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ORIGINAL PAPER

In vitro and in situ screening systems for morphological and phytochemical analysis of *Withania somnifera* germplasms

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Abstract We report, for the first time for *Withania somnifera,* the use of a modified in vitro system for morphological and phytochemical screening of true to type plants as compared with those grown in a conventional in situ system. Eleven germplasms of cultivated *W. somnifera* from different regions of India were collected to examine chemotypic variation in withaferin A (WA). Methods were developed to optimize WA extraction. The maximum concentration of WA was extracted from manually ground leaf and root material to which 60 % methanol was added followed by sonication in a water bath sonicator. Variation in WA concentration in whole plants was observed amongst the different germplasms. In the in vitro system, the concentration of WA ranged between 0.27 and 7.64 mg/g dry weight (DW) and in the in situ system, the range in concentration was between 8.06 and 36.31 mg/g DW. The highest amount of WA found in leaves was 7.37 and 41.42 mg/g DW in the in vitro and the in situ systems respectively. In roots, the highest WA concentration was 0.27 mg/g DW in the in vitro and 0.60 mg/g DW in the in situ system. There are distinct advantages in using the in vitro grown plants rather than those grown in the in situ system including the simplicity of design, efficient use of space and nutrition and a system which is soil and contaminant free. The proposed in vitro system is therefore ideal for utilization in molecular, enzymatic and biochemical studies.

Keywords In vitro · In situ · *Withania somnifera* · Withaferin A · Withanolides · HPLC

Abbreviations

ESI	Electron spray ionization
DW	Dry weight
HPLC	High performance liquid chromatography
HPLC–	High performance liquid chromatography
MS	mass spectrometry
MS	Murashige and Skoog
PDA	Photo diode array
WA	Withaferin A

Introduction

Crude extracts of *Withania somnifera* (*W. somnifera*) consist of rich repositories of phytochemicals (Chatterjee et al. 2010). Steroid alkaloids and lactones isolated from the various parts of the plant primarily consist of withanolides, which are considered to exhibit pharmacological activities. Among withanolides, WA, is well known for its medicinal properties (Kushwaha et al. 2012; Lee et al. 2010, 2012; Maitra et al. 2009; Mayola et al. 2011; Min et al. 2011; Mohan et al. 2004; Yang et al. 2011). *Withania somnifera* has been an important source of formulation for traditional medicines for centuries (Kaileh et al. 2007a). The use of *W. somnifera* in traditional medicine (Ayurveda) has prompted exploration into analysis and isolation of its phytochemical constituents (Chatterjee et al. 2010;

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Chaurasiya et al. 2008; Ganzera et al. 2003; Khajuria et al. 2004; Sharma et al. 2007b). The phytochemical constituents of both crude as well as purified extracts have been studied for their efficacy in in vitro and in vivo models to understand the mechanism behind their pharmacological actions (Aalinkeel et al. 2010; Mayola et al. 2011; Nakajima et al. 2011; Vaishnavi et al. 2012).

Withania somnifera is found naturally in subtropical and semi-temperate regions within dry areas. This specie also occurs widely in the Middle East, Africa, Pakistan, India, and the eastern Mediterranean region (Kumar et al. 2007, 2011; Patra et al. 2004). In India, wild plants of *W. somnifera* are distributed in the north-western region of Himachal, Jammu and Punjab and it is commercially cultivated in Madhya Pradesh, Rajasthan, Andhra Pradesh and Uttar Pradesh (Anon 1976; Kothari et al. 2003). Studies have shown considerable genotypic and phenotypic variation among wild and cultivated species (Atal 1975; Kual 1957). Reports have described genetic diversity based on morphometric and molecular markers in correlation with withanolide markers (Misra et al. 1998; Dhar et al. 2006, 2008; Jain et al. 2007; Kumar et al. 2007). Amplified fragment length polymorphism and selectively amplified microsatellite polymorphic loci DNA marker systems for analysing genetic relationships between *W. somnifera* genotypes have been documented (Negi et al. 2000, 2006). Biochemical and molecular studies have also been undertaken to analyse the metabolic pathways required for the synthesis of withanolides (Madina et al. 2007; Senthil et al. 2010; Sharma et al. 2007a). Due to genetic diversity in *W. somnifera*, compositional standardization of different herbal formulations is difficult, which has led to continuous commercial exploitation of the plant (Sangwan et al. 2004). It is thus necessary to select the best germplasm across the geographical range (Dhar et al. 2006; Kumar et al. 2007; Negi et al. 2006; Ramesh Kumar et al. 2011, 2012; Scartezzini et al. 2007).

Studies on withanolides to date have been mainly based on the conventional in situ pot grown plants in green houses or on plants grown in the field. Plants grown under such conditions show seasonal variation in growth, development and metabolite production along with different harvesting issues related to soil adherence to roots, damage to roots while washing and contamination of roots by soil parasites and root-rotting fungi. In view of these disadvantages and to address the heightened interest in withanolides for end-user benefits different variations in the in vitro system of plant growth have been proposed. These methods have included a variety of tissue culture methods (Wadegaonkar et al. 2006; Kulkarni et al. 2000; Manickam et al. 2000; Rani and Grover 1999; Roja et al. 1991; Sen and Sharma 1991) shoot cultures (Sangwan et al. 2007) and suspension cultures (Ciddi 2006; Nagella and Murthy 2010). Each of these methods of propagation was associated with a closed system supplemented with phytohormones.

The aim of the present study was to develop a modified in vitro system suitable for growth of true to type plants that would enable analysis of variation in germplasms on the basis of morphological and phytochemical parameters. The new in vitro system is based on the use of compartmentalized sterile containers that have been found effective for analyzing interactions of roots with microorganisms (Voets et al. 2009).

