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Metabolite Profiles of Arsenic Tolerant Plants Regenerated from Stem Calli of *Andrographis paniculata* (Burm.f.) Nees using HPLC and 1D ^{1}H NMR

[1]J. Vijayakumar, [2]P. Ponmanickam, [1]P. Samuel, [1]B. Pavithra, [1]A. Manjula, [1]S. Aswathi and [1]G. Shobana Rathi

[1]Department of Biotechnology, Ayya Nadar Janaki Ammal College, Sivakasi - 626 124, Tamil Nadu, India
[2]Department of Zoology, Ayya Nadar Janaki Ammal College, Sivakasi - 626 124, Tamil Nadu, India

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Corresponding Author:
J. Vijayakumar
Department of Biotechnology,
Ayya Nadar Janaki Ammal College, Sivakasi - 626 124,
Tamil Nadu, India
Email: georgejarsvijay2004@yahoo.com

Abstract: *In vitro* culture provide a suitable condition for regeneration of arsenic tolerant plants from stem calli of *Andrographis paniculata* (Burm.f.) Nees. The regenerated plants could be valuable material applicable to soil remediation. *In vitro* culture of stem explants on MS basal salts, 3.0% sucrose, 0.8% agar medium fortified with various concentrations of As_2O_3 (0.0-9.0 μM) along with NAA (3.5 mg L^{-1}) and KIN (1.0 mg L^{-1}) influenced resistant callus formation. Growth of callus was slightly inhibited with increased resistance up to 22% (53±0.8 mg fwt and 27±1.5 mg dwt) on 7.0 μM As_2O_3 selective medium. The resistant callus line inoculated on MS medium supplemented with different concentrations of As_2O_3 along with BA (2.5 mg L^{-1}) and NAA (3.0 mg L^{-1}) induced the development of shoots. Shoot organogenesis was slightly inhibited by arsenic metal stress. However the growth tolerance has increased up to 14.5% on medium with 7.0 μM As_2O_3. The rate of adventitious rooting of plantlets was gradually decreased with more tolerance (11.1±1.4 rootlets per plantlets) in 7.0 μM As_2O_3 selected media. After acclimatization, about 40% of plants were survived as arsenic tolerance than control plants in pots containing soil treated with 7.0 μM As_2O_3 solutions. The level of arsenic detectability was 0.96 ppb and 4.67 ppb in control and 7.0 μM As_2O_3 treated plants, respectively by AAS analysis. Moreover, the production of Andrographolide was found quite high (4.41 mg/g) in tolerant plants grown at 7.0 μM As_2O_3 treatment than control by HPLC analysis. 1D ^{1}H NMR profile revealed the metabolic changes significantly in control and 7.0 μM As_2O_3 treated plant samples. This is the first report confirming the suitability of *in vitro* selection for obtaining of vigorous and proliferative clones of *A. paniculata* plants tolerant to elevated arsenic concentration.

Keywords: *Andrographis paniculata* (Burm.f.) Nees, Medicinal Plant, Andrographolide, Indirect Organogenesis, Heavy Metal, Arsenic Trioxide and Metabolites

Introduction

The worldwide issue of rising consequence for bionomical, evolutionary and environmental basis is heavy metals toxicity (Sharma *et al*., 2010). The risk assessment of metal contaminants requires the details on pollutants pools of chemically reactive and existence in the soil environment (Wenzel *et al*., 2001). Recently, arsenic has received great attention because of its constant toxicity and increasing the level of deposits into the environment in countries such as Bangladesh, China and India (Meharg, 2004). Arsenic is represented as crystalline metalloid and exists in the environment in several forms and oxidation states. The toxic mobility of arsenic in the environment depends on chemical and species forms (Pongratz, 1998). Arsenic content in soil causes great concern with respect to plant uptake and subsequent entry into wildlife and food chains of human.

Arsenic speciation is occurred in the environment as organic and inorganic forms, but the interconversion between the number of arsenic species regulated by abiotic and biotic processes. The strong phytotoxicity symptoms of arsenic occur in plants, but their level is higher in root than shoot biomass (Tang and Miller, 1991; Carbonell *et al*., 1998). Gupta *et al.* (2008) study also demonstrated that the increase in root length shoed the tolerance ability of *Artemisia annua* against arsenic.

A. paniculata (Burm.f.) Nees is an herbaceous medicinal plant, commonly known as king of bitters belongs to the family Acanthaceae. It is mostly grown well in the plains of India, Pakistan and Sri Lanka. It is an erect and branched annual herb. Plant body is enormously bitter in taste. The whole plant body is more useful in wounds, hyperdipsia, ulcers, chronic fever, burning sensation, inflammations, cough, bronchitis, pruritis, haemorrhoids, leprosy, intestinal worms and vitiated conditions of pitta, flatulence, dyspepsia, colic, diarrhea, malarial and intermittent fevers (Warrier *et al*., 1993). The andrographolide is an active diterpene lactone compound in *A. paniculata* (Patarapanich *et al*., 2007). *In vitro* propagation is the proven method for regeneration of *A. paniculata*. In earlier, *in vitro* plant regeneration via micropropagation (Purkayastha *et al*., 2008; Karuppusamy and Kalimuthu, 2010; Jindal *et al*., 2015) and somatic embryogenesis (Martin, 2004) have been reported in *A. paniculata*. Now a day, *in vitro* selection and somaclonal variation techniques are utilized for attaining plant genotype tolerance to the abiotic stress like high salinity, drought, heavy metal stress, acid soil and disease tolerance over biotic stresses (Ahmed *et al*., 1996; Yusnita *et al*., 2005). *In vitro* selection is an effective method to alter the plant with desired character through applying a selective agent on media (Bulk, 1991). These *in vitro* techniques have useful in culturing of metal tolerant plants that can be essential in raising the yield of secondary metabolites (Saba *et al*., 2000; Narula *et al*., 2004). The heavy metal toxicity causes a range of physiological and biochemical changes (Maitra and Mukherji, 1979; Wickliff and Evans, 1980) and the potentiality of these toxic elements in altering the quality and quantity of various plant products of medicinal importance (Zheljazhov and Fair, 1996). The tolerance to toxic metal has also been accounted to involve variance in the structure and function of membranes or differential gene expression in different biochemical pathways (Foy *et al*., 1978). Dhankher *et al.* (2002) demonstrated that that *Arabidopsis thaliana* grown on arsenic revealed a greater fresh shoot weight, indicating that growth can be improved in the presence of heavy metals. Recently, the effects of heavy metals and uptake of arsenic metal from contaminated soil was evaluated in *Pteris vittata* (L.) plants (Fayiga *et al*., 2007). Though there are very limited reports on *in vitro* regeneration of heavy metal tolerant medicinal plants originated from India. To the best of our knowledge, no reports were found on the *in vitro* regeneration of arsenic tolerant *A. paniculata* plants. Therefore, the present study aimed to examine the effects of arsenic on regeneration of plants from stress resistant callus line under aseptic culture condition and also to evaluate the metabolite profile changes in *A. paniculata* plants.

