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PIXE-based quantification of health-proactive trace elements in genetically transformed roots of a multi-medicinal plant, *Sida acuta* Burm.f.

Somanatha Jena · Lopamudra Sahu · Dinesh K. Ray · Sagar K. Mishra · Pradeep K. Chand

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Abstract Inorganic element composition of genetically transformed hairy root cultures (HRCs) of a medicinal plant, *Sida acuta* was determined using PIXE technique. HRCs had a higher accumulation of pro-health trace elements compared to natural roots. It was estimated that < 160 g d.wt. of a selected rhizoclone could suffice to provide nearly all tested essential elements catering to *per diem* requirement of the human body. The ideal multi-elemental profile ushers a new possibility of integrating hairy root extracts in modern therapeutics ("rhizotherapy") as complementary medicine or a dietary nutraceutical supplement.

Keywords Hairy roots · Particle induced X-ray emission (PIXE) · *Sida acuta* Burm.f. · Trace elements

Introduction

Particle induced X-ray emission (PIXE) is a widely used nuclear analytical technique based on the measurements of characteristic X-rays which are induced by the energetic proton beam (MeV energy scale) directed onto the surface of a sample held under vacuum/air [1]. This technique is non-destructive, accelerator-based, simultaneous multi-elemental and suitable for quantitative elemental characterization of a wide range of complex materials, including biological samples, especially containing lower-middle Z (atomic number) elements [2]. Additional advantages of this technique encompass its reliability, high sensitivity (below ppm low level detection) for several elements and its appropriateness for routine analytical work. While most of the other techniques (ICP-OES, AAS, GF-AAS) demand extensive (multi-step) and destructive sample preparation procedures, in PIXE analysis, minimal sample preparation is required; hence external sources of contamination resulting in erroneous results can be avoided.

It is, indeed, interesting to note that minerals necessary for healthcare are accumulated in the plants as components of their growth and development [3]. Essential trace elements, not only play a role in plant nutrition but also, being cofactors of enzyme activity, they influence physiological functions in biological systems in general [4]. A number of macro- and micronutrients are known to influence the biochemical processes and metabolism in humans [5]. A study of these major and trace elements, native to medicinal plants, has revealed that they play a key role in combating various human diseases [6]. Trace elements once absorbed in the body get deposited in the liver through the blood stream and are carried to different parts of the body to participate in various biochemical reactions. Hence, changes in the concentrations of these trace elements may pose various clinical and pathological disorders. The deficiency or excess of trace element concentrations in the cancer tissues of different vital organs (kidney/testis) is believed to vary [7]. Trace elements are also necessary for the maintenance and regulation of gene, cell and membrane functions [8].

S. Jena · L. Sahu · P. K. Chand (✉)
SFC Applied Microbiology, Post-Graduate Department of Botany, Utkal University, Vani Vihar, Bhubaneswar 751 004, Odisha, India
e-mail: pkchanduubot@rediffmail.com

D. K. Ray
Ion Beam Laboratory, Institute of Physics, Bhubaneswar 751 004, Odisha, India

S. K. Mishra
University Department of Pharmaceutical Sciences, Utkal University, Vani Vihar, Bhubaneswar 751 004, Odisha, India

Due to the adverse effect of synthetic medicines, plant based drugs are gaining importance in the last few decades. Plants have long been used in the Indian traditional system of medicine for various human ailments and diseases. The medicinal properties of these plants are due to chemical constituents present in them in the form of secondary metabolites, such as alkaloids, terpenoides, flavonoids, quinones, saponins, phytosterols, amines, glycosides etc.; and besides, the trace elements, which play an important role in the formation of these compounds [9, 10]. Knowledge of the elemental content in these medicinal plants and their therapeutic implication can be valuable as some of these elements are closely related to human healthcare [11]. Therefore, a correlation between the elemental profile of a given medicinal plant and its traditional curative usage is desirable [12]. Based on these information, the dosages of the drugs prepared using such plant extracts can be determined for recommendation against different diseases.

Sida acuta Burm.f., is a Malvaceous weed that frequently dominates improved pastures, waste and disturbed places on roadsides. The plant is native to Mexico and Central America but has spread throughout the tropics and subtropics, including the hotter parts of India. Root of the plant is used as a bitter tonic, stomachic, diaphoretic and antipyretic [13]. Roots are also reported to be useful in treating nervous and urinary diseases, blood, bile and liver disorders [14], skin diseases, snake bites, leucorrhoea, tuberculosis, diabetes, and as anti-rheumatic and antipyretic [15].

Several biotechnological approaches such as de-differentiated cell suspension cultures have been exploited to enhance the content of bioactive phytochemicals. However, success in this direction has not been satisfactory because of low levels of accumulation of secondary compounds and somaclonal variation accountable for biochemical and genetic instability of cells. Hence, an inclination towards transformed organ cultures, especially transformed 'hairy root' cultures has gained importance, being genetically and biosynthetically more stable with respect to the cell suspensions [16]. In this perspective, a phyto-pathogenic Gram-negative soil bacterium, namely *Agrobacterium rhizogenes,* naturally causing the 'hairy root' disease mostly in dicotyledonous plants, holds significance. The disease occurs as a result of the expression of T-DNA genes following their conjugational transfer from the large Ri plasmids of *A. rhizogenes* to the infected plant genome. This T-DNA region harbours the characteristic oncogenic sequences encoding auxin biosynthesis (*iaa*M, *iaa*H) and root induction (*rol*A-D). Following signal transduction events the T-DNA borne genes are integrated into the host root genome and finally over-expressed which results in excessive auxin production by transformed roots. This natural "genetic engineer" has become an important component of plant biotechnology; hairy root cultures being exploited for the production of pharmaceutically important compounds from various plant species of medicinal value [17]. Mostly these extraordinary in vitro root cultures are being utilized for obtaining useful organic compounds, especially secondary metabolites [18]; however, their inorganic element content has been given little consideration. Owing to the multiple medicinal properties of *Sida acuta*, the present investigation was targeted at inducing and establishing transformed hairy root cultures of this plant species and determining their trace element content. Such genetically transformed root cultures could be harnessed as a sustainable source of essential minerals aiming at effective drug development.