Materials and methods

Plant material procurement, seed sterilization and germination

Seeds of 11 germplasms of *W. somnifera* were obtained from cultivated sources from ten states of India (Fig. 1; Table 1). For surface sterilization, 100 seeds of each germplasm were washed in running tap water followed with soaking in 0.1 % w/v mercuric chloride in water for 5 min. The seeds were then rinsed five times with sterilized distilled water to remove traces of mercuric chloride. Then to promote germination the seeds were incubated in sterilized distilled water for 3 days at 25 ± 2 °C under white light (47 μmol m^{-2} s^{-1} photosynthetic photon flux density) with a 16 h photoperiod. Fifty sterilized, soaked seeds of each germplasm were washed again in sterilized distilled water and then placed on 30 ml Murashige and Skoog media (Murashige and Skoog 1962) in petri plates (90 mm). Seeds were incubated at 30 °C in the dark for germination. Plates were transferred to continuous light after seed germination and were maintained at 25 ± 2 °C with a 47 μmol m^{-2} s^{-1} photosynthetic photon flux density.

Growth of plants

In vitro system establishment of plants

For plant growth in the in vitro system, 30 ml of semi-solid MS medium (pH 5.8) with 0.25 % phytagel (Sigma, Bangalore, India) without any phytohormones was placed in each 90 mm diameter Petri dish and the dish closed with a lid that had a 2–3 mm hole in the center. Roots of a 3 week old seedling were inserted through the hole in the lid so that the roots touched the growth medium surface and the aerial part of the seedling was kept outside the Petri plate. Any gap between the hole in the lid and the seedling stem was sealed with sterilized silicon grease and a plastic film (Parafilm, Tarsons, New Delhi, India) was used to seal the perimeter of the plate. The Petri plate was covered with an opaque black card with a diameter of 110 mm with a slit

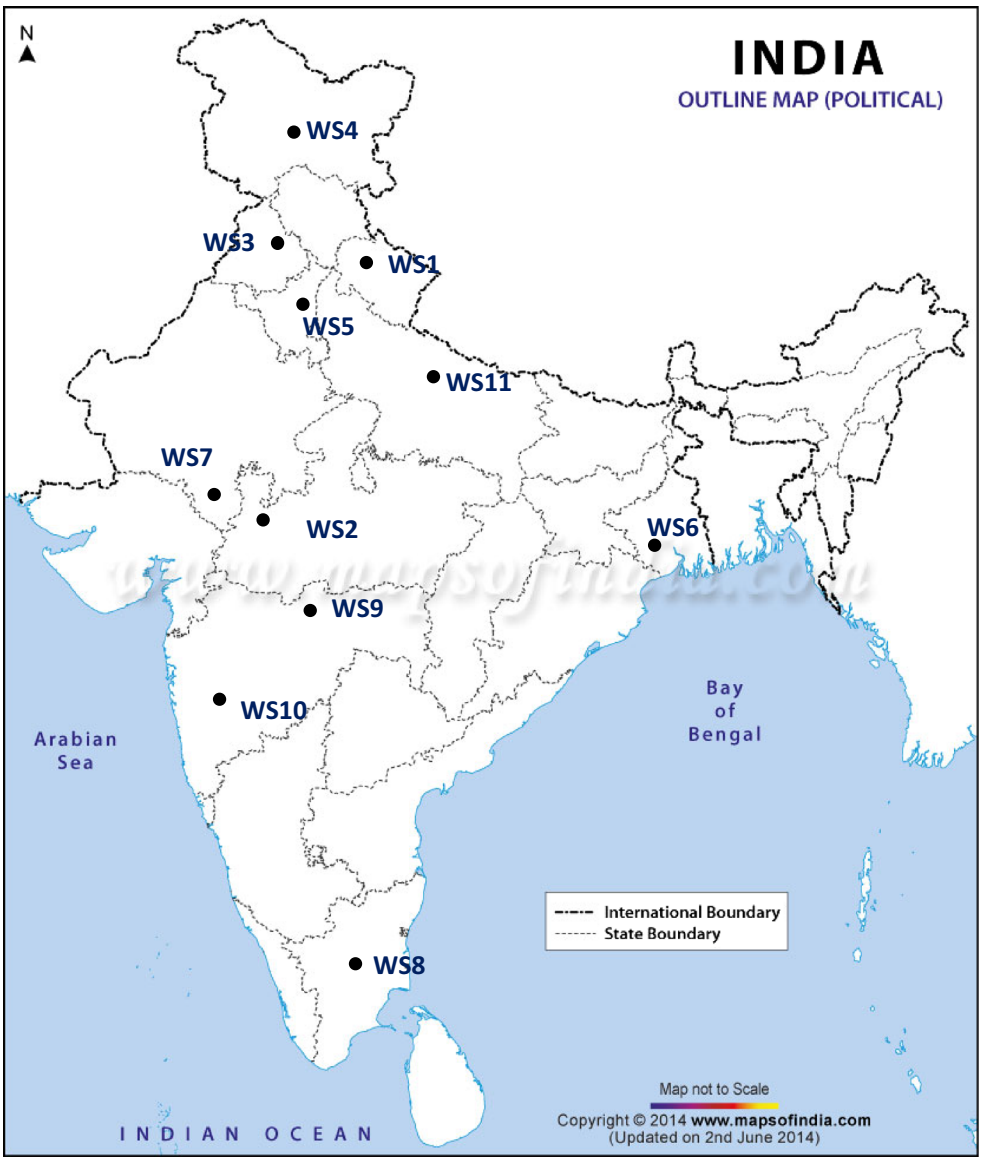

Fig. 1 Map of India showing sites of germplasm collection. *W. somnifera* germplasms are coded as *WS*. Arabic numbers following *WS* code represents different places of the collected germplasms in India

allowing the card to be placed around the stem of the plant shielding the roots from direct light (Fig. 2). Plants of each germplasm were grown in replicates of five at 25 ± 2 °C and 47 µmol m^{-2} s^{-1} photosynthetic photon flux density with a 16 h photoperiod.