Materials and Methods

Preparation of Plant Materials

The seeds of Nilavembu (*A. paniculata* (Burm.f.) Nees) were procured from MPCP (Medicinal Plants Conservation Parks), Sevaiyoor, Kariapatti, TN, India. Seeds were surface cleaned with three drops of 10% *Teepol* solution and kept under running tap water for 10 min. Then the seed were brought to laminar air flow chamber for further sterilization. The seeds were subjected to 70% alcohol (v/v) treatment for 30 sec, followed by 2 -3 min soaking in 0.1% mercuric chloride (w/v) solution and then washed thrice with sterile distilled water. Then, seeds were inoculated aseptically on culture tubes (25×150 mm) each containing 15 mL MS basal medium (Murashige and Skoog, 1962) which was capped with non-absorbent cotton plugs. Seeds were germinated at 25ºC with a 16-h photoperiod for 14 days. The 7-9 days old stem explants were carefully excised from the apex and basal part of *in vitro* seedlings plants.

Induction of As_2O_3 Resistant Callus

The stem explants were cut into 0.5-1.0 cm long segments and wounded with the help of sterile surgical blade and inoculated horizontally on MS basal salts, 3.0% (w/v) sucrose and 0.8% agar (w/v) medium fortified with optimum level of NAA (3.5 mg L^{-1}) and KIN (1.0 mg L^{-1}) along with different concentrations of As_2O_3 (0.0, 1.0, 3.0, 5.0, 7.0 and 9.0 μM). All explants were incubated under 36 μmol $m^{-2}s^{-1}$ with a 16-h photoperiod provided by cool white fluorescent tube at 25±2°C. Every subculture was done after 2 weeks of interval. Each treatment was replicated thrice. Callusing efficiency was explained as the percentage of explants that produced callus. The average fresh weight and dry weight of As_2O_3 resistant callus was calculated at each treatment after 60 days of culture.

In vitro Regeneration of As_2O_3 Tolerant Plants

Approximately 100 mg of control and resistant calli were isolated from stem explants and cultured on MS basal salts, 3.0% sucrose (w/v), 2.5 mg L^{-1} BA and 3.0 mg L^{-1} NAA medium fortified with different concentrations of As_2O_3 (0.0-9.0 μM) for the regeneration of tolerant microshoots. Shoot clumps developed from organogenic calli were subcultured after 2 weeks of interval and maintained in As_2O_3 selective medium for further proliferation of tolerant microshoots.

Data on shoot organogenesis was recorded after 45 days of culture. The plantlets were excised from shoot clumps of control and As_2O_3 resistant calli and transferred to half strength MS basal salts, 1.5% sucrose (w/v), 0.8% agar (w/v) medium supplemented with 2.0 mg L^{-1} IBA and different levels of As_2O_3 (0.0-7.0 µM). All the cultures were maintained at 25±2°C under 36 µmol $m^{-2}s^{-1}$ under 16-h photoperiod with white fluorescent light. Data on root induction was recorded after 30 days of culture initiation. The regenerated plantlets were carefully taken out from the culture tubes and washed in running tap water to eliminate gelling agents from the roots. The healthy plants were successfully transplanted onto 6.0 cm diameter plastic cups containing the sterile red soil, garden soil and sand mixture (1:2:1). Each pot was covered with clean polythene bag to control relative humidity (85-95%) and maintained under aseptic condition for the initial 7 days. The control and arsenic tolerant plants were frequently supplied with 7.0 µM As_2O_3 solutions and transferred to greenhouse condition. The rate of survival was noticed after 15, 30 and 45 days of transfer to soil.

Analysis of Arsenic in Plant Samples by AAS

Approximately, 30-40 days old *in vitro* raised control and As_2O_3 tolerant plants from stem calli of *A. paniculat*a were dried under shade at ambient temperature for 15 days, ground into powder with a mechanical grinder and homogenized using mortar and pestle. The samples were subsequently stored in separate bottles 10 mL concentrated nitric acid (HNO_3) (ultrapure 65%) was added to 1 g of both control and As_2O_3 tolerant plant samples and allowed to stand overnight at room temperature. The samples were then heated at 120°C for 4-h, after that the temperature was increased to 140˚C. The process was continued at this temperature until about 1 mL of acid remained. The liquidity was filtered in a 50 mL flask and diluted to the mark after cooling. The modified method of Batty *et al.* (2000) and Wei and Theil (2000) were followed for extraction of samples. The stock for standard solutions of Arsenic containing 1000 ppm of metal were prepared by dissolving appropriate quantities and dried in distilled water. Calibration standards of 1.0 ppb, 2.0 ppb and 3.0 ppb of Arsenic element were prepared by proper dilution of the stock solutions. The control and As_2O_3 tolerant plant samples were taken for arsenic analysis of arsenic content by Atomic Absorption Spectrometry (AAS).

Sample Extraction and Analysis of Andrographolide by HPLC

Preparation of Solvents and Andrographolide Standard

Methanol and HPLC grade water were used as reagents and solvents for chromatographic analysis. Methanolic movable phase and the samples were filtered through 0.45µm membrane filter. Ultrasonicator was used for degassing of mobile phase. The purity of Andrographolide (99%, pure, Sigma) was used as a standard. 1ml of andrographolide compound was prepared by the dissolving 2.0 mg of andrographolide compound in 5 mL of methanol (100%) (v/v) before analysis. It was stored at 4°C for further analysis and maintained steady for at least 30 days.