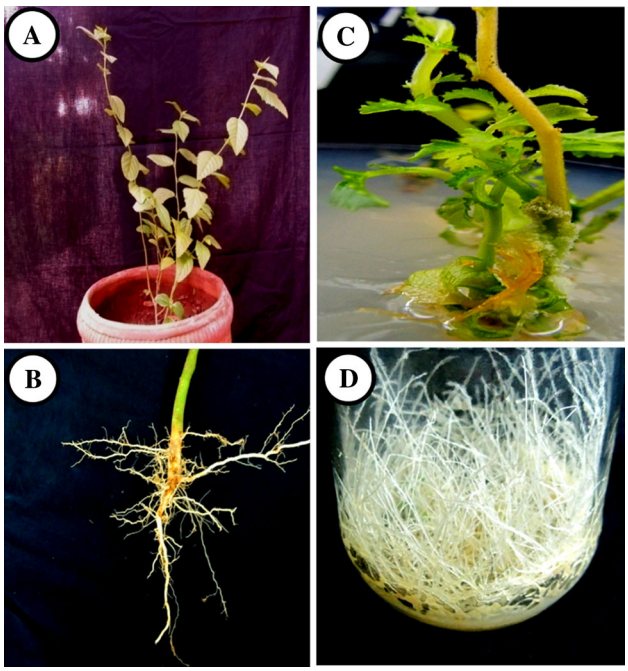

Fig. 1 Root tissue sources of *Sida acuta* for PIXE analysis **A** A 2-month-old seed-derived plant used as explant donor **B** Natural root system of the explant-donor plant **C** An in vitro–raised non-transformed plant with roots **D** A well proliferated hairy root somaclone (rhizoclone ST5F4 induced by genetic transformation via co-cultivation of an internode explant with *Agrobacterium rhizogenes* A4T strain) in agar-solidified MS0 after 45 days of culture

Materials and methods

Induction and proliferation of transformed root cultures

Plant material

Sida acuta Burm.f. plants were grown from seeds in earthenware pots (Fig. 1a, b) and maintained in the

Department of Botany, Utkal University, Bhubaneswar (India). Nodal segments were collected and cleansed by placing them under running tap water (20 min). Explants were treated with 7.5 % (v/v) Lizol (Reckitt Benckiser, India) for 20 min and thereafter rinsed in autoclaved tap water (4–5 changes). This was followed by surface-disinfection with 0.1 % (w/v) mercuric chloride (8 min) and rinsing with autoclaved distilled water (4–5 changes). Nodal segments were inoculated into 300 ml screw-capped glass jars (Excel Corporation, India), containing full-strength Murashige and Skoog (1962) medium (MS) [19] fortified with 1 mg BA l^{-1} (20 ml per jar). Multiple shoots developed in vitro were cut into single node pieces (0.8–1.0 cm) and sub-cultured in the same medium at a periodic interval of 4–6 weeks for successive multiplication to establish axenic shoot cultures. In vitro-raised shoots were rooted in MS augmented with 0.25 mg l^{-1} IBA (Fig. 1c). Cultures were maintained inside a culture room (25 ± 1 °C, 35–40 μmol m^{-2} s^{-1} photon flux densities [PFD], 60 % R.H).

Agrobacterium rhizogenes strain and culture media

A wild type strain of *A. rhizogenes* viz. A4T possessing an agropine type pRiA4 (kind gift from Dr. D. Tepfer, Laboratoire de Biologie de la Rhizosphere, Institut National de la Rechereche Agronomique, Versailles, Cedex, France) was used for transformation events. The strain was grown in MYA medium (5 g l^{-1} yeast extract, 0.5 g l^{-1} casamino acids, 8 g l^{-1} mannitol, 2 g l^{-1} $(NH_4)_2SO_4$, 5 g l^{-1} NaCl; pH 6.6). Prior to autoclaving, the pH of the culture medium was adjusted and then the medium was cooled to 40 °C in a water bath. Thereafter, 2 ml of 1 M $MgSO_4$, $7H_2O$ and 50 mg l^{-1} rifampicin from the respective filter-sterilized stock solutions were added. In order to infect the explants, a loopful of bacteria from a single colony was inoculated into 20 ml liquid medium in a 50-ml Erlenmeyer flask and incubated on a reciprocal shaker (120 rpm) at 28 °C. Acetosyringone (Sigma, USA; 100 mM stock solution in DMSO) was added to the culture medium at a final concentration of 100 μM prior to bacterial inoculation. *A. rhizogenes* cultures were re-grown inside an incubator shaker (N-Biotek, NB-205QF, Korea; 120 rpm, 28 °C). Bacterial suspensions were grown overnight (16–18 h) to an A_{660} (OD 660 nm) ≅ 0.6 and diluted to a density of 10^9 cells ml^{-1} to be used as inoculum for co-cultivation of explants for transformation.

Transformation

Stem internodal segments (3–4 cm) from outdoor-grown in vivo plants were surface-disinfected prior to transformation, where as those from in vitro grown axenic shoot cultures (4-week-old) were used directly. The tips of the internodal segments were sliced tangentially with a scalpel blade and the cut surfaces were inoculated by means of a sterile hypodermic needle with 10–20 μl of overnight-grown culture of *A. rhizogenes*. Two methods were used for inoculation. In the first method, the explants were injected with bacterial suspension directly by a hypodermic syringe, while in the other they were pre-wounded by pricking manually with sterile needle and then immersed in the bacterial suspension for 15–20 min. Another third method was used which involved immersion of explants in the *A. rhizogenes* suspension without any prior wounding or pricking. All the explants treated with *A. rhizogenes* were plunged into screw-capped jars containing agar-gelled (0.8 % w/v) MS medium without added growth regulators (MS0, 20 ml per jar) and were kept inside the culture room (25 ± 1 °C) under diffused light with a low PFD (10–15 μmol m^{-2} s^{-1}). Subsequent to co-cultivation (2–8 days), explants were transferred onto MS0 medium fortified with a bactericidal antibiotic, cefotaxime (Sigma, USA; 500 μg ml^{-1}). Similar explants wounded with the hypodermic needle without any bacteria were used as control cultures and maintained under the same conditions of light and temperature as for inoculated cultures.