In situ establishment of plants

Fifty sterilized, soaked seeds of each germplasm were washed in sterilized distilled water and then sown in sterilized soil in pots at 25 ± 2 °C. For the in situ system, 40-celled hyco trays (Balaji Beej Bhandhar, New Delhi, India) with each cell dimension of 90 × 40 mm (height × width) were filled with 30 g of sterilized sandy loam soil. Three week old plants in replicates of 5 were grown at 25 ± 2 °C in the green house. Plants were watered daily and nutrient solution (Hoagland and Arnon 1950) was added to each hycotray cell every 15 days.

Morphological comparisons between plants grown in the in vitro and the in situ system

Growth and development of plants under the in vitro and the in situ system conditions were assessed using the following morphological parameters: plant height, leaf shape and time of flowering. A ruler with 1 mm graduations was used to assess plant height from the base of the stem at soil level to the apical meristem. Leaf shape and time of flowering was observed during the harvest. Each parameter was assessed at the time of harvest.

Table 1 The location of the germplasm collection from different regions in India

Germplasms	Name of state	Latitude	Longitude
WS1	Uttarakhand	30.31694N	78.03219E
WS2	Madhya Pradesh	24.45000N	74.87000E
WS3	Punjab	30.90097N	75.85728E
WS4	Jammu and Kashmir	34.08366N	74.79737E
WS5	Haryana	28.45950N	77.02664E
WS6	West Bengal	21.83856N	87.43145E
WS7	Rajasthan	24.03000N	74.78000E
WS8	Tamil Nadu	13.05970N	80.22523E
WS9	Maharashtra	20.70000N	77.00000E
WS10	Maharashtra	18.52043N	73.85674E
WS11	Uttar Pradesh	26.84651N	80.94668E

Plant harvesting and dry weight determination

Harvesting of plant material was undertaken at the early flowering stage (Fig. 3) when flowers were present in congested clusters (cymose inflorescence). In the in vitro and the in situ systems, early flowering stage was attained between 58 and 72 days for plants with the obovate type leaf shape and between 120 and 142 days for plants with the ovate type leaf shape and plants were subsequently harvested. For the in vitro system grown plants the aerial part of the plant was removed from the roots, washed and blot dried on blotting paper. The media attached to roots was deionized using 10 mM sodium citrate buffer (Doner and Becard 1991) by placing the root system in the buffer at 25 °C for 30 min at 100 rpm in an incubator shaker (Kuhner Shaker, Basel, Switzerland). Roots were collected using a sieve (52 British Standard Sieve, Industrial Wire Netting Co., New Delhi. India) washed with distilled water and then blot dried. For the in situ system harvesting, whole plants were removed from the hyco trays. The aerial part of the plant was separated from the roots, washed and blot dried on blotting paper and the roots were also washed and blot dried. All roots and aerial parts from the two growth conditions were separately wrapped in blotting paper and dried in a hot air oven (Salvis, Thermo Center Oven, Rotkreuz, Switzerland) at 30 °C. After 1 week, DW of aerial parts and roots were taken continuously every alternate day until it was constant for three consecutive days. Dry weights of the aerial part and roots of all germplasms were recorded.

Optimization of extraction protocol

For optimization of the extraction protocol two germplasms were randomly selected from those growing in the in situ system. For this purpose a 1-year-old plant of germplasm *W. somnifera* 5 and *W. somnifera* 6 were selected. The plant was harvested and 50 leaves were separated from the plant. Leaves were then washed in running water and dried on blotting paper. The dried leaves were then wrapped in blotting paper and any remaining moisture was removed by drying in a hot air oven at 30 °C. After 1 week the DW of the aerial parts and roots was recorded as described previously. Dried leaves were then ground to a powder using a mortar and pestle and a 50 mg subsample was taken and used in each of three replicates.

Leaf subsamples were extracted using a range of methanol (Analytical Grade, Merck, Mumbai, India) in distilled water in ratios of v/v 0:100, 20:80, 40:60, 60:40, 80:20 and 100:0. Grinding, sonication, and a combination of grinding and sonication of the powdered sample in extraction solvents were used as the three different extraction platforms. In the grinding method, 10 ml of each extraction solvent ratio was added to the mortar prior to grinding. Ground samples were then placed in 30 ml centrifuge tubes and subjected to centrifugation (Heraeus Biofuge Stratos, Thermo Scientific) at 10,000 rpm at 25 °C for 5 min. Supernatant was collected and the pellet was resuspended in 10 ml of solvent. The resuspension step was repeated three times and all the supernatants were pooled.

In the sonication method, a waterbath sonicator (B3510E-DTH, Branson, Danbury, Connecticut, US) with an operating frequency of 42 kHz was used with the cleaning tray removed. The position of highest sonication within the waterbath was identified using aluminum foil (100 × 50 mm) placed within the sonicator at different positions. Powdered sample was placed in a 50 ml glass test tube and 10 ml of each extraction solvent ratio was added and sonicated for 15 min at 25 °C. Sample tubes were suspended into the sonicator at a distance of 4 cm from the base. The temperature of the waterbath was maintained at 25 °C using crushed ice. Sonicated samples were centrifuged at 10,000 rpm at 25 °C for 5 min. The supernatant was collected and pellet was resuspended in 10 ml of the solvent. This final step was repeated three times and all the supernatants were pooled.