HPLC was adapted for the estimation of andrographolide from 30-40 days old *in vitro* control and arsenic tolerant plants from stem calli of *A. paniculata* grown on MS medium with 2.5 mg L^{-1} BA and 3.0 mg L^{-1} NAA along with 7.0 µM As_2O_3 treatments. The samples were shade dried at ambient temperature for 15 days, ground into powder with a mechanical grinder. The samples were subsequently stored in separate sample bottles for further study. The modified method of Victório *et al.* (2009) was applied for the extraction of plant material. The samples (2 gm each) were extracted with 20 ml methanol at room temperature for 24-h with occasional shaking. The rotary evaporator was used to concentrate sample under reduced pressure to give a gummy residue. The residue was dissolved and suspended in methanol. This concentrated solution was diluted with methanol and filtered through a 0.45 µm nylon filter into HPLC vials. The diluted samples were used for injection in HPLC. The presence of andrographolide was determined using a C18 reverse phase column with methanol as mobile phase at 0.2 µl/min flow rate and detected by UV detector at 266 nm. The data were reported and processed by millennium 32 software from Waters (Milford, MA, USA).

Sample Analysis by 1D ^1H NMR

Preparation of Samples and Model Solutions

The 30-40 days old dried samples of *in vitro* control and arsenic tolerant plants from stem calli cultured on 7.0 µM As_2O_3 treatment was ground well in mechanical grinder and included with 1.2 mL of methanol-d4, 0.3 mL of potassium dihydrogen phosphate buffer and 150 µL of 33% deuterium oxide (D_2O) (pH. 6). Then, the samples were vortexed for 10 sec. After that, the extracts were centrifuged at 16,000g for 10 min at 4°C. The supernatant was evaporated and dried in a speed-vacuum concentrator at room temperature and frozen at −80°C until 1D ^1H spectrum NMR analysis. The chemicals of NMR reference, 2,2,3,3-d4-3-(trimethylsilyl) propionic acid sodium salt (TSP) was purchased from Hi-Media, Mumbai and prepared model solutions in D_2O at standard level before spectra recording. This protocol used for analyzing metabolites from plant samples were based on Saiman *et al.* (2012) method with some modification.

1D ^{1}H NMR Spectra Recording Condition

The pH of sample solution was adjusted to the desired value by adding 5 µL of Sodium deuteroxide (NaOD). After measuring pH, an aliquot of 0.8 ml supernatant was transferred into a 5 mm diameter 1D ^{1}NMR ultra-glass tube. A conserved coaxial capillary containing a solution of 2,2,3,3-d4-3-(trimethylsilyl) propionic acid sodium salt (TSP) was served as external chemical shift and quantification reference and fixed in the NMR tube. One dimensional pulse acquire the NMR spectra results were recorded at 25°C on a 400 MHz Bruker DMX 400 spectrometer working at proton NMR frequency of 400.13 MHz and equipped with a 5 mm cryoprobe. The spectra were referenced by fixing the ^{1}H δ of the TSP methyl groups. The assignment of signal was obtained with a database created by setting the standard level of pH on chemical shifts (δ) and multiplicity of 1D ^{1}H NMR resonances and confirmed by spiking representative samples with reliable standards.

Data Analysis

All experiments were performed with Complete Randomized block Design (CRD) and different factorial with types of hormones as independent variables. Average of fresh weight and dry weight of stem calli, number of shoots, length of shoot, number of leaf, number of root and length of roots obtained during initial culture and subsequent transfers were tabulated. The different data on callus induction, regeneration, estimation of Arsenic Andrographolide and other metabolites in both control and metal tolerant plants were subjected to ANOVA test. Mean separation and significance was carried out using Duncan's Multiple Range Test (DMRT) using SPSS (version 12.0) software package in an experimental practice.

Results

Production of Arsenic Resistant Callus

Calli were initiated from the wounding site of stem explants on MS basal salts, 3.0% sucrose, 0.8% agar medium fortified with different concentrations of As_2O_3 metal (0.0- 9.0 µM) along with optimum level of NAA (3.5 mg L^{-1}) and KIN (1.0 mg L^{-1}) after 2-3 weeks of culture (Fig. 1A). In this case, about 100% callusing (229±0.5 mg fwt/96.3±1.5 mg dwt) of calli was observed from stem explants after 60 days of culture in control experiment. The stress resistant calli were proliferated by subculturing on fresh medium. The callusing frequency was decreased in different concentrations of As_2O_3 tested medium. As_2O_3 at 1.0 µM influenced 72.5% resistant calli (201±1.8 fwt/88.1±1.0 dwt) formation whereas 3.0 µM As_2O_3 treated medium induced 55.7% callusing (160±1.3 mg fwt/69.3±1.5 mg dwt). As_2O_3 at 5.0 µM stimulated 40% resistant calli (110±2.3 mg fwt/52.1±0.3 dwt) while 9.0 µM of As_2O_3 influenced minimum of 3.2% resistant calli (9±1.9 mg fwt/2.1±1.5 mg dwt) from stem explants culture. Although 7.0 µM As_2O_3 stress induced 22% resistant callus line (53±0.5 mg fwt/27±1.5 mg dwt) after 60 days of culture (Table 1; Fig. 1B).

Effect of Arsenic on Shoot Organogenesis

Calli subcultured on media supplemented with different levels of As_2O_3 (0.0-9.0 µM) along with 2.5 mg L^{-1} BA and 3.0 mg L^{-1} NAA stimulated the conversion from non-organogenic into organogenic type of resistant calli. In control experiment, stem calli induced 92.4% shoot organogenesis (7.3 number of plantlets) was recorded after 45 days of culture. Stem derived calli cultured on 1.0 µM As_2O_3 treatment media induced 71.9% shoot organogenesis (6.5 number of plantlets). About 44% shoot organogenesis (4.7 number of plantlets) was produced at 3.0 µM As_2O_3 treatment while 5.0 µM As_2O_3 influenced 30% shoot regeneration (3.3 number of plantlets). Although 14.5% shoot organogenesis (1.6 number of plantlets) was noticed in 7.0 µM As_2O_3 selective medium after 45 days of culture. Shoot length was decreased from 1.5-0.6 cm and the number of leaf induction was ranged from 8.3-3.1 per plantlet at 0.0-7.0 µM As_2O_3 treated medium, respectively. Shoot induction was not observed from stem calli on 9.0 µM As_2O_3 treated medium (Table 1; Fig. 1C).

