), putrescine. The differences of relative levels of the metabolites assigned by GC-MS were investigated and the biochemical changes through metabolic pathway were shown in fig 2. Compared with the leaves grown *in vitro* conditions (1MIL and 1.5MIL), relatively higher levels of myo-inositol in 2MFL were observed.

The relative levels of each metabolite were obtained by dividing the percentage area of each metabolite by the percentage area of the internal standard. Diff

ANOVA was performed to assess the statistical significance of differences between samples ($p<0.05$). Data are mean values with error bars representing standard deviation values. Different letters in bars represent the difference of statistical significance of metabolites levels.

Among amino acids, Asparagine, Aspartic acid, glutamine, glycine, proline, serine and threonine were present significantly in higher intensities in leaves harvested from *in vitro* condition in comparison to field grown condition. Similarly, citric acid, Gluconic acid, malic and

γ-amino butyric acid among organic acids category were also recorded to be present in elevated intensities among *in vitro* leaf samples.

Significantly, higher levels of GABA in the leaves grown *in vitro* (1MIL and 1.5MIL) than that of the leaves grown field (2MFL and 5MFL) were observed. The levels of soluble sugars, including fructose and glucose, were significantly different between the field grown and *in vitro* cultured samples.

Principal component analysis (PCA)

The PCA derived score plot of *W. somnifera* leaves is indicated in fig. 3 (A) where PC 1 explained 50.9% of the total variation in the data, while PC 2 explained 17.7%. There were clear differences between the field (2MFL and 5MFL) and *in vitro* (1MIL and 1.5MIL) conditions along PC 1. A general separation from the field samples with 2MFL and 5MFL was observed. However, the discrimination between *in vitro* samples with 1MIL and 1.5MIL was not satisfactory.

According to loading (fig. 3 (B), the relatively higher proline and myo-inositol accumulation among the field grown leaf samples determined to be a distinguishing factor from that of *in vitro* grown leaf samples. And also higher levels of uracil, glutamic acid and tryptophan were observed in 2MFL and 5MFL. On the other hand, glucose and mannose were recorded to be abundant in 1MIL and 1.5MIL, which are soluble sugars that cause differentiation between the leaves grown *in vitro* and the field. In addition, elevated levels of γ-aminobutyric acid were shown in 1MIL and 1.5MIL accompanied by depleted levels of glutamic acid was observed.

DISCUSSION

Our results showed that relatively higher levels of myo-inositol in 2MFL were observed compared to leaves grown *in vitro* conditions (1MIL and 1.5MIL). It has been reported that myo-inositol could serve as substrates for the productions including $_D$-ononitol and $_D$-ponytail that have tolerance to drought or high salt conditions [20, 21]. Therefore, the concentration of myo-inositol is important for osmotic regulation. Plant cells, like some animal tissues, have the capacity to synthesize myo-inositol from D-glucose [22] via the enzyme L-myo-inositol 1-phosphate synthase, which utilizes D-glucose 6-phosphate as substrate [23, 24]. The L-myo-inositol 1-phosphate formed is then dephosphorylated to yield Myo-inositol. Moreover, it was reported by Nelson *et al.* [25] that higher accumulation of myo-inositol in cytosol was seen during salt stress. Field grown plant are adversely affected by salinity, a major environmental stress that limits agricultural production [26].

Table 1: A GC-MS based metabolic profiling of 70% methanol extracts of *W. somnifera* leaves