In the combination method, both the above methods were used. To each sample in the mortar 10 ml of extraction solvent ratio was added and the sample ground. Ground extract was then sonicated for 15 min at 25 °C with sample as described previously, followed with centrifugation of extracts and subsequent pooling of supernatants. For removal of pigments and fatty acids from leaves pooled supernatants for each of the three methods were subjected to three rounds of liquid–liquid partitioning in 10 ml of Hexane (Analytical Grade, Merck, Mumbai, India) using a 100 ml separating funnel (Borosil, New Delhi, India). The methanol phase was collected, pooled and subjected to liquid–liquid partitioning with 10 ml of chloroform (Analytical Grade, Merck, Mumbai, India) to

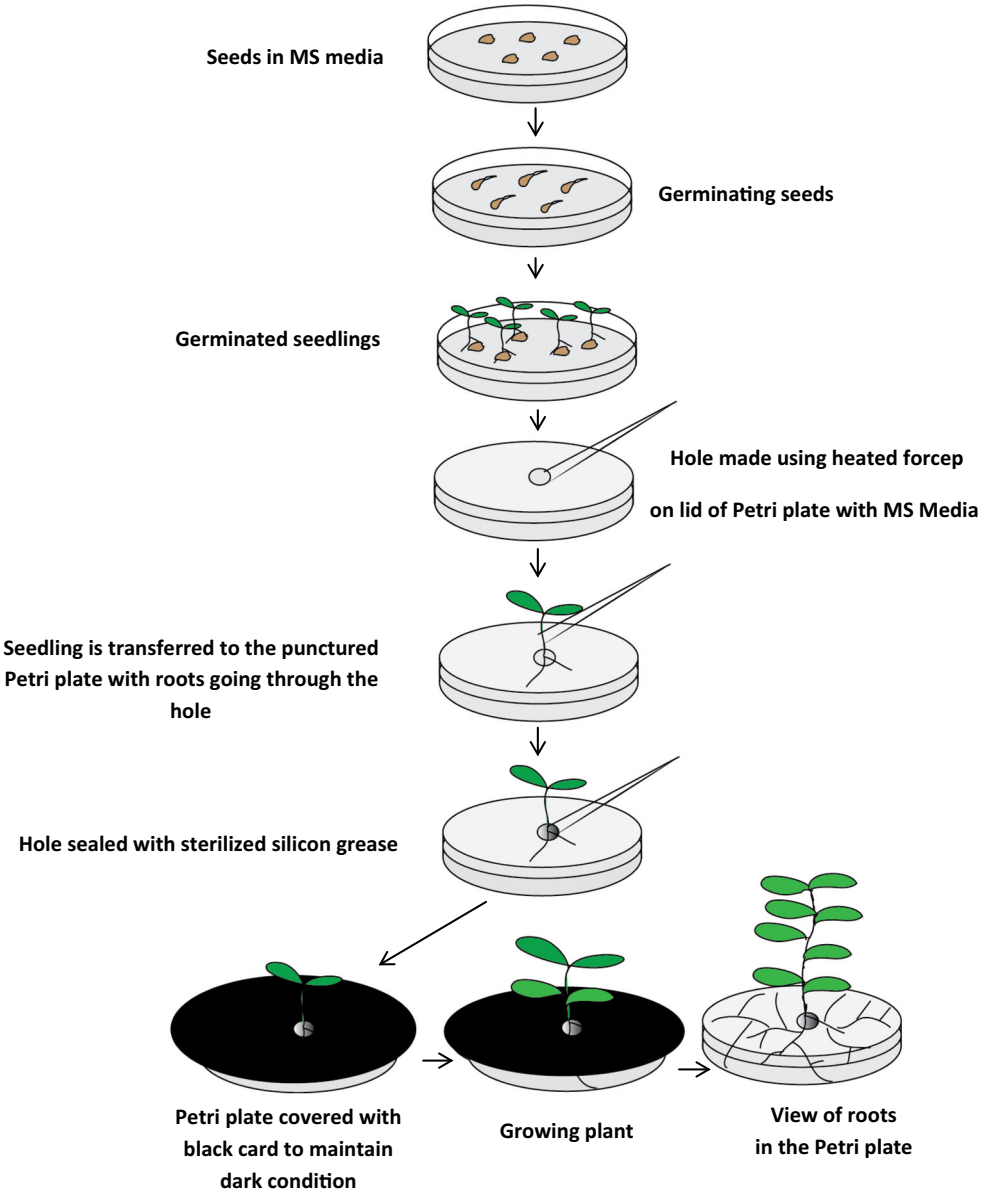

Fig. 2 Diagrammatic presentation of the in vitro system establishment in Petri plate. Seeds of *W. somnifera* at seedling stage were transferred to Petri plates with roots to grow in the media and aerial part in open environment resembling a true to type system

partition WA into the chloroform layer. The pooled chloroform phase was evaporated to dryness using rotary evaporator (Rotavapor, CH-9230, Buchi, Flawil, Switzerland). Dried extract was resuspended in 2 ml of 100 % methanol (HPLC Grade, Merck, Mumbai, India) which was then filtered (0.22 µm Millipore, Merck, Mumbai, India) before subjecting to HPLC analysis.

Sample preparation and HPLC analysis

Leaves and roots from plants at early flowering stage for both the in vitro and the in situ system were extracted using a combination extraction method with 60:40 (methanol:water) as the extraction solvent but skipping the hexane liquid–liquid partitioning step for roots due to the absence of pigmentation. The 50 mg of leaves and roots were used for extraction and samples were prepared from three plants of each germplasm. All extracts prepared were analyzed using HPLC. A calibration curve for WA standard (Sigma, USA) was made using 10, 20, 30, 40, 50 and 60 µg/g of WA. Peak areas were plotted against the corresponding concentration. The calibration curve showed a high coefficient of determination ($r^2 = 0.9989$) by linear equation of $y = 23462x + 31.667$. HPLC–PDA of the filtered extract was carried out on a Shimadzu, CBM-20A, with a C18 Phenomenex column (Gemini®250 × 4.6 mm, 5 µm) and mobile phase of water (HPLC Grade, Merck, Mumbai, India) containing solvent A as 0.1 % acetic acid (HPLC Grade, Merck, Mumbai, India) and solvent B as methanol (HPLC Grade, Merck, Mumbai, India) containing 0.1 %