Root Induction and Acclimatization Response

The isolated individual plantlets (0.6-1.5 cm length) cultured on half strength MS basal salts, 1.5 % sucrose, 0.8% agar medium fortified with As_2O_3 (0.0-9.0 µM) and optimum level of IBA (2.0 mg L^{-1}) influenced the adventitious rooting after 30 days of culture. In control, the cutting edge of plantlets produced 37.9 numbers of rootlets (1.7 cm in length) on half strength MS basal medium fortified with IBA (2.0 mg L^{-1}). The *in vitro* selection of 1.0 µM As_2O_3 induced 25.4 number of rootlets (1.5 cm in length) whereas 3.0 µM As_2O_3 treatment developed 19.9 number of rootlets (1.2 cm in length). Plantlets cultured on 5.0 µM As_2O_3 produced 14.5 number of rootlets (1.0 cm in length). However, plantlets with 11.1 number of rootlets (0.8 cm in length) was recorded on 7.0 µM As_2O_3 tested media 30 days after of culture initiation (Table 1; Fig. 1D). As_2O_3 at 9.0 µM treatment was not suitable to induce shoot and root development in stem calli. The *in vitro* raised control and As_2O_3 tolerant plants were survived in pots containing soil supplied with the optimum level of As_2O_3 (7.0 µM) solution and adapted to the normal environmental condition (Table 1; Fig. 1D and E). In

this case, about 40% survival was noticed in As_2O_3 tolerant plants 45 days after transfer to soil (Table 2; Fig. 1D and E), but *in vitro* control plants showed only 19% survival in pots containing soil. Further, the arsenic tolerant plants were grown well and adapted to open soil under greenhouse condition (Fig. 1F).

Analysis of Arsenic in Plant Samples by AAS

The arsenic concentration in control and As_2O_3 tolerant plants were analysed by AAS. The results showed that the level of Arsenic was increased when plants were exposed to As_2O_3 stress ranged from 0.0-9.0 µM. In this case, about 0.96 ppb Arsenic was recorded in control plants. The tolerant plants on 1.0 µM As_2O_3 treated medium showed only 1.57 ppb of Arsenic while 3.0 and 5.0 µM As_2O_3 selective medium influenced the accumulation of 2.88 ppb and 3.50 ppb Arsenic in tolerant plants, respectively. However, maximum of 4.67 ppb Arsenic was accumulated in 7.0 µM As_2O_3 treated plants (Table 3; Fig. 2).

Analysis of Andrographolide in Plant Samples by HPLC

The HPLC mobile phase was standardized to get a better resolution of the peak spot for andrographolide. Spectral studies showed the identical similar pattern of the peaks for both standard andrographolide and test samples. The peak area of standard Andrographolide was eluted at 2.871 min (Fig. 3A). Total amount of Andrographolide was estimated by considering of retention time and peak area. The powder samples of *in vitro* control and As_2O_3 tolerant plants were extracted with methanol to quantify the Andrographolide content by HPLC. The amount of Andrographolide calculated in *in vitro* control plant extract of *A. paniculata* was 1.84 mg/g. About 2.18 mg/g Andrographolide content was determined in 1.0 µM As_2O_3 tolerant plants. *In vitro* As_2O_3 (3.0 µM) tolerant plants showed 2.95 mg/g Andrographolide while 3.52 mg/g Andrographolide was determined from 5.0 µM As_2O_3 stress tolerant plants. However, tolerant plants grown at 7.0 µM As_2O_3 noticed maximum of 4.41 mg/g of Andrographolide (Table 4; Fig. 3B and C).

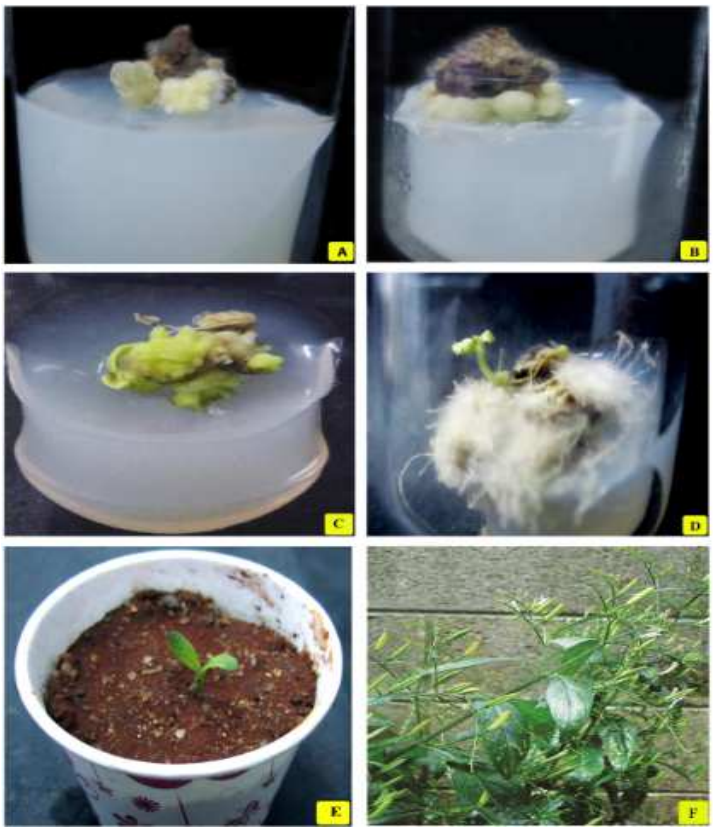

Fig. 1: Effect of As_2O_3 on tolerant plant regeneration from stem calli of *A. paniculata* (Burm.f.) Nees, (A,B) Initiation and proliferation of resistant calli on 7.0 µM) As_2O_3 and +3.5 mg L^{-1} NAA+1.0 mg L^{-1} KIN treatment media after 60 days culture, (C) Shoot organogenesis on 7.0 µM As_2O_3+2.5 mg L L^{-1} BA+3.0 mg L L^{-1} NAA treatment media after 45 days of culture, (D) Adventitious root induction on 7.0 µM As_2O_3+IBA (2.0 mg L^{-1}) treatment media after 30 days culture, (E) Survival of Arsenic (As) tolerant plants on plastic pots containing autoclaved soil mixture, (F) Adaptation of Arsenic (As) tolerant plants 90 days after transfer to soil under greenhouse condition