Compound	RT (min)	Relative intensity			
		2MFL	5MFL	1MIL	1.5MIL
Alcohols					
Glycerol	10.25	24.15±2.81^{a}	5.84±0.71^{b}	6.81±0.47^{b}	6.49±1.04^{b}
Myo-inositol	33.42	73.20±7.97^{a}	36.10±5.36^{b}	29.93±2.24^{bc}	22.07±3.48^{c}
Xylitol	22.21	0.49±0.10^{a}	1.94±0.38^{b}	0.28±0.03^{a}	ND
Amino acids					
Asparagine	20.77	5.86±0.95^{a}	23.83±3.33^{b}	40.54±2.81^{c}	34.79±7.11^{c}
Aspartic acid	16.60	3.13±0.59^{ab}	2.26±0.52^{a}	6.44±0.52b^{c}	6.84±2.61^{c}
Glutamic acid	19.32	5.64±1.15^{a}	5.97±1.17^{a}	3.37±0.97^{b}	3.62±0.72^{b}
Glutamine	23.89	2.09±0.06^{a}	13.46±3.13^{b}	23.19±3.70^{c}	10.32±5.97^{b}
Glycine	10.97	1.73±0.23^{a}	1.77±0.50^{a}	3.37±0.27^{b}	3.82±0.65^{b}
Histidine	28.13	ND	ND	0.63±0.06	0.54±0.07
Homoserine	14.78	0.36±0.06^{a}	0.62±0.11^{a}	2.06±0.14^{b}	1.73±0.39^{b}
Isoleucine	10.69	3.07±0.48^{a}	5.09±1.77^{ab}	4.94±0.35^{ab}	6.85±1.35^{b}
Lysine	28.35	0.43±0.07^{a}	1.04±0.24^{a}	2.71±0.20^{b}	2.41±0.55^{b}
Proline	10.75	49.80±9.39^{a}	138.03±17.71^{b}	70.49±9.39^{a}	44.10±9.61^{a}
Serine	12.48	4.35±0.68^{a}	8.61±1.36^{ab}	11.53±0.90^{bc}	17.59±4.90^{c}
Threonine	13.13	4.91±0.64^{a}	10.74±1.39^{b}	13.17±0.93^{b}	15.42±3.70^{d}
Tryptophan	36.38	0.78±0.12^{a}	5.88±1.01^{b}	ND	ND
Organic acids					
Citric acid	25.28	11.55±2.19^{a}	19.32±3.43^{b}	20.26±2.05^{b}	9.00±1.43^{a}
Fumaric acid	12.25	0.13±0.01^{a}	0.51±0.10^{b}	0.46±0.06^{b}	0.37±0.06^{a}
Galactonic acid	30.55	ND	ND	0.47±0.12	0.60±0.10
Glucaric acid	32.00	ND	ND	1.14±0.19^{a}	0.29±0.04^{b}
Gluconic acid	30.70	2.20±0.60^{a}	0.64±0.11^{b}	1.11±0.17^{b}	0.55±0.10^{b}
Glucuronic acid	42.08	0.81±0.11^{a}	0.55±0.09^{a}	4.49±0.39^{b}	1.70±0.27^{c}
Glyceric acid	11.68	2.95±0.37^{a}	2.15±0.32^{b}	1.97±0.13^{bc}	1.37±0.21^{c}
Malic acid	15.82	15.03±2.27^{a}	33.54±5.60^{b}	49.02±4.13^{c}	25.22±4.37^{ab}
Malonic acid	31.36	0.34±0.11^{a}	0.21±0.05^{ab}	0.21±0.02^{ab}	0.13±0.02^{b}
Pyruvic acid	15.24	0.11±0.02^{a}	0.12±0.02^{a}	0.37±0.01^{b}	0.37±0.06^{b}
Rythronic acid	17.69	1.95±0.25^{a}	1.31±0.18^{b}	1.07±0.08^{bc}	0.66±0.11^{c}
Succinic acid	11.27	5.09±0.55^{a}	5.61±0.86^{a}	2.88±0.20^{b}	2.15±0.31^{b}
α-Aminoadipic acid	22.09	0.31±0.04^{a}	0.24±0.04^{a}	0.29±0.02^{a}	0.70±0.14^{b}
γ-Aminobutyric acid	16.77	13.57±1.58^{a}	21.40±8.52^{b}	58.86±9.20^{c}	31.37±6.07^{b}
Purine					
Xanthine	31.26	ND	ND	1.17±0.15^{a}	0.05±0.01^{b}
Pyrimidine					
Uracil	11.81	0.49±0.06^{a}	0.22±0.03^{b}	0.11±0.01^{c}	0.28±0.05^{d}
Sugars					
Fructose	24.76	11.69±1.31^{a}	10.33±1.73^{a}	88.15±7.09^{b}	34.09±7.45^{c}
Galactose	28.22	2.62±0.35^{a}	2.37±0.39^{a}	4.59±0.42^{b}	2.26±0.34^{a}
Glucose	28.87	140.81±16.22^{a}	48.72±6.10^{b}	294.08±23.45^{c}	258.70±40.19^{c}
Mannose	27.46	12.51±1.67^{a}	7.49±0.85^{a}	40.39±3.70^{b}	22.83±3.35^{c}
Melibiose	54.25	15.83±2.05^{a}	1.41±0.23^{b}	9.81±0.60^{c}	7.24±1.06^{c}
Xylose	26.87	14.17±2.16^{a}	3.09±0.34^{b}	30.91±3.01^{c}	17.50±2.80^{a}
Other					
Putrescine	22.49	0.23±0.03^{a}	0.21±0.04^{a}	0.72±0.07^{b}	0.81±0.16^{b}