Establishment of transformed root cultures

As a result of infection with *A. rhizogenes* the individual roots (2.0 cm), that developed along the sites of inoculation, were excised and each transferred to 50 × 11 mm transparent plastic TPX Petri dish (Tarsons, India) each containing 8.0-10 ml of MS0 medium supplemented with 0.25 mg l^{-1} IBA in addition to the bactericidal antibiotic (500 μg ml^{-1} cefotaxime). Root cultures were incubated at 25 ± 1 °C and 10–15 μmol m^{-2} s^{-1} PFD and sub-cultured every 3–4 weeks onto a fresh medium (10–20 ml) contained in screw-capped glass jars (300-ml, Excel Corporation, India.). Subsequently, roots were maintained in MS0 medium free of the antibiotics.

Polymerase chain reaction

Different fast-growing hairy root somaclones (rhizoclones) were selected and template DNA was isolated from them according to a modified CTAB extraction protocol [20]. Root DNA was isolated from the naturally grown seed-derived explant-donor plant (Fig. 1b) to serve as a negative control. A4T was used as a source of plasmid DNA to serve as a positive control. The plasmid DNA was isolated using the alkaline-lysis mini-prep method (Minipreps DNA Purification Systems, Promega, USA). The oligonucleotide primers (Bangalore Genei, Bangalore, India), were based on the known nucleotide sequence of the T_L-DNA of *A. rhizogenes* A4 [21] and designed for amplification of *rol*C

[22]. The 20/19-mer oligos used for PCR amplification of the *rol*C gene of A4 were 5′-GCA CTC CTC ACC AAC CTT CC-3′ and 5′-ATG CCT CAC CAA CTC ACC A-3′, a combination of which was expected to give a predictable amplicon size of 257 bp. Primers used for amplification of agropine synthase gene (*ags*) were 5′-CGG AAA TTG TGG CTC GTT GTG GAC-3′ and 5′-AAT CGT TCA GAG AGC GTC CGA AGT T-3′ designed according to Tiwari et al. (2007) to give a predictable amplicon size of 1600 bp. The optimum PCR mixture (25 µl) contained 1U Taq DNA polymerase, 10 mM Tris–HCl (pH = 8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µl of each forward and reverse primer (10 pM) and 50 ng of template DNA. The cyclic DNA amplification was performed using the following program of the thermal cycler (Applied Biosystems 9700, CA, USA): initial template denaturation at 95 °C for 2.5 min, annealing at 55 °C for 1 min and extension (copy-synthesis) at 72 °C for 3 min for the first cycle followed by 36 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s and extension at 72 °C for 50 s. The final cycle consisted of an additional step of extension at 72 °C for 10 min prior to hold at 4 °C. The amplified product was separated by 1 % (w/v) agarose gel electrophoresis in 1 × TAE buffer (0.04 M Tris–acetate, 1 mM EDTA, pH 8) at 80 V for 45 min. The gel was subsequently stained with ethidium bromide solution (0.5 µg ml^{-1}) for 15 min by continuous gentle shaking on a slow shaker, followed by destaining in double-distilled water (30 min). The gel was then photographed using a Gel Documentation unit (Bio-Rad, USA).

PIXE- analysis of multi-elemental content

Instrumentation

At the Ion Beam Laboratory of the Institute of Physics (Department of Atomic Energy, Government of India), Bhubaneswar, India, PIXE technique was carried out using the 3 MV horizontal Tandem Pelletron accelerator (9SDH-2, NEC, USA) for elemental analysis of the root samples [23]. The samples were made into pellets and mounted on a multi-faced target holder which was placed at 45° to the beam direction. The target holder was mounted on an insulated stand and was surrounded by a cylindrical electron suppressor held at negative potential with respect to the target (Fig. 2 a, b). Inside the PIXE chamber, the proton beam was collimated to a diameter of 2 mm on the target in vacuum (10^{-6} Torr) [24]. Integrated charge on the thick sample was measured using a current integrator, which was connected to the target holder. Proton beams (3 MeV) with 20 nA beam current was used to bombard the targets. To detect the characteristic X-rays emitted from the targets, a Si(Li) detector (Ortec, SLP-06165-opt-0.5) with a resolution of 165 eV at 5.9 keV with a

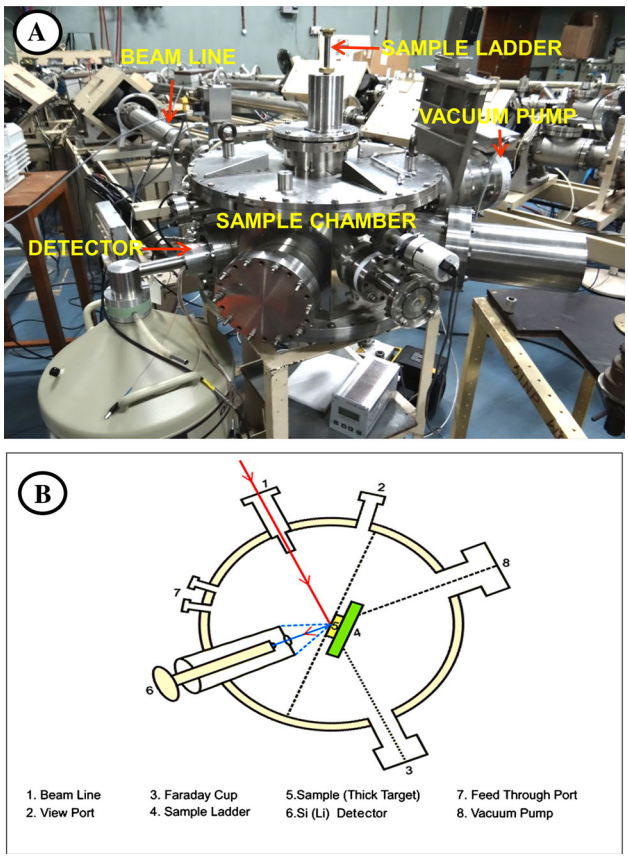

Fig. 2 PIXE set up in the Ion Beam Laboratory of the Institute of Physics (Department of Atomic Energy, Government of India) at Bhubaneswar, Odisha, India **A** Instrument assembly **B** Ray diagram

thin beryllium window (0.0127 mm), placed at 90° to the beam direction, was used. The X-rays exit the scattering chamber through a 95 µm Mylar window before entering the detector. During data collection, no extra/other X-ray absorbers were used between the detector and incoming X-rays. Spectra were recorded using a multi-channel analyser (MCA) calibrated with ^{55}Fe X-ray source.