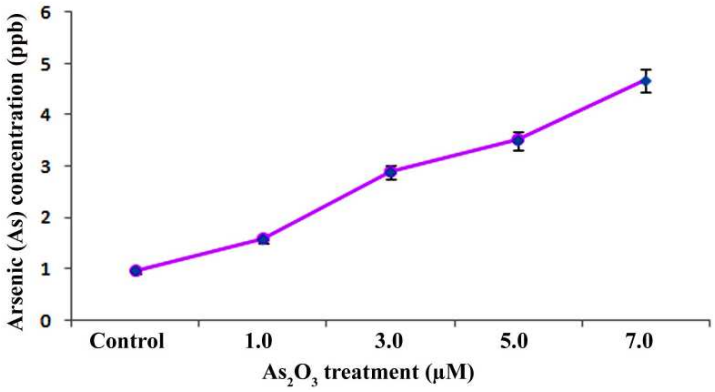

Fig. 2: Analysis of Arsenic (As) from *in vitro* control and As_2O_3 tolerant plants of *A. paniculata* (Burm.f.) Nees by AAS

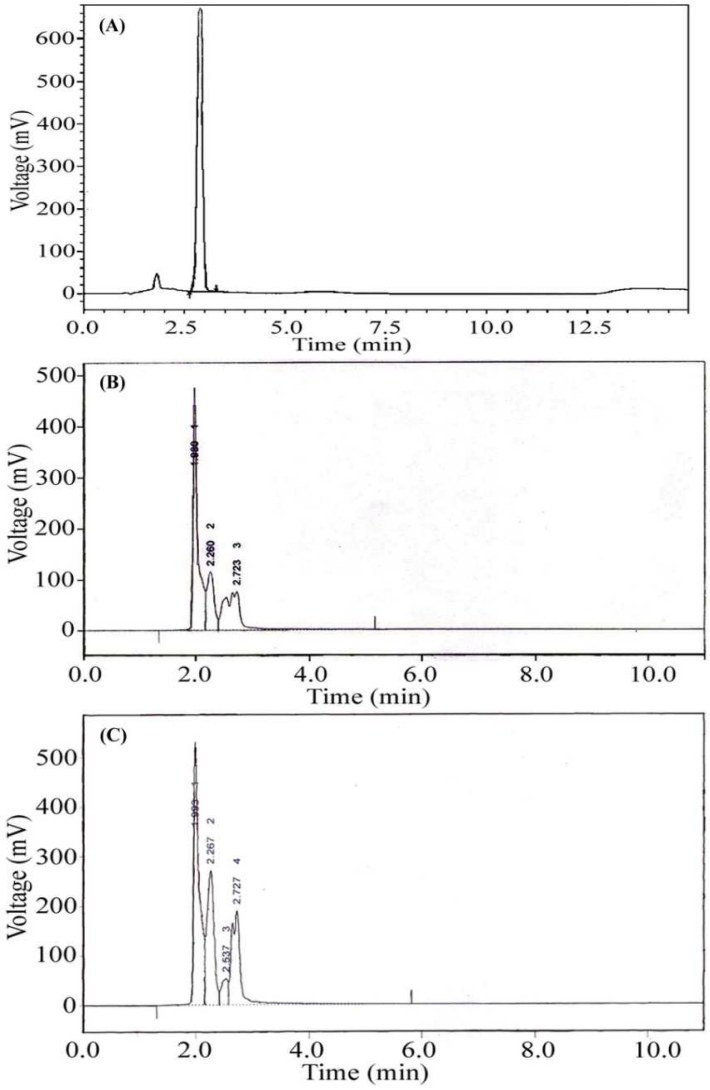

Fig. 3: HPLC chromatogram of andrographolide compound in different samples of *A. paniculata* (Burm.f.) Nees. (A) HPLC standard chromatogram of andrographolide compound, (B) HPLC chromatogram of andrographolide present in *in vitro* control plant grown in 2.5 mg L^{-1} BA+3.0 mg L^{-1} NAA fortified media, (C) HPLC chromatogram of andrographolide present in tolerant plant samples grown 7.0 µM As_2O_3+2.5 mg L^{-1} BA + 3.0 mg L^{-1} NAA treatment media

Table 1: Effect of As_2O_3 stress on plant regeneration from stem derived calli of *Andrographis paniculata* (Burm. f.) Nees. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test (p<0.05). *mg fresh weight, [#]mg dry weight

	As_2O_3 concentrations (µM)					
Culture condition	0.0	1.0	3.0	5.0	7.0	9.0
Resistant callus line (MS+3.5 mg L^{-1} NAA+.0 mg L^{-1} KIN)	*229±0.5a [#]96.3±1.5] -100%	201±1.8a [#]88.1±1.0] -72.50%	160±1.3a [#]69.3±1.5] -55.70%	110±2.3a [#]52.1±0.3] -40%	53±0.5a [#]27±1.5] -22%	9±1.9a [#]2.1±1.5] -3.20%
Number of shoots (MS+2.5mg L^{-1} BA+3.0 mg L^{-1} NAA)	7.3±2.9cd	6.5±1.8bc	4.7±2.0cd	3.3±1.1cd	1.6±0.7cd	0.0±0.0b
Shoot organogenesis(cm)	(92.4%)	(71.9%)	(44%)	(30%)	(14.5%)	0%
Shoot length	1.5±1.2ef	1.3±0.9de	1.1±2.5de	0.8±2.0e	0.6±1.3de	0.0±0.0b
Number of leaf per plantlets	8.3±0.9c	6.1±1.5c	5.0±3.1c	4.4±0.9c	3.1±1.0c	0.0±0.6b
Number of roots (MS+2.0 mg L^{-1} IBA)	37.9±0.6b	25.4±2.5b	19.9±1.2b	14.5±1.7b	11.1±1.4b	0.0±0.0b
Root length (cm)	1.7±1.0e	1.5±1.2d	1.2±2.5d	1.0±2.0d	0.8±1.0d	0.0±0.0b