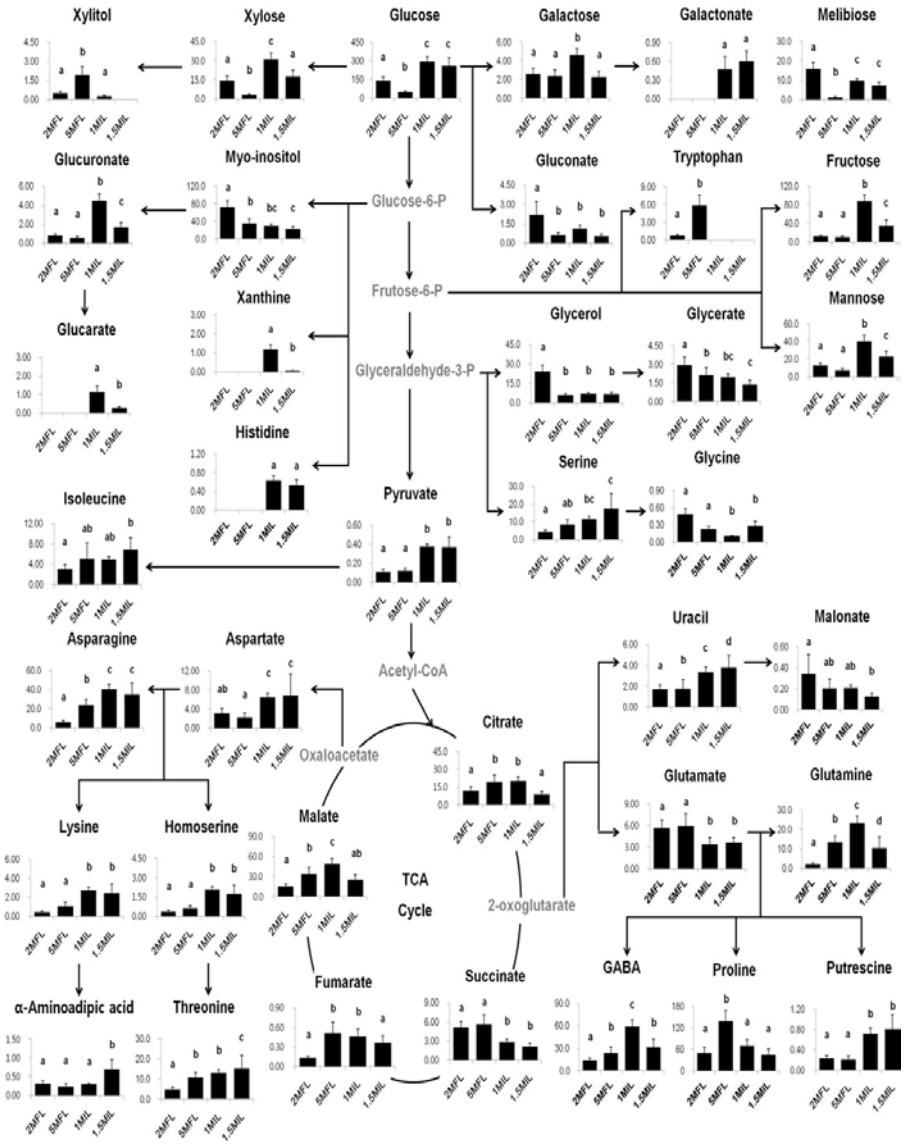


Fig. 2: Schematic diagram of the metabolic pathway and relative levels of the major compounds detected in *W. somnifera*. This was modified from pathways presented in KEGG database(http://www. genome. jp/kegg/)