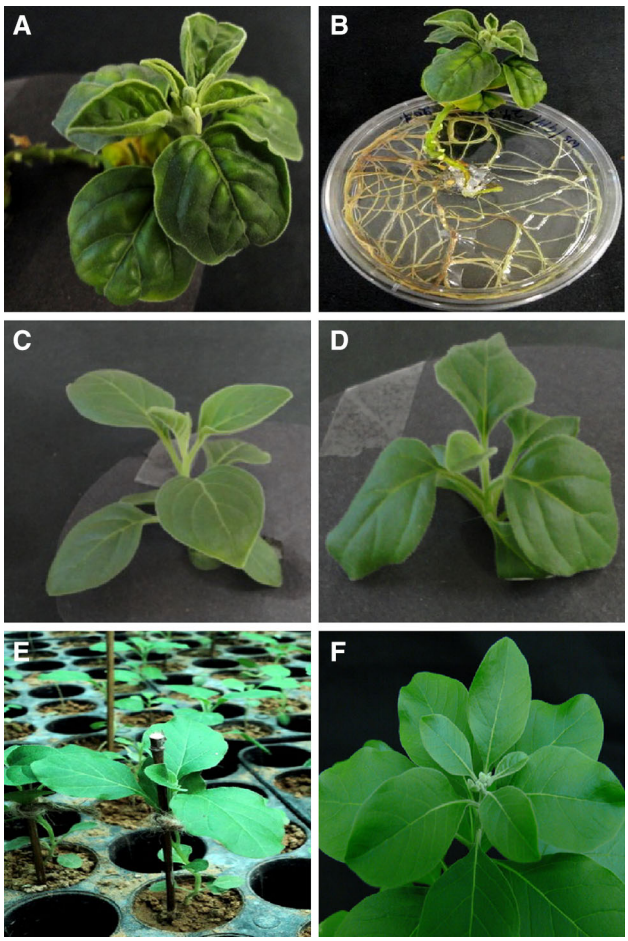

Fig. 3 Plants of *W. somnifera* established and grown in the in vitro and the in situ system. **a**, **b** The in vitro system plants growing in Petri plate. **a** Flower bud initiation and **b** roots in the Petri plate with exhausted media. **c**, **d** Obovate and ovate leaf shapes respectively in the in vitro system. **e**, **f** Plants in the in situ system growing in hycotrays

acetic acid (HPLC Grade, Merck, Mumbai, India). Gradient programming of the solvent system was performed at 27 °C, first at 40 % B changed to 60 % B at 15 min, maintained for the next 2.0 min, changed to 75 % B at 30 min and then to 95 % B at 39 min and then to 100 % B at 40 min. The solvent composition was maintained until the run time reached 45 min. The flow rate of 1.0 ml/min was kept throughout the program. All the gradient segments were linear. The wavelength scan range of the PDA was set to 190–350 nm. Chromatograms were recorded at 227 nm. WA quantification was performed using the peak area of the sample chromatogram in the regression equation of the WA standard calibration curve.

HPLC–MS

An Agilent Technologies 6210 MSD TOF mass spectrometer was used in positive electrospray ionisation (ESI) mode for mass spectral analysis. The analysis conditions were: drying gas (N_2) flow rate and temperature (7 l min^{-1}, 350 °C), nebuliser gas (N_2) pressure (30 psi), capillary voltage 3.0 kV, vaporizer temperature 350 °C, and cone voltage 60 V. MS data acquisition was carried out using Agilent MassHunter Workstation Acquisition for TOF/Q-TOF [B.02.00 (B1128)] and data analysis was carried out using Agilent MassHunter Qualitative Analysis (version B.03.01).

Statistical analysis

All data was analyzed using a commercial software package (SPSS Statistics 21, IBM). One way analysis of variance (ANOVA) was used to determine WA concentration in different germplasms. Statistical significance was determined at the $p < 0.05$ level using the Tukey post hoc test.

Results

Establishment and growth of *W. somnifera* in the in vitro and the in situ systems

In vitro system growth conditions

In the in vitro system conditions (Fig. 3a, b), plants were healthy with the typical alternate leaves of two distinct shapes depending on the germplasm source analysis of the morphological parameters at the time of harvest (Table 2). *W. somnifera* germplasms 1, 2, 3, 9 and 10 had an obovate (Fig. 3c) leaf shape and these germplasms had early flower bud initiation between 62 and 72 days. The shape of the leaves was ovate (Fig. 3d) in *W. somnifera* germplasms 4, 5, 6, 7, 8 and 11 and with flower bud initiation observed between 123 and 142 days. Plant height was observed to be highest in *W. somnifera* 2 (10.33 cm) and lowest in *W. somnifera* 5 (6.93 cm) and dry weight was highest in germplasms 2, 5 and 9 (0.25 g) and the lowest in *W. somnifera* 4 (0.20 g), but the differences in dry weight among the germplasms were not statistically significant.

In situ system growth conditions

In the in situ system (Fig. 3e, f) plant height (Table 3) was highest in *W. somnifera* 10 (35 cm) with the lowest observed in *W. somnifera* 5 (7 cm) while dry weight was highest in *W. somnifera* 10 (0.37 g) followed by *W. somnifera* 1 and 3 (0.35 g). However, there was no significant difference observed in their dry weight. *W. somnifera* germplasm with the lowest dry weight was 6 (0.11 g). Flower bud initiation was observed in two time frames. In germplasms showing obovate type leaf shape (58–68 days), flower bud initiation

Table 2 Morphometric parameters of *W. somnifera* germplasms in the in vitro system

Germplasms	Height (cm)	Dry weight (g)	Flower bud initiation (days)	Leaf shape
WS1	08.53 ± 0.35 bc	0.22 ± 0.01 a	062.67 ± 1.45	Obovate
WS2	10.33 ± 0.12 a	0.25 ± 0.05 a	071.67 ± 1.67	Obovate
WS3	07.66 ± 0.24 cde	0.24 ± 0.01 a	064.00 ± 2.08	Obovate
WS4	07.50 ± 0.26 de	0.20 ± 0.02 a	125.70 ± 1.20	Ovate
WS5	06.93 ± 0.09 e	0.25 ± 0.01 a	125.00 ± 2.52	Ovate
WS6	08.13 ± 0.13 bcd	0.22 ± 0.01 a	142.00 ± 1.00	Ovate
WS7	07.10 ± 0.10 e	0.21 ± 0.02 a	142.70 ± 1.20	Ovate
WS8	08.40 ± 0.21 bcd	0.24 ± 0.02 a	135.00 ± 2.52	Ovate
WS9	08.50 ± 0.21 bc	0.25 ± 0.03 a	072.00 ± 1.53	Obovate
WS10	07.46 ± 0.18 de	0.21 ± 0.01 a	063.33 ± 1.86	Obovate
WS11	08.93 ± 0.07 b	0.23 ± 0.09 a	123.70 ± 1.45	Ovate