Table 2: Effect of As_2O_3 on survival of *in vitro* raised plants in pots containing soil mixture. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test (p<0.05)

		Survival of plants under greenhouse condition		
Response	Mean no. of plants	15 days	30 days	45 days
Control plants	26.7±2.0b	53.40%	27%	19%
As_2O_3 tolerant plants	42.5±1.4a	85%	55%	40%

Table 3: Analysis of Arsenic (As) in control and tolerant plants of *A. paniculata* (Burm. f.) Nees by atomic absorption spectrometry. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test (p<0.05) *ppb-parts per billion

S. No	Control/tolerant plant samples in As_2O_3 treatment	Amount of Arsenic (As) (ppb)*
1	0.0 µM	0.96±1.3cd
2	1.0 µM	1.57±0.7c
3	3.0 µM	2.88±2.5b
4	5.0 µM	3.50±2.0ab
5	7.0 µM	4.67±1.7a

Table 4: Analysis of Andrographolide in control and As_2O_3 tolerant plants from stem calli of *A. paniculata* (Burm.f.). Nees by HPLC. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test (p<0.05)

As2O3 (µM)	Andrographolide (mg/g)
0.0	1.84±0.9d
1.0	2.18±2.0cd
3.0	2.95±1.0c
5.0	3.52±1.5b
7.0	4.41±0.5a

Table 5: Metabolite assignment from control and 7.0 µM As_2O_3 tolerant plants of *A. paniculata* (Burm.f.) Nees by 1D ^{1}H NMR. Values are mean of three repeated experiments. Mean within a column followed by the same letters are significantly different according to one way ANOVA and Duncan's multiple range test (p<0.05)

Aliphatic amino acids and other metabolite region	Control plants [δ ppm]	Assignment compounds	As_2O_3 (7.0 µM) tolerant plants [δ ppm]	Assignment compounds
1	1.030±1.5e	Valine	1.031±0.9d	Valine
2	1.046±0.5de	Valine	1.047±1.3cd	Valine
3	1.062±1.0d	Isoleucine/Isobutanol	-	-
4	2.507±2.0c	Glutamine/Succinic acid	2.508±1.0c	Glutamine/Succinic acid
5	2.994±1.1bc	Aspartic acid/2-DMG	2.997±0.4bc	Aspartic acid
6	3.169±0.7b	Choline/EDTA	3.172±1.6b	2-Oxoglutarate/Choline
7	3.342±0.5ab	GABA/Glycine/Proline	3.346±1.0ab	Proline/Taurine
8	3.904±1.6a	Glycine/Glucose	3.993±1.2a	Glycine

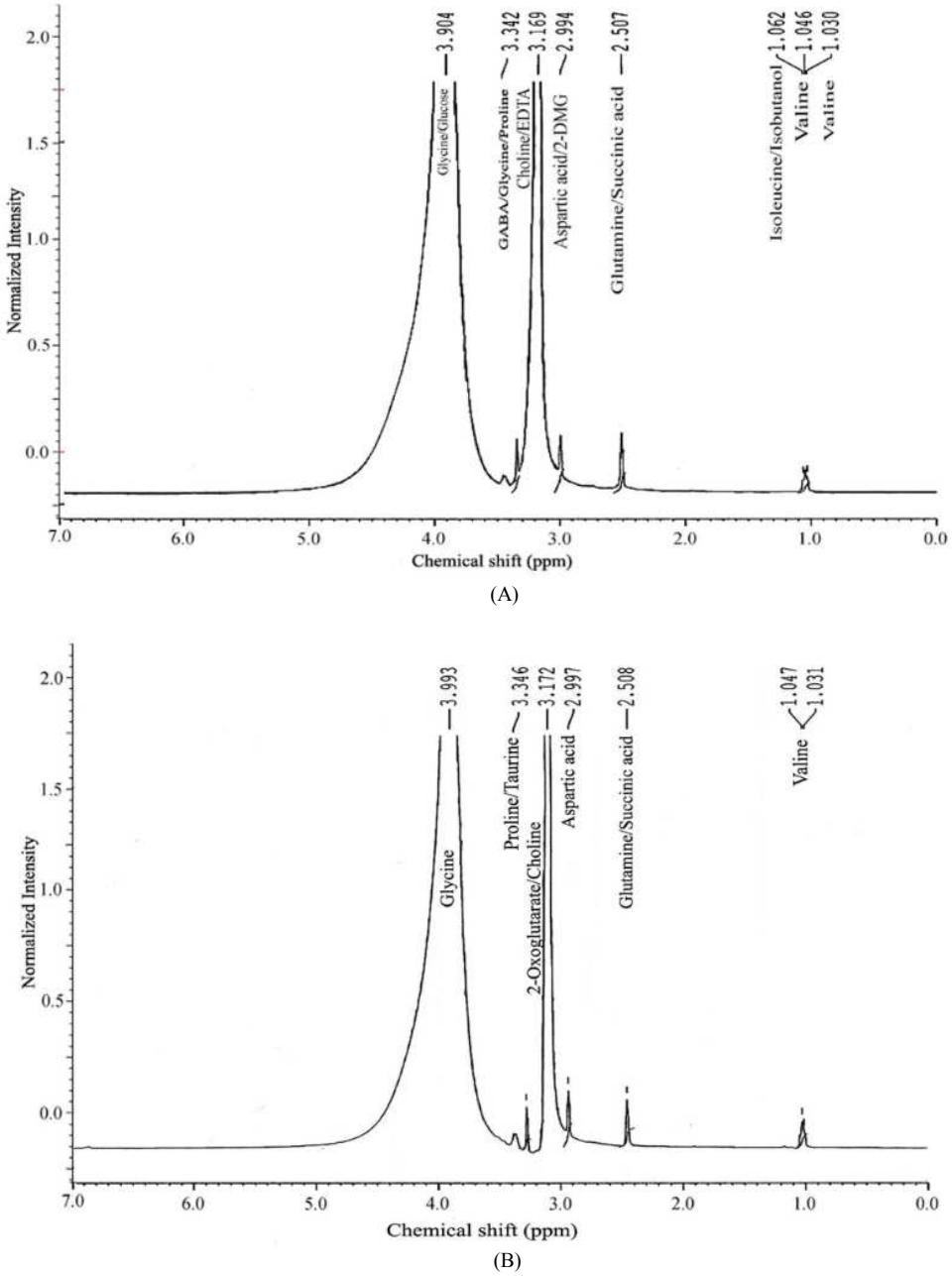

Fig. 4: Representative 1D 1H-nuclear Magnetic Resonance (NMR) spectra of *in vitro* control (A) and Arsenic (7.0 µM As_2O_3) tolerant (B) plant extracts