In the present study, intensity of GABA was recorded to be significantly higher in *in vitro* leaf tissues. GABA was reported to be rapidly induced in a variety of plant tissues under several abiotic environmental conditions that contain mechanical stimulation, damage, cold or heat shock, hypoxia, cytosolic acidification, darkness, water stresses, and hormonal changes [27]. In addition, Roberts [28] reported that GABA plays a signaling role in plant development and in stress responses.

The levels of soluble sugars, including fructose and glucose, were significantly different between the field grown and *in vitro* cultured samples. The sugar levels of *in vitro* cultured leaf samples were much higher than those of field grown leaf samples. Li *et al.* [29] observed similar result of high sugar levels released by peanut root cultures and concluded that accumulation of sugar molecules could provide the abundant nutrition for growth and development. Similarly, the level of putrescine was significantly different between the field grown and *in vitro* cultured samples. In plant cells, the diamine putrescine (Put), constitutes the major polyamines. They occur in the free form or as conjugates bound to phenolic acids and other low molecular weight compounds or to macromolecules such as proteins and nucleic acids. As such, they stimulate DNA replication, transcription and translation. They have been implicated in a wide range of biological processes in plant growth and development, including senescence, environmental stress [30-32].

PCA identifies and ranks major sources of variance within the data sets and allows clustering of biological samples into both expected and unexpected groups based on similarities and differences in the measured parameters [33]. PCA also revealed discrete metabolic phenotypes for the different organs and led to the identification of metabolite markers for each. The first component PC 1 accounted for 50.9% of the variance and allowed the distinction of young and mature field grown leaf samples. The PC 2 subsequent components covered a sum of 17.7% of variance, but did not yield distinctions between the samples that could be linked to organ age or plant growth conditions [33].

These comparisons were motivated by sample classifications made evident from PCA analysis: comparison of samples cultured *in vitro* as well as field. The differences could be due to the samples harvested at different time point and from different environmental conditions [33]. [34] identified the variation in the antioxidant potential of different tissues of *N. indicum* plant using PCA accompanied by hierarchical cluster analysis (HCA). As a result of the PCA score plots, it could be concluded that the metabolic profile by GC-MS provide much differentiation between *in vitro* and field samples and various metabolites, including myo-inositol, proline and soluble sugars produced to adapt environment stress was identified as important factors determining discrimination between the samples grown *in vitro* and field.

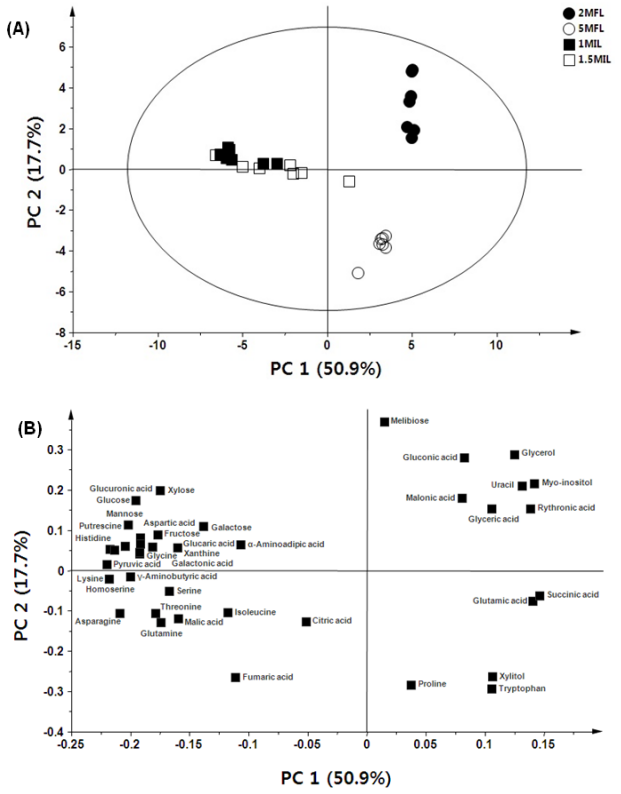


Fig. 3: PCA score plots

Principal component analysis (PCA) of the metabolomes derived from *in vitro* cultured and field grown leaves of *W. somnifera*. (A) PCA derived score plots. (B) PCA-derived loading plots. PC: principal component.