Values with same letters are not statistically different. Data reported as mean ± SE for three samples

Table 3 Morphometric parameters of *W. somnifera* germplasms in the in situ system

Germplasms	Height (cm)	Dry weight (g)	Flower bud initiation (days)	Leaf shape
WS1	33.67 ± 0.33 ab	0.35 ± 0.08 a	060.67 ± 0.67	Obovate
WS2	30.67 ± 0.33 c	0.26 ± 0.04 abc	068.00 ± 1.53	Obovate
WS3	20.67 ± 0.88 d	0.35 ± 0.01 a	058.33 ± 0.88	Obovate
WS4	09.00 ± 0.58 g	0.12 ± 0.02 bc	122.00 ± 1.53	Ovate
WS5	07.00 ± 0.58 g	0.18 ± 0.02 abc	120.00 ± 0.00	Ovate
WS6	07.00 ± 0.01 g	0.11 ± 0.01 c	139.70 ± 0.88	Ovate
WS7	12.33 ± 0.67 f	0.13 ± 0.01 bc	132.70 ± 1.20	Ovate
WS8	22.67 ± 0.33 d	0.32 ± 0.01 ab	133.00 ± 2.08	Ovate
WS9	31.33 ± 0.33 bc	0.32 ± 0.04 ab	068.33 ± 1.67	Obovate
WS10	35.00 ± 0.00 a	0.37 ± 0.07 a	060.33 ± 0.88	Obovate
WS11	17.33 ± 0.67 e	0.20 ± 0.03 abc	120.00 ± 0.58	Ovate

Values with same letters are not statistically different. Data reported as mean ± SE for three samples

Table 4 WA concentration (mg/g) DW in *W. somnifera* germplasm 5 grown in pots in the in situ system using different extraction methodologies

Treatments of methanol (%)	Grinding (mg/g) DW	Sonication (mg/g) DW	Combination (mg/g) DW
Control (water)	7.33 ± 0.17 b	07.66 ± 0.23 c	07.86 ± 0.36 d
20	4.13 ± 0.10 c	09.85 ± 0.34 b	09.96 ± 0.32 c
40	3.94 ± 0.09 c	10.63 ± 0.07 ab	10.71 ± 0.07 b
60	9.15 ± 0.04 a	11.37 ± 0.10 a	12.39 ± 0.13 a
80	2.66 ± 0.05 e	04.03 ± 0.05 d	04.84 ± 0.09 e
100	0.58 ± 0.01 b	0.552 ± 0.03 e	01.04 ± 0.02 f

Concentrations with same letters are not statistically different. Data reported as mean ± SE for three samples

occurred earlier to germplasms with ovate type leaf shape (120–139 days).

WA extraction and optimization protocol

The extraction protocol that used the combination of grinding followed with sonication resulted in the highest WA concentration extracted (Table 4). Among the different protocols, methanol used at 60 % produced maximum extraction efficiency with the combination extraction protocol yielding the highest levels of WA (12.39 mg/g DW). In the protocols used, it was observed that methanol at 20 % (4–9 mg/g DW) and 40 % (3–10 mg/g DW) produced more WA than methanol at 80 % (2–4 mg/g DW). Extraction using 100 % methanol resulted in the lowest yields of WA. Water-based extraction alone yielded 7–8 mg/g DW. Using the grinding, sonication and combination extraction protocols with 60 % methanol 9.15, 11.37 and 12.39 mg/g DW of WA were produced respectively, with the combination extraction protocol yield found to be statistically significant.

It was observed that the concentration of WA in combination (grinding followed with sonication) and sonication (alone) methods produced similar concentrations for when 20, 40 and 80 % methanol quantities were used. Also similar concentrations were observed when extraction was performed using water as a control for extraction in both the methods. However, an increase in concentration of WA was observed in 60 % methanol:water when the combination

method was used as compared to sonication method. In all the extraction platforms used with different methanol to water ratios, 60 % showed the highest concentration yield when grinding and sonication methods were used in combination and was thus used for extraction of all subsequent experimental samples.

WA in the in vitro and the in situ systems

HPLC was utilized with WA standards in order to confirm the concentration of WA within each sample set (Fig. 4). For all extractions, WA was confirmed via high resolution HPLC-ESI-TOF MS. In the in vitro system the concentration of WA in leaves was between 0.26 and 7.37 mg/g DW while roots yielded between 0.01 and 0.27 mg/g DW (Table 5). The highest concentration found in leaves of germplasms *W. somnifera* 1, 2 and 10 with the lowest level in *W. somnifera* 5. In roots, the germplasm that produced the highest in leaves also produced the highest in roots with the exception of *W. somnifera* 11 which produced 0.21 mg/g DW. The lowest concentration of WA was found in roots of *W. somnifera* 4 and 5 (0.01 mg/g DW). On the basis of recovery per plant, it was observed that *W. somnifera* germplasms 1, 2, 3 and 10 in the in vitro system produced WA in the range between 1.6 and 1.8 mg with DW of the whole plant in the range of 0.2–0.25 g (s). In *W. somnifera* germplasms 4, 5, 6 and 7 though the DW of the plant was in the range 0.2–0.25 g (s), WA content was less than 0.4 mg (Fig. 5).