Analysis of Metabolite in Plant Samples by 1D ^1H NMR

1D ^{1}H-NMR is adequate to produce metabolomic data of plant sample within a short period. The NMR signals are directly relative to the self-determining characteristic of a compound. The absolute metabolite concentration can be estimated by comparison of the peak intensity with an internal standard. In this case, a typical 1D 1H-NMR spectrum was very functional to show the signals with chemical shifts (δ) regions of interest to predict the preliminary metabolites of *in vitro* control and As_2O_3 tolerant plant samples of *A. paniculata.* For *in vitro* control plant sample, aliphatic amino acid region displayed the resonances of assignment compounds, valine (doublet at δ 1.030 ppm and δ 1.046 ppm), isoleucine/isobutanol (singlet at δ 1.062 ppm), glutamine/succinic acid (singlet at δ 2.507 ppm), aspartic acid/ Dimethyl glycine (2-DMG) (singlets at δ 2.994 ppm), choline/EDTA (singlets at δ 3.169

ppm), γ-aminobutyric acid (GABA)/proline (singlet at δ 3.342 ppm) and glycine (doublet at δ 3.342 ppm and δ 3.904 ppm). As_2O_3 (7.0 µM) tolerant plant samples showed the signals of valine (doublet at δ 1.031 ppm and 1.047, Glutamine/Succinic acid (singlet at δ 2.508 ppm), aspartic acid (singlet at δ 2.997 ppm), 2-oxoglutarate/choline (singlets at δ 3.172 ppm), proline/taurine (singlets at δ 3.346 ppm) and glycine (singlet at δ 3.993 ppm). The signal of isoleucine, isobutanol, glutamine, 2-DMG, EDTA and γ-aminobutyric acid (GABA) were not recorded in As_2O_3 (7.0 µM) tolerant plant samples (Fig. 4A and B). The chemical shifts of 1D ^{1}H-NMR assignment compounds are summarized in Table 5.

Discussion

Arsenic Resistant Callusing Response

Callus initiation can be started from the wounding site of explant due to the effect of exogenous growth regulators. The callus tissues could be a valuable biological material to its genetic stability and the polyploidization under certain culture condition (Botau *et al*., 2005). The current study was optimized the culture conditions to develop a competent callus tissues from stem explants of *A. paniculata* in NAA (3.5 mg L^{-1}) and KIN (1.0 mg L^{-1}) tested medium as control experiment. Similarly, Martin (2004) reported the callus induction from internode explants of *A. paniculata* in NAA and KIN tested medium. The supplementation of NAA and KIN induced frequency of callusing in *Ipomoea aquatic* forsk (Prasad *et al*., 2006) and *Cleome spinosa* Jacq (Qin *et al*., 2012). In this case, the stem explants cultured on different concentrations of As_2O_3 (0.0 - 9.0 µM) along with optimum level of NAA (3.5 mg L^{-1}) and KIN (1.0 mg L^{-1}) induced frequency of resistant callus production after 60 days of culture in treatment experiment. The resistant callus production was slightly decreased by increasing the level of As_2O_3 in the medium. The reduction of callus growth could be due to arsenic accumulation in undifferentiated cells. However, 7.0 µM As_2O_3 was found to be better concentration in development of 22% resistant callus line after 60 days of culture. The production of resistant callus line was very poor (3.2%) at 9.0 µM As_2O_3 treated media.

In Vitro Regeneration of Arsenic Tolerant Plants

Arsenic speciation is existing in the environment as inorganic and organic forms by biotic and abiotic processes. The arsenic concentration in soils causes extensive symptoms that correspond to plant uptake and subsequent entry into human food chains and wildlife (Meharg and Whitaker, 2002). Recently, regeneration of tolerant plants through *in vitro* selection pressure is a very important technology which has received attention as an innovative and cost-effective methods and alternative to the more established treatment method for elimination of heavy metals. The present study was utilized an *in vitro* selection method to regenerate As_2O_3 tolerant plants from stem calli of *A. paniculata*. The shoots can regenerate at basal edge of explant through indirect pathway after callus formation (García-Luis *et al*., 2006). Here, microshoots induced on MS medium fortified with 2.5 mg L^{-1} BA and 3.0 mg L^{-1} NAA was found to be optimal for shoot proliferation in control experiment. Similarly, shoot organogenesis was reported from calli of *Aegle marmelos* (Arya *et al*., 1981), *Momordica dioica* (Nabi *et al*., 2002) and *Rauwolfia serpentina* (Tomar and Tiwari, 2006) cultured on BA and NAA fortified medium. Further, *in vitro* selection of As_2O_3 influenced shoot organogenesis from resistant stem calli of *A. paniculata* plants. The shoot induction frequency was decreased when the As_2O_3 concentration was enhanced in the medium. In this case, As_2O_3 at 7.0 µM was found to be greatest for regenerating 14.5% arsenic tolerant microshoots from stem calli after 45 days of culture while resistant calli were failed to grow further and necrosed in 9.0 µM As_2O_3 treated medium after 5-7 days of culture. Adventitious roots were formed directly from shoot base without development of intervening callus on media fortified with 2.0 mg L^{-1} IBA in control experiment. The obtained results are in concurrence with Purkayastha *et al*. (2008; Jindal *et al*., 2015) reports in *A. paniculata* plants. In the course of treatment experiment, the root induction was gradually decreased at various levels of As_2O_3 along with 2.0 mg L^{-1} IBA treated media after 30 days of culture. However, the tolerant roots were developed from healthy looking plantlets in 7.0 µM As_2O_3 treated media and found superior as hyperaccumulator of Arsenic metal and showed more tolerance while As_2O_3 at 9.0 µM induced the strong inhibition of growth and development of shoots and roots. The accumulation of Arsenic in tolerant plants induces reactive oxygen species production that can lead to the synthesis of antioxidant metabolites and enzymes. Modification of glutathione production pathway has been shown to increase arsenic tolerance in plants. In other hand, the rate of arsenic accumulation permits the plant to detoxify the incoming Arsenic before stuffing of the defense systems (Finnegan and Chen, 2012). The arsenic treated plants produce phytochelatins which offer protection against heavy metals in tolerant plants (Cobbet and Goldsbrough, 2002). The accumulated organic or inorganic forms of arsenic metals are detoxified in the soil by plants through phytoremediation process. Speciation can provide very useful information for understanding the accumulation, transformation and detoxification mechanism of arsenic in plants (Cai and Braids, 2001). In a similar fashion, arsenic tolerance and detoxification mechanisms has been reported in *Pteris vittata* plants (Zhang *et al*., 2002). Although the present investigation reports that the