CONCLUSION

First time on record, we compared the metabolite profiles between leaf tissues cultured in *in vitro* and field grown conditions. The results of this study highlighted that *in vitro* leaf cultures able to accumulate metabolites in similar fashion as that of field grown tissues. Hence, we conclude the potential utilization of *in vitro*-cultured *W. somnifera* leaves as alternative resources to field-grown leaves for the production of useful metabolites such as γ-amino butyric acid and putrescine, as they exhibited relatively higher intensities in a shorter cultivation period. This study implies that *in vitro* cultured leaves of *W. somnifera* can be used for the development of biopharmaceuticals or functional foods.

ABBREVIATION

GC-MA, Gas chromatography–Mass spectrometry; GABA, γ aminobutyric acid; 1MIL, 1 mo *in vitro* leaf; 1.5MIL, 1.5 mo *in vitro* leaf; 2MFL, 2 mo field grown leaf; 5MFL, 5 mo field grown leaf; PCA, Principal component analysis.

ACKNOWLEDGEMENT

This work was supported by Mid-career Researcher Program (NRF-012R1A2A2A02011748) through NRF grant funded by the MSIP and WOS-A Program of the Department of Science and Technology [SR/WOS-A/lS-532/2011(G)], New Delhi,

CONFLICT OF INTERESTS

Conflict of interest declared none.