Table 5 WA concentration (mg/g) DW in leaves and roots of different germplasms in the in vitro system

Germplasms	Leaves (mg/g) DW	Roots (mg/g) DW
WS1	7.15 ± 0.76 a	0.24 ± 0.01 a
WS2	7.37 ± 1.69 a	0.27 ± 0.01 a
WS3	6.04 ± 0.47 ab	0.09 ± 0.01 b
WS4	0.48 ± 0.05 c	0.01 ± 0.01 d
WS5	0.26 ± 0.02 c	0.01 ± 0.01 d
WS6	0.83 ± 0.03 c	0.02 ± 0.01 cd
WS7	0.90 ± 0.03 c	0.08 ± 0.01 bc
WS8	5.10 ± 0.49 ab	0.14 ± 0.01 b
WS9	4.96 ± 0.20 ab	0.13 ± 0.01 b
WS10	7.17 ± 0.56 a	0.23 ± 0.01 a
WS11	3.06 ± 0.19 bc	0.21 ± 0.03 a

Concentrations with same letters are not statistically different. Data reported as mean ± SE for three samples

Germplasm *W. somnifera* 10 was the highest yielding germplasm in the in situ system with 41.42 mg/g DW in leaves followed by *W. somnifera* 1 and *W. somnifera* 2 which yielded 35.50 and 35.71 mg/g DW respectively (Table 6). Concentrations in roots were however the highest in roots of *W. somnifera* germplasm 2 (0.60 mg/g DW) followed by *W. somnifera* 1 (0.52 mg/g DW) and the lowest in *W. somnifera* 5. WA in roots of *W. somnifera* 10 and 11 were in the range of 0.20–0.26 mg/g DW. The experiment conducted in the in situ system was found to follow almost the same trend as in the in vitro system with

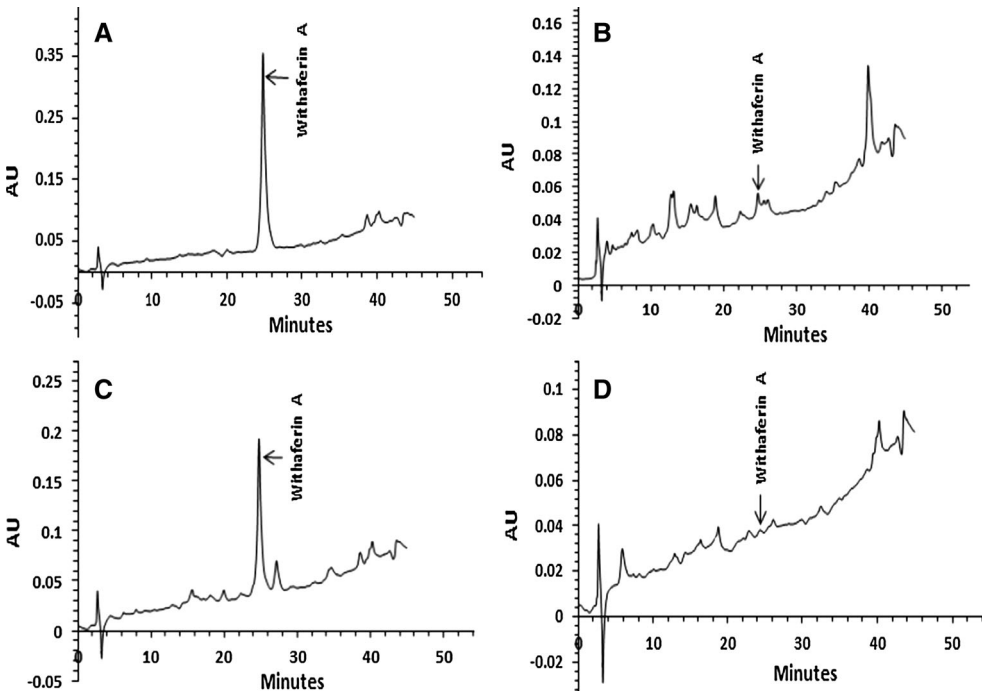

Fig. 4 HPLC Chromatogram of leaves and roots extracts. WA is detected at 24th min. **a**, **b** Chromatograms are extracts of leaves and roots from the in vitro system, respectively. **c**, **d** Chromatograms are extracts of leaves and roots from the in situ system, respectively

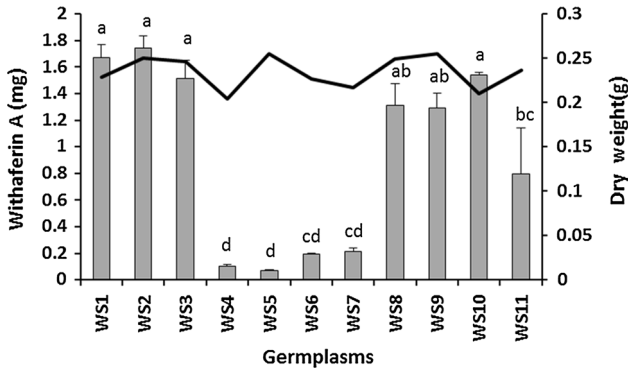

Fig. 5 Recovery of WA (mg/plant) in different germplasms in the in vitro system with respect to their dry weight (g). *Bars* represent recovery of WA in milligrams and *line* represents dry weight. *Bars with same letters* are not statistically different. Data reported as mean ± SE for three samples

Table 6 WA concentration (mg/g) DW in leaves and roots of different germplasms in the in situ system

Germplasms	Leaves (mg/g) DW	Roots (mg/g) DW
WS1	35.50 ± 2.96 ab	0.52 ± 0.01 ab
WS2	35.71 ± 1.99 ab	0.60 ± 0.01 a
WS3	29.08 ± 1.85 bc	0.25 ± 0.03 c
WS4	10.03 ± 1.30 e	0.09 ± 0.01 d
WS5	08.05 ± 0.25 e	0.01 ± 0.01 d
WS6	12.50 ± 1.23 de	0.07 ± 0.01 d
WS7	09.88 ± 0.98 e	0.25 ± 0.02 c
WS8	29.04 ± 3.24 bc	0.44 ± 0.03 b
WS9	22.33 ± 0.56 c	0.29 ± 0.03 c
WS10	41.42 ± 0.09 a	0.20 ± 0.01 c
WS11	20.37 ± 1.54 cd	0.26 ± 0.03 c