Sample preparation

Roots from a selected fast-proliferating transformed hairy root somaclone of *S. acuta* (rhizoclone ST5F4; Fig. 1d), along with those obtained from an in vitro non-transformed plant (Fig. 1c) and the natural roots (Fig. 1b) of the seed-grown explant-donor plant, were washed carefully with autoclaved distilled water and kept in deep freezer at −20 °C for 24 h to drain out the moisture content. The root samples were lyophilized at −40 °C for 8 h and the dried samples were powdered using an agate mortar and pestle. For PIXE analysis, pellets (13 mm diameter) were prepared for which 150 mg of powdered samples were mixed with 150 mg of high pure graphite powder (1:1; Alfa Aesar, purity 99.9999 %). Thick targets were prepared by pressing

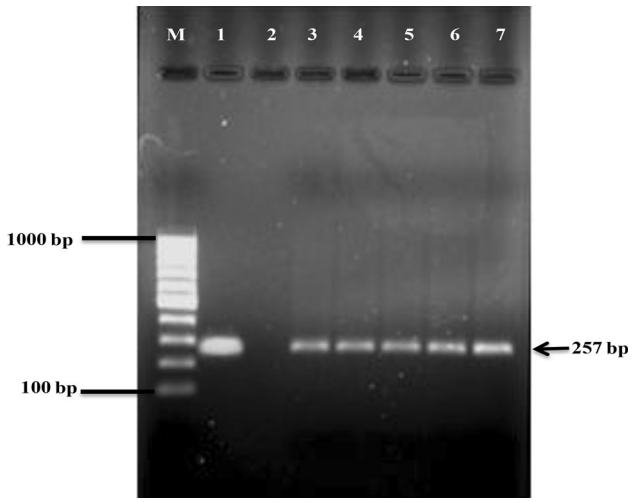

Fig. 3 PCR amplification of *rol*C gene in rhizoclones resulted from transformation with *A. rhizogenes* A4T strain. *M* 100 bp DNA ladder, *Lane 1* Plasmid DNA from A4T (Positive control), *Lane 2* Root DNA from the naturally grown explant-donor plant (Negative control), *Lane 3–7* DNA from selected transformed rhizoclones ST2D1, ST3E3, ST5F4, ST7G6 and ST8H7 respectively

the above mixture in a hydraulic press [25]. In a similar fashion, pellets were also made from a certified Standard Reference Material (SRM) i.e. apple leaves (NIST-1515) obtained from the National Institute of Standards &Technology (NIST), USA. Prior to irradiation of the sample, pellets of NIST–SRM were irradiated as standards for calibration, quantification and verification of results.

Data analysis

GUPIX-2000 software package [26] was used to carry out the PIXE spectral analyses. GUPIX is versatile software package for fitting PIXE spectra for thin, thick, intermediate and layered specimens. This package has provision to convert the X-ray peak intensities into elemental concentrations using a standardization technique involving fundamental parameters and pre-determined instrumental constants. This provides a non-linear least square fitting of the spectrum, together with subsequent conversion of the fitted X-ray peak intensities into elemental concentrations which were expressed on dry weight basis (mg element per kg d. wt. test sample). The certified and measured values of elemental concentrations of "Apple leaves Standard" (NIST-1515) are presented (Table 1).

Statistical analysis

All transformation experiments were set up in a completely randomized design (CRD). Each treatment consisted of 10 replicate jars, each containing 4–6 internodes. Each experiment was repeated three times. For determination of multi-element content, data from three separate experiments each with 5–7 replicates were subjected to analysis of variance (ANOVA) for a completely randomized design. Duncan's new multiple range test (DMRT) was used to separate the mean of significant effect.

Results

Genetic transformation and root development

Root initiation occurred from the inoculated sites on stem internodes. Explants from seed-grown plants from outdoor exhibited a superior response to *A. rhizogenes* treatment than those obtained from in vitro-grown shoot cultures. Not

Table 1 Certified and measured values of elemental concentrations (μg g^{-1}) of standard: Apple leaves (NIST-1515)

Elements	Certified value	Measured value			
		R-1	R-2	R-3	Mean ± SE
Potassium (K)	16,100 ± 200	16,116	15,992	16,222	16,110 ± 66.463
Calcium (Ca)	15,260 ± 150	15,248	15,271	15,301	15,273 ± 15.344
Manganese (Mn)	54 ± 3.0	52.8	55.1	54.2	54.03 ± 0.669
Iron (Fe)	83 ± 5.0	84.4	85.1	81.6	83.70 ± 1.069
Nickel (Ni)	0.91 ± 0.12	1.02	0.88	0.92	0.94 ± 0.042
Copper (Cu)	5.64 ± 0.24	5.62	5.77	5.51	5.63 ± 0.075
Rubidium (Rb)	10.2 ± 1.5	11.1	10.4	9.7	10.40 ± 0.404
Strontium (Sr)	25 ± 2.0	24.8	24.5	27.1	25.46 ± 0.821
Vanadium (V)	0.26 ± 0.03	0.25	0.28	0.24	0.25 ± 0.012
Selenium (Se)	0.050 ± 0.009	0.054	0.047	0.051	0.05 ± 0.002
Zinc (Zn)	12.5 ± 0.3	12.2	12.7	12.3	12.4 ± 0.153

Data basing on three replicates (R-1, R-2, R-3) were recorded using the PIXE set-up at the Ion Beam Laboratory, Institute of Physics, Bhubaneswar, India (*Department of Atomic Energy, Government of India*)

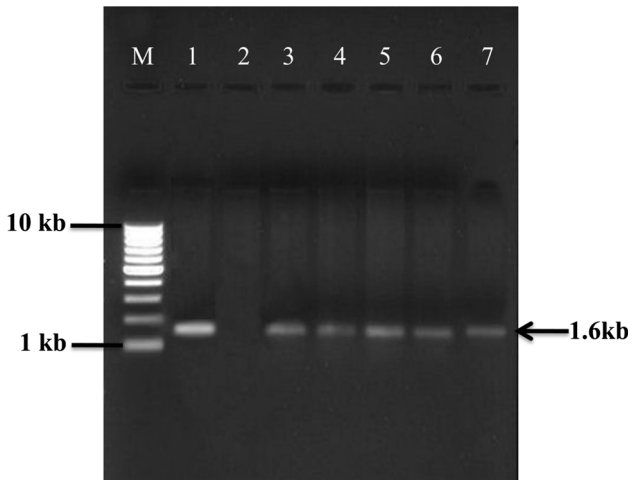

Fig. 4 PCR amplification of *ags* gene in rhizoclones resulted from transformation with *A. rhizogenes* A4T strain. *M* 1 kb DNA ladder, *Lane 1* Plasmid DNA from A4T (Positive control), *Lane 2* Root DNA from the naturally grown explant-donor plant (Negative control), *Lane 3–7* DNA from selected transformed rhizoclones ST2D1, ST3E3, ST5F4, ST7G6 and ST8H7 respectively

only that an early response of rhizogenesis (13–16 days) was elicited, but also a higher transformation efficiency (36.98 %) with an average of 5.9 roots per explant were scored from in vivo internodal segments. The aerial roots differed from the *in natura* roots in demonstrating a characteristic negative geotropism with a tendency to grow towards light and away from the culture medium. The roots were ageotropic and mostly plagiotropic (growing parallel to the culture medium). Roots showed prolific growth and grew as closely interwoven masses over the surface of the culture medium and along the sides of the culture vessel (Fig. 1d). The uninfected control explants, without any bacterial treatment, failed to produce roots at the inoculated sites.

PCR amplification for detection of *rol*C and ags genes in the transformed rhizoclones

Genomic DNA isolated from several independent transformed hairy root clones revealed the expected amplicon size of 257 bp specifying A4 *rol*C gene (Fig. 3). Additionally, transformation was also confirmed by using the primer designed for *ags* (agropine synthetase) gene which gave the predictable amplicon size of 1.6 kb (Fig. 4). This confirmed the presence of *rol*C and *ags* gene, borne on the pRi T_L-DNA or pRi T_R-DNA respectively of *A. rhizogenes*, in the recipient plant nuclear genome via genetic transformation. However, no amplification product was detected in DNA from roots of naturally grown plants when subjected to PCR amplification with the gene primers specific to the *rol*B or *ags* gene sequences.

Multi-elemental content

Of several hairy root somaclones (rhizoclones) resulted via genetic transformation of *S. acuta* Burm.f., a fast-proliferating rhizoclone ST5F4 was selected for inorganic elemental quantification using PIXE technique. The contents were compared with that of roots of the naturally grown explant-donor plant and in vitro non-transformed roots (Table 2). The representative spectra of natural roots and in vitro non-transformed/transformed root cultures were comparable as depicted in the superimposed layout (Fig. 5). Fourteen different elements viz. K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Br, Rb and Sr were detected in the processed pellets in different concentrations (μg g^{-1}). All the elements tested in transformed root cultures were found to be either *at par* in contents or in most cases higher with respect to the root samples from in vitro non-transformed cultures and the naturally grown explant-donor plant. Two macronutrients namely K and Ca were detected in markedly high quantities whilst Fe was available in quantity just above the trace level (100 μg g^{-1}) in all root types. Toxic elements such as As and Pb, though detected in the natural root sample at a very low level (0.05 ± 0.002 and 0.010 ± 0.003 μg g^{-1} respectively), they were neither found in the in vitro non-transformed roots nor in transformed hairy root cultures. The levels of some therapeutically important trace elements (e.g. Cr, Mn, Fe, Co, Cu, Zn, Se), that were detected in lower concentrations in natural roots were relatively high in in vitro non-transformed roots. Interestingly, in the transformed roots these trace elements were detected in elevated amounts (Table 2). For instance, there was a 30-fold increase in Se content in hairy root cultures of *S. acuta* compared to that in natural roots of in vivo donor plant.

There was a progressive trend in the elemental concentration of the transformed root cultures. K and Ca had values of 22,800 and 16,300 μg g^{-1} respectively which were higher than that of in vitro transformed root cultures and natural roots. With respect to the trace elements the detected values were arranged in decreasing order as Fe (480.92 μg g^{-1}), Zn (164.22 μg g^{-1}), Mn (148.27 μg g^{-1}), Rb (24.86 μg g^{-1}), Sr (19.40 μg g^{-1}), Br (9.56 μg g^{-1}), Cr (9.13 μg g^{-1}), Cu (7.53 μg g^{-1}), V (2.97 μg g^{-1}), Se (0.60 μg g^{-1}), Ni (0.52 μg g^{-1}) and Co (0.19 μg g^{-1}). In this study, transformed roots had a general trend of having accumulated higher levels of trace elements except nickel (Ni).

The quantity of transformed roots required to provide the suggested *per diem* (daily) dose of major and trace elements for human body, referred to as "Reference nutrient intake" (RNIs) or "Recommended dietary allowance" (RDA) as per British Nutrition Foundation and Food and Drug Administration (FDA), USA respectively were estimated and given in Table 3. Evident in this table, as

Table 2 PIXE-based estimation of elemental content (μg g^{-1}) in transformed hairy roots (rhizoclone ST5F4), non-transformed in vitro roots and natural roots of the seed-grown explant-donor plant of *Sida acuta*

Elements	Natural roots	In vitro non-transformed roots	In vitro transformed hairy roots
Potassium (K)	21,500 ± 5.42[c]	22,100 ± 4.37[b]	22,800 ± 2.78[a]
Calcium (Ca)	15,400 ± 4.63[c]	16,200 ± 3.54[b]	16,300 ± 2.66[a]
Vanadium (V)	1.62 ± 0.1[c]	1.88 ± 0.02[b]	2.97 ± 0.03[a]
Chromium (Cr)	8.16 ± 0.10[c]	8.54 ± 0.05[b]	9.13 ± 0.13[a]
Manganese (Mn)	130.55 ± 1.06[c]	140.65 ± 1.65[b]	148.27 ± 3.48[a]
Iron (Fe)	441.29 ± 14.88[c]	450.54 ± 3.54[b]	480.92 ± 5.99[a]
Cobalt (Co)	0.13 ± 0.064[b]	0.14 ± 0.011[b]	0.19 ± 0.013[a]
Nickel (Ni)	02.34 ± 0.07[a]	1.71 ± 0.03[b]	00.52 ± 0.03[c]
Copper (Cu)	04.89 ± 0.37[c]	5.54 ± 0.12[b]	07.53 ± 0.17[a]
Zinc (Zn)	152.88 ± 4.13[c]	156.51 ± 3.99[b]	164.22 ± 2.19[a]
Selenium (Se)	0.02 ± 0.005[b]	Nd	0.60 ± 0.001[a]
Bromine (Br)	08.56 ± 2.88[c]	08.88 ± 1.31[b]	09.56 ± 1.50[a]
Rubidium (Rb)	21.06 ± 1.72[c]	22.61 ± 1.21[b]	24.86 ± 4.80[a]
Strontium (Sr)	17.60 ± 2.60[c]	18.7 ± 2.02[b]	19.40 ± 2.80[a]

Data pooled from a total of 3 experiments each with 5–7 replicates Values (Mean ± SE) within rows (between columns) followed by different alphabets (a, b & c) in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

Nd Not detected

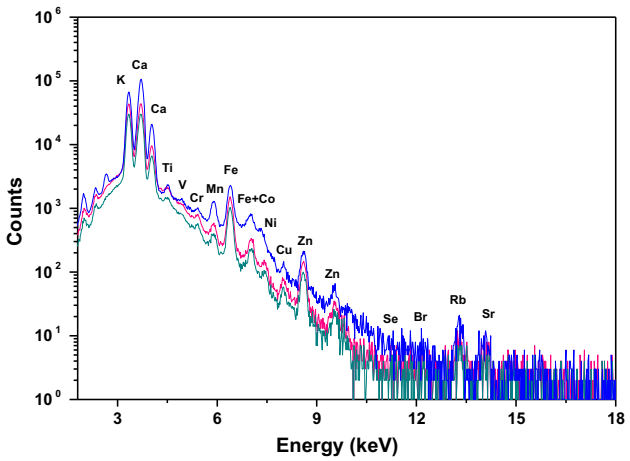

Fig. 5 The superimposed spectra of 45 day-old genetically transformed hairy root somaclone (rhizoclone ST5F4) (*blue*), in vitro non-transformed roots (*red*) and natural roots of seed-grown explant-donor plant (*green*). (Color figure online)

low as 2.74 and 3.61 g d.wt. of a fast-growing transformed root somaclone of *Sida acuta* (rhizoclone ST5F4) would suffice to cater to *per diem* requirement of chromium (Cr) in adult women and men respectively. Likewise, in terms of fulfilling the body necessity for iron (Fe) on daily basis in men/women 18.09/30.77 g d. wt. of the same rhizoclone would be adequate. Similarly, the selected rhizoclone at a range of varying quantities i.e. 13–34, 42–58, 100–125, and 160 g d. wt holds the potential to provide the recommended doses for daily consumption of Mn, Zn, Se and Cu respectively. The *per diem* body requirement for adult persons for two major elements, Ca and K can be met by the ST5F4 rhizoclone of *S. acuta* at approximately 43 g d. wt and 154 g d. wt respectively.

Discussion

Presently, the use of herbal medicines to relieve and treat many human diseases is an increasing trend around the world due to their mild features and low side effects [27]. Trace elements constitute the structural components of some key secondary metabolites which are compounds responsible as pharmaceutically active principle present in medicinal plants [28]. Various physico-chemical factors are known to affect elemental concentration of the plants, such as the (i) altered state of cellular metabolism during in vitro cultivation under the influence of exogenous growth regulators, environmental conditions (illumination, temperature, humidity) to which such cultures were subjected and (ii) preferential absorbability of a particular plant or in vitro culture for the corresponding element from the respective planting substrate [29]. Information on elemental concentrations of the medicinal plants could determine the dosage of drugs to be prepared using such plant extracts. Certain metals such as zinc, iron, copper, chromium, and cobalt are essential for the human body and become toxic only at exceedingly high concentrations, while a few others such as lead, cadmium or arsenic are exclusively toxic.

Table 3 Transformed hairy roots as a source of inorganic elements based on *per diem* requirement of human body

Element	*Per diem* element requirement of healthy adult (men/women)	Element content *per* 100 g d.wt. transformed hairy root culture (rhizoclone ST5F4)	Estimated daily intake (d.wt.) of transformed root tissue (rhizoclone ST5F4) for *per diem* element requirement
Potassium (K)	3,500 mg[a] (healthy adult)	2,280 mg	153.51 g (healthy adult)
Calcium (Ca)	700 mg[a] (healthy adult)	1,630 mg	42.94 g (healthy adult)
Chromium (Cr)	33 μg men[b]	0.913 mg	3.61 g men
	25 μg women[b]		2.74 g women
Manganese (Mn)	2–5 mg[b] (healthy adult)	14.827 mg	13.49–33.72 g
Iron (Fe)	8.7 mg men[a]	48.092 mg	18.09 g men
	14.8 mg women[a]		30.77 g women
Cobalt (Co)	0.13 mg[b] (healthy adult)	0.019 mg	684.21 g (healthy adult)
Copper (Cu)	1.2 mg[a] (healthy adult)	0.753 mg	159.36 g (healthy adult)
Zinc (Zn)	9.5 mg men[a]	16.422 mg	57.85 g men
	7 mg women[a]		42.62 g women
Selenium (Se)	60 μg women[a]	0.060 mg	100 g women
	75 μg men[a]		125 g men

[a] "Reference Nutrient Intake" (RNIs) as per British Nutrition Foundation, UK

[b] "Recommended Dietary Allowance" (RDA) as per Food and Drug Administration (FDA), USA

Although atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectroscopy (ICP-MS) have been routinely used for elemental analysis, destructive chemical methods involving sample dissolution procedure and inability for routine analysis of samples in solid form explain their major disadvantage [30]. AAS, albeit a common and popular technique, requires a complicated time-consuming and destructive sample preparation procedure. Though it is possible to perform multi-elemental analysis without destroying the matrix using multi-elemental lamps, however, lamps with 4 or more elements are not recommended for all applications. In inductively coupled plasma atomic emission spectroscopy (ICP-AES) the extensive use of standards, while in electron probe microanalysis (EPMA), the comparatively high detection limit ($\geq$200 ppm) due to bremsstrahlung X-rays generated during slowing down of the primary element beam are considered to be their major deficiencies [2]. Laser ablation combined with ICP-MS (LA-ICP-MS) technique appears to be fascinating because it is rapid, highly sensitive, having low background and mostly non-destructive; however, the unavoidable requirement for extensive use of standards [31, 32] renders it unsuitable for analysis of heterogeneous materials [30].

X-ray fluorescence (TRXRF and EDXRF) technique, albeit earned popularity being non-invasive and reliable, have shortcomings too. Although TRXRF permitted a better low-level detection (LLD) for the high-Z elements above Fe, PIXE is more suitable having a better LLD for low-Z elements (up to Fe [33]). A comparative picture of the use of PIXE and EDXRF has been provided by Benyaich et al. [34]. They plotted the variations of the intrinsic and effective sensitive ratios (S_{PIXE}/S_{XRF}), versus the atomic number of the emitting target element; computed for K lines for $Z \leq 42$ and for L lines for $Z > 42$. From this, they inferred that PIXE is much more sensitive than XRF for the low energy X-ray lines due to the higher ionization cross-section of protons, which balances the strong self-absorption occurring at low energies, (with MDL values falling down to few atomic ppm), while XRF is more sensitive at high X-ray energies (with MDL values ranging 0.6–4.0 atomic ppm). Investigation by Nayak et al. [30, 35, 36] have demonstrated the suitability of PIXE for the analysis of lower middle Z elements ($19 < Z < 31$ from K X-rays; Ba from L X-rays) and of EDXRF for higher middle Z elements ($30 < Z < 45$ from K X-rays; Pb from L X-rays). It is advocated that the two techniques PIXE and XRF are complementary, the former being more sensitive for lighter elements (low energy X-ray emitters), while the latter for heavier elements. For EDXRF, there is requirement of a relatively high amount of powdered material (500 mg) compared to that needed for PIXE analysis (150 mg). Because of smaller pellet size (13 mm diameter) in PIXE analysis compared to that in EDXRF (2.54 cm diameter pellets), more number of samples can be accommodated in the sample holder for analysis using the former technique. Time required for data processing and generation of spectra per pellet is shorter (30 min) in PIXE method compared to EDXRF in which double the duration (60 min) is warranted.

PIXE has been used to estimate the trace elemental concentrations in different types of biological samples by earlier workers [7, 37]. This technique has been quite interesting to the biologists as it is non-destructive, rapid, sensitive and at the same time, it facilitates quantitative analysis of samples without necessitating chemical pretreatment [38]. PIXE has been successfully applied to determine the elemental composition (Cl, K, Ca, Ti, Cr, Mn, Fe, Ni, Cu, Zn, Br, Rb and Sr) of some anti-diabetic plants [39]. In the present investigation, PIXE technique

has provided a highly sensitive tool for detecting and estimating the multi-elemental composition of *Agrobacterium*-mediated transformed hairy roots, in vitro non-transformed root cultures and natural roots of a medicinal plant, *Sida acuta*. The major elements such as K and Ca were quite high in the transformed and in vitro non-transformed root cultures as well as roots of naturally grown plant. Studies on high levels of K and Ca have been reported in different medicinal plants [40]. K along with Na is vital to life and plays an important role in the transmission of the action potential in nerve cells. K^+ ions play a key role in functioning of the neurons (brain and nerve), and in influencing osmotic balance between cells and the interstitial fluid, with their distribution mediated in all animals by the Na^+/K^+-ATPase pump. This ion pump uses ATP to pump three sodium ions out of the cell and two potassium ions into the cell, thus creating an electrochemical gradient over the cell membrane [41]. Potassium aids in preventing muscle contraction and sends all nerve impulses in animals through action potentials. As hypothesized by Lockless et al. [42] ion channels and pumps in cell membranes can distinguish between the K^+ and Na^+ ions, actively pumping or passively allowing one of the two ions to pass, while blocking the other. K functions as a cofactor for activating the enzymes required for normal growth and muscle function. Standard concentrations of K are required for optimal secretion of insulin. Deficiency of potassium in blood (referred to as hypopotassemia/hypokalemia), resulting from vomiting, diarrhoea or increased diuresis might cause muscle weakness, paralytic ileus, ECG abnormalities, decreased reflex response and in severe cases respiratory paralysis, alkalosis and cardiac arrhythmia [43, 44]. Calcium (Ca) is one of the most important macronutrient in the human body that strengthens bones and teeth. Besides, Ca is required for numerous intracellular functions including neuromuscular, systemic and cardiac function, and regulation of heartbeat, blood clotting (hemostasis), cellular permeability, muscular contraction, transmission of nerve impulses and enzymatic activity [45]. Ca deficiency causes impaired bone mineralization and can lead to osteomalacia in adults [46].

In addition to K and Ca, PIXE analysis also revealed the presence of several micro-nutrients including trace elements such as V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Rb and Sr in transformed roots, in vitro non-transformed root cultures and natural roots. Of these, selenium (Se) content was 30-fold higher in hairy root cultures of *S. acuta* compared to that in roots of in vivo donor plant. Se is an essential micronutrient which acts as a component of two unusual amino acids, selenocysteine (Se-Cys) and selenomethionine (Se-Met). It functions as a cofactor for the reduction of antioxidative enzymes, including glutathione peroxidases and certain forms of thioredoxin reductase. Se and various selenoproteins have antioxidant effects that reduce the biological impact of ROS [47]. The antioxidant property of selenoproteins is important as they reduce the levels of hydroperoxides [48, 49]. Se enters human and animal systems in the form of selenite or selenomethionine, that consequently become metabolized to various products including methylated derivatives, of which the monomethylated forms were shown to possess anti-cancer properties [50]. Se deficiency is known to have lead to hypothyroidism, cardiac disease, and a weakened immune system [51].

Vanadium (V) is an important trace element that helps in maintaining glucose levels, lowering high blood pressure and to enhance athletic performance [52]. In several studies on animals including human beings, V has been shown to have facilitated reduction in blood sugar levels and also increase in insulin sensitivity in those with type 1 and type 2 diabetes [52]. V is also required for various cellular metabolism, formation of bones and teeth, inhibition of cholesterol synthesis, improved glucose tolerance, growth and reproduction.

Copper (Cu), bound to many amino acids [53], is also an essential nutrient involved in various energy metabolisms and is an important constituent for the synthesis of haemoglobin, myoglobin, cytochromes and some peptide hormones. It is also needed for proper utilization of Fe and protection against oxidative damage to cells. Cu plays a significant role in electron transfer reactions due to its redox cycling ability. It helps in the production of chemicals that regulate blood pressure and promote healing [54]. In addition, Cu plays a key role in Fe metabolism and its deficiency results in fragile bone cotises and spontaneous rupture of major vessels. It also acts as a cofactor for various oxidase enzymes which participate in the stabilization of matrices of connective tissue, oxidation of ferrous ion, synthesis of neurotransmitters, conferral of pigment to hair and skin, assertion of immune system competence, generation of oxidative energy and protection from free radicals [40]. Deficiency of Cu might result in a weakened energy production, abnormal glucose and cholesterol metabolism, increased oxidative damage, increased tissue iron (Fe) accrual, altered structure and function of circulating blood and immune cells, abnormal neuropeptide synthesis and processing, aberrant cardiac electrophysiology, impaired myocardial contractility, and persistent effects on neurobehavior and the immune system [55]. Zinc (Zn) is an important microelement which aids in the proper functioning of numerous enzymes [56], in insulin metabolism and acts as an efficient antioxidant [57]. It is involved in the synthesis of nucleic acids, protein synthesis, growth inflammatory syndromes, testosterone secretion and cerebral function. Zn deficiency has been reported in patients receiving prolonged zinc-free parenteral nutrition

and causes hyperglycemia, mental apathy, a moist eczematoid dermatitis, especially around the mouth, and loss of hair. Zn along with Cu forms the scavenging enzyme, superoxide dismutase (Cu–Zn superoxide dismutases) that protects against the formation of free ROS and lowers their activity in plasma and biological fluids [58]. A change in the concentration of Cu and Zn at the plasma level can cause certain diseases; an imbalance in Cu:Zn ratio is a better indicator of infection, vascular complications, and prognosis of diseases than Zn or Cu alone [59, 60]. In elderly people Cu:Zn ratios in blood plasma are coupled with high levels of interleukin-6 (IL-6), a potent pleiotropic cytokine that regulates cell growth and differentiation and plays an important role in immune response. Thus, Cu:Zn ratio may be useful as a functional inflammatory/nutritional biomarker for overall good health, or conversely frailty [61]. Cu and Zn have an effect on immune functions and the plasma levels of these elements are altered in inflammatory states. A high serum Cu level triggers oxidative stress, which promotes the inflammatory response [62] whereas a reduced Zn concentration limits the antioxidant response, contributing to inflammation [63]. Thus, the transformed hairy root cultures of *Sida acuta*, with a relatively low Cu:Zn ratio observed in the present study, might serve a healthy ingestion to enhance the natural defence system in aged persons, thereby reducing the risk of inflammation.

Iron (Fe) is a component of haemoglobin, myoglobin of cytochromes and many enzyme cofactors (heme and Fe–S clusters), and plays a key role in oxygen and electron transport. Iron deficiency is a common affliction, resulting in Fe-deficiency anemia characterized by a low number of oxygen-carrying cells in the blood stream. As Fe is essential for the production of these cells, its deficiency can limit their production; as a result, the amount of oxygen supply to the blood becomes limited [52].

The role of chromium (Cr) is relevant as it is a critical cofactor in insulin action. For example, it activates the enzyme phosphoglucomutase and increases the activity of insulin; hence, chromium deficiency would lead to decrease in glucose tolerance and increase the risk of cardiovascular diseases [27]. Chromodulin, which is a naturally occurring biologically active form of chromium, plays an important role in carbohydrate and lipid metabolism.

Manganese (Mn) is an essential trace element necessary for all forms of life on earth. It is present in all tissues, and is a requisite for various metabolic reactions [64]. It plays a vital part in bone and cartilage development and wound healing. Mn^{2+} is the key component of various metalloenzymes and activates many metal-enzyme complexes such as arginase, pyruvate carboxylase and glutamine synthase. On activation by Mn, the enzymes bind the protein directly or by acting through an intermediate interaction with a substrate, such as ATP, resulting in a conformational change and activation of the enzymes. Mn^{3+} is found in the enzymes as manganese catalase and manganese superoxide dismutase, both of which breakdown oxidants using Mn^{3+} in their reactive catalytic center [65].

Cobalt (Co), being an integral component of Vitamin B12, is an essential nutrient for non-ruminant animals and humans. Vitamin B12 is a cofactor for two enzymes, methionine synthase which methylates homocysteine to form methionine, and methylmalonyl coenzyme A (CoA) mutase which converts L-methylmalonyl Co-A, formed by the oxidation of odd-chain fatty acids, to succinyl