REFERENCES

1. Archana R, Namasivayam A. Antistressor effect of *Withania somnifera.* J Ethanopharmacol 1999;64:91–3.
2. Davis L, Kuttan G. Effect of *Withania somnifera* on DMBA induced carcinogenesis. J Ethanopharmacol 2001;75:165–8.
3. Kumar A, Kaul MK, Bhan MK, Khanna PK, Suri KA. *Withania somnifera* (L.) Dunal (Solanaceae). Genet Resour Crop Evol 2007;54:655–60.
4. Gupta GL, Rana AC. *Withania somnifera* (Aswagandha): a Review. Pharmacognosy 2007;1:129–36.
5. Sharma S, Dahanukar S, Karandikar SM. Effect of long-term administration of the roots of ashwagandha and shatvari in rat. Indian Drugs 1985;29:133–9.
6. Chatterjee S, Srivastava S, Khalid A, Singh N, Sangwan RS, Sidhu OP, *et al*. Comprehensive metabolic fingerprinting of *Withania somnifera* leaf and root extracts. Phytochemistry 2010;71:1085-94.
7. Ray AB, Gupta M. Withanosteroids, a growing group of naturally occurring steroidal lactones. Prog Chem Org Nat Prod 1994;63:2–106.
8. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazon J. Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. Molecules 2009;14:2373-93.
9. Jayaprakasam B, Nair MG. Cyclooxygenase-2 enzyme inhibitory withanolides from *Withania somnifera* leaves. Tetrahedron 2003;59:841-9.
10. Ichikawa H, Takada Y, Shishodia S, Jayaprakasam B, Nair MG, Aggarwal BB. Withanolides potentiate apoptosis, inhibit invasion,and abolish osteoclastogenesis through suppression of nuclear factor-EB (NF-EB) activation and NF-EB–regulated gene expression. Mol Cancer Ther 2006;5:1434-45.
11. Praveen N, Naik PM, Manohar SH, Murthy HN. Distribution of withaolide A content in various organs of *Withania somnifera* (l.) Dunal. Int J Pharma Bio Sci 2010;1:1-5.
12. Sharada M, Ahuja A, Suri KA, Vij SP, Khajuria RK, Verma V, *et al*. Withanolide production by *in vitro* cultures of *Withania somnifera* (L.) and its association with differentiation. Biol Plant 2007;51:161-4.
13. Sivanandhan G, Arun M, Mayavan S, Rajesh M, Mariashibu TS, Manickavasagam M, *et al.* Chitosan enhances withanolides production in adventitious root cultures of *Withania somnifera* (L.) Dunal Ind Crops Prod 2012;37:124-9.
14. Abraham A, Kirson I, Glotter E, Lavie DA. Chemotaxonomic study of *Withania somnifera* (L.) dunal. Phytochemistry 1968;7:957–62.
15. Singariya P, Kumar P, Mourya KK. Identification of new bioactive compounds by GC-MS and estimation of physiological and biological activity of kala dhaman (*Cenchrus setigerus*). Int J Pharm Biol Arch 2012;3:610-6.
16. Stashenko EE, Martínez JR. GC-MS Analysis of Volatile Plant Secondary Metabolites. In: Gas Chromatography in Plant Science. Salih B. (Eds.); 2012. p. 402-20.
17. Murthy HN, Dijkstra C, Anthony P, White DA, Davey MR, Power JB, *et al.* Establishment of *Withania somnifera* hairy root cultures for the production of withanolide A. J Integr Plant Biol 2008;50:975–81.
18. Styczynski MP, Moxley JF, Tong LV, Walther JL, Jensen KL. Systematic identification of conserved metabolites in GC/MS data for metabolomics and biomarker discovery. Anal Chem 2007;79:966-73.
19. Erisson L, Johansson E, Kettaneh-Wold N, Trygg J, Wikstrom C. Multi-and megavariate data analysis. In: Kingston SJ. (Ed.). Basic principles and applications, Umetrics Academy Umea, Umea, Sweden; 2006. p. 195-9.
20. Adams P, Thomas JC, Vernon DM, Bohnert HJ, Jensen RG. Distinct cellular and organismic responses to salt stress. Plant and Cell Physiology 1992;33:1215-23.
21. Vernon DM, Bohnert HJ. A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum*. EMBO J 1992;11:2077-85.
22. Loewus FA, Kelly S. Conversion of glucose to inositol in parsley leaves. Biochem Biophys Res commun 1962;7:204-8.
23. Funkhouser EA, Loewus FA. Purification of myoinositol 1-phosphate synthase from rice cell culture by affinity chromatography. Plant Physiol 1975;56:786-90.
24. Loewus MW, Loewus FA. D-Glucose-6-phosphate cycloaldolase: Inhibition studies and aldolase function. Plant Physiol 1973;51:263-6.
25. Nelson DE, Koukoumanos M Bohnert HJ. Myo-Inositol-dependent sodium uptake in ice plant. Plant Physiol 1999;119:165–72.

26. Läuchli A, Grattan S. Plant growth and development under salinity stress. In: Advances in molecular breeding toward drought and salt tolerant crops. Jenks MA, Hasegawa PM, Jain SM. (Eds.). Dordrecht, Springer; 2007. p. 1–32.
27. Bouche F, Fromm H. GABA in plants: just a metabolite? Trends Plant Sci 2004;9:110-5.
28. Roberts MR. Does GABA act as a signal in plants? Plant Signaling Behav 2007;2:408-9.
29. Li XG, Zhang TL, Wang XX, Hua K, Zhao L, Han ZM. The composition of root exudates from two different resistant peanut cultivars and their effects on the growth of soil-borne pathogen. Int J Biol Sci 2013;9:164–73.
30. Tiburcio AF, Campos JL, Figueras X, Besford RT. Recent advances in the understanding of polyamine functions during plant development. Plant Growth Regul 1993;12:331-40.
31. Galston AW, Kaur-Sawhney R, Altabella T, Tiburcio AF. Plant polyamines in reproductive activity and response to abiotic stress. Bot Acta 1997;110:197-207.
32. Bais HP, Ravishankar GA. Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue Organ Cult 2002;69:1-34.
33. Desbrosses GG, Kopka J, Udvardi MK. *Lotus japonicus* metabolic profiling: development of gas chromatography-mass spectrometry resources for the study of plant-microbe interactions. Plant Physiol 2005;137:1302–18.
34. Dey P, Chaudhuri TK. Antioxidant capacity of *N. indicum*: a correlation study using principal component analysis and multivariate statistical approach. Int J Pharm Pharm Sci 2013;5:931-7.