in vitro culture of *A. paniculata* is efficient in taking up arsenic from media and found suitable to show higher survival rate in As_2O_3 contaminated soil.

Measurement of Arsenic in Tolerant Plants by AAS

The accumulation of arsenic in control and tolerant plant samples were compared by AAS analysis. It is the most commonly used method for arsenic speciation by element detection (Rajaković *et al*., 2013). The arsenic content was significantly increased in As_2O_3 treated plants than control. However, about 4.67 ppb arsenic was found to be highest in *in vitro* tolerant plants on 7.0 µM As_2O_3 treatment while *in vitro* control plants showed only 0.96 ppb arsenic. Similarly, the presence of arsenic was quantitatively estimated from the leaves and stem bark of ten medicinal plants (Atinafu *et al*., 2015).

Quantification of Andrographolide by HPLC

The quality of chemical substances from the herbal extracts can be guaranteed by applying of suitable analytical methods for identification, determination and quantification of the active elements. Earlier, many researchers have also been involved to estimate the amount of Andrographolide in the active constituents of *in vivo* grown *A. paniculata* plants (Sharma *et al*., 1992; Jain *et al*., 2000; Srivastava *et al*., 2004; Chen *et al*., 2007; Raina *et al*., 2007). Further, *in vitro* studies indicated that the accumulation of 2.35 mg/g andrographolide from hairy root culture of *A. paniculata* in IBA (5.0 µM) tested medium was estimated (Marwani *et al*., 2015). In the present observation, the production of andrographolide was gradually increased in tolerant plants treated with different levels of As_2O_3. However, 4.41 mg/g andrographolide was estimated to be highest in *in vitro* tolerant plants treated with 7.0 µM As_2O_3 when compared to control.

Metabolomic Analysis by 1D ^{1}H-NMR

NMR-based metabolomics analysis is a very popular analytical method in terms of the quality control of medicinal plants (Kim *et al*., 2010). It provides the overall profile for the assessment of different proton-containing soluble metabolites. The signals of NMR assignments were made based on the earlier findings (Choi *et al*., 2006; Leiss *et al*., 2009). In this study, the stacked 1D ^{1}H NMR spectra (δ 1.030-3.993 ppm) of *in vitro* control and tolerant plant samples of *A. paniculata* from 7.0 µM As_2O_3 treatment were analysed with the expansion of intensity of aliphatic amino acid, organic acids and other metabolite regions. By visual inspection, 1D ^{1}H NMR-based metabolomics profile revealed the changes of metabolite pattern significantly in both samples. There are 12 metabolites present in *in vitro* control plants grown in MS medium fortified with NAA (3.5 mg L^{-1}) and KIN (1.0 mg L^{-1}). These are valine, isoleucine, isobutanol, glutamine, succinic acid, aspartic acid, Dimethyl glycine (2-DMG), choline, EDTA, γ-aminobutyric acid (GABA), proline and glycine. Of these metabolites, the signals of isoleucine, isobutanol, Dimethyl glycine (2-DMG), EDTA, γ-aminobutyric acid (GABA) and glucose were not detected in tolerant plants grown at 7.0 µM As_2O_3 selected medium. The metabolites such as 2-Oxoglutarate and Taurine alone were present in As_2O_3 tolerant plants, but absent in *in vitro* control plants of *A. paniculata*. Similarly, the different samples of *Glycyrrhiza* species were examined by 1D ^{1}H NMR-based metabolomics analysis (Yang *et al*., 2010). Anand *et al.* (2011) also reported the various bioactive chemical compounds by NMR spectral analysis in *Zehneria scabra.* The results showed that the usage of 1D ^{1}H NMR for comparing metabolic profiles of *in vitro* culture samples can be useful for understanding the biochemical relationships (Mahmud *et al*., 2014).

The results of present study suggest the impact of As_2O_3 on regeneration of arsenic tolerant plants from stem callus line of *A. paniculata* through *in vitro* selection pressure. The arsenic content was significantly increased in 7.0 As_2O_3 treated plants than control by AAS analysis. The arsenic level in tolerant plant was found suitable and showed within the WHO permissible levels and safe to be exploited in herbal drug formulation. Further, the As_2O_3 treatment has great potential in enhancing biosynthetic pathway and significantly increased the Andrographolide content in arsenic tolerant *A. paniculata* plants than control. It is noted that 1D ^{1}H NMR-based spectral comparison can be a valuable tool for understanding the distinct amino acid, organic acid and other metabolite differences among *in vitro* raised control and tolerant plants due to As_2O_3 stress. Moreover, this efficient and reliable protocol of *in vitro* selection of As_2O_3 offered less costly and environment-friendly phytoremediation method for regeneration of high frequency tolerant plants of *A. paniculata* to detoxifify Arsenic metal present in culture media and contaminated soil.

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Author's Contributions

J. Vijayakumar: Performed all the experiments and worked on the study plan and design, data analysis and interpretation and wrote the manuscript.

P. Ponmanickam: Participated and was involved to co-ordinate the data analysis in the manuscript.

P. Samuel: Involved in the data analysis & interpretation.

G. Shobana Rathi: Performed all the experimental methods.

B. Pavithra: Participated in all experiments and was involved in scientific discussion.

A. Manjula: Participated in all experiments, and coordinated the study and was involved in scientific discussion.

S. Aswathi: Participated in all experiments and was involved in Critical revision.

Ethics

The authors declare that they have no conflict of interest.

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