Concentrations with same letters are not statistically different. Data reported as mean ± SE for three samples

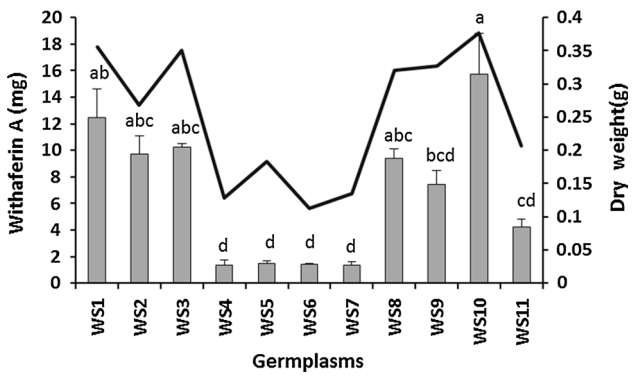

Fig. 6 Recovery of WA (mg/plant) in different germplasms in the in situ system with respect to their dry weight (g). *Bars* represent recovery of WA in milligrams and *line* represents dry weight. *Bars with same letters* are not statistically different. Data reported as mean ± SE for three samples

respect to leaf shape, time taken for flower bud initiation and WA concentration. All extractions for WA was confirmed via high resolution HPLC-ESI-TOF MS. Recovery per plant in the in situ system was observed to be highest in *W. somnifera* 10 germplasm (16 mg) with 0.35 g as dry weight followed with *W. somnifera* 1, 2, 3 and 8 in the range between 9 and 13 mg. WA content in *W. somnifera* 4, 5, 6 and 7 was <0.05 mg (Fig. 6). On the basis of recovery, *W. somnifera* 1, 2 and 10 are germplasms with high WA content.

Discussion

Withania somnifera, a plant of the Solanaceae family with rich repository of important withanolides (Chatterjee et al. 2010) was the subject of the present study. Roots and leaves of the plant are the richest tissues that contain withanolides and that have been prescribed in traditional systems of medicine (Kaileh et al. 2007a). Various studies on the phytochemical analysis of *W. somnifera* have been reported (Ramesh Kumar et al. 2011, 2012). In our study, 11 different *W. somnifera* germplasms were analyzed on the basis of their morphological and biochemical characteristics with WA as the candidate molecule to generate a screening profile for high yielding germplasm in the in vitro and in situ systems. We have found that germplasms showed differences in their various growth parameters and metabolite concentrations.

To address the question of optimum yield, an easy, fast and efficient extraction platform has been developed by combining extraction methodologies. Sonication mediated extraction of dried samples and microwave-assisted extraction of powdered samples using different solvents had previously been used (for example, Sharma et al. 2007b; Mirzajani et al. 2010) but a combination of extraction techniques with respect to varying methanol concentrations has not been studied. Here, we have found grinding followed by sonication extraction platform to be an effective and straight forward approach for WA extraction. Grinding of plant material using mortar and pestle is one of the traditional methods that have been followed for disruption of cells. Common extraction methods such as extraction of WA in a ground sample followed by percolation in solvents (Dhar et al. 2006; Scartezzini et al. 2007; Kumar et al. 2011) for hours is often not reliable with time and physical efficiency. Thus, grinding sample in solvent at specific concentration followed with sonication which disrupts plant cells has been used in our study.

Variation in WA concentration in the different germplasms collected and grown has been observed in our study. Concentration of WA in leaves from the in vitro

system was 12-fold higher in the maximum WA producing germplasm than that previously reported (Dewir et al. 2010) and twofold less in the lowest producing germplasm in the same report. Similarly, the concentration of WA in roots as found by Dewir et al. (2010) in the in vitro grown plants was similar in the lowest producing and 20 times higher in the highest producing germplasms. The observed increase in metabolite content in leaves may be due to environmental variation in the original cultivated regions of the plants (seeds used were from the plants grown in the various regions), the differences in growth systems being used and the true to type condition of the aerial plant parts being exposed to air and light. Other than whole plants, callus cultures grown in MS media have been shown to produce 0.10 mg/g in control and 3.88 mg/g DW when phytohormones and elicitors were used in the media (Sivanandhan et al. 2013). In the in vitro system used in our study, the WA concentration was 7.64 mg/g DW in the highest producing germplasms.

The concentration of WA was found to be higher in the in situ system as compared to the in vitro system, which was likely due to nutrient and moisture limitations in the latter system. Replenishment of nutrient and moisture may provide increased concentrations of WA in the in vitro system. An interesting aspect of our in situ system was the increased concentration of WA in comparison with other published reports. The concentration of WA in cultivated leaves and roots as reported by Kumar et al. (2011) was respectively 8.20 and 0.18 mg/g DW. Similarly, WA concentration growing in wild and cultivated *W. somnifera* leaves as in earlier reports fall in the range between 5 and 13 mg/g DW for the whole plant (Dhar et al. 2006; Kumar et al. 2007, 2011). The concentration in leaves in the highest producing germplasms in our in situ system was 2–3 folds higher in the highest WA producing germplasms and in the same range in the low producing germplasms. In roots similar concentrations were found in the lowest yielding germplasm and a tenfold increase in the highest producing germplasms (Dewir et al. 2010). We propose that the higher yield may be due to our in situ system being used with the combined extraction methodology.

In summary, the newly developed in vitro system used here was found to be very useful for studying various parameters of plant grown under controlled conditions. The in vitro system described opens the way for a new approach for phytochemical screening and provides an efficient system for molecular and enzymatic studies. The system reduces the effort required for wide-scale manual harvesting, removal of soil or other substrates, reduces the chance of microbial contamination and provides an enhanced production of useful secondary metabolites such as WA in comparison with other published methods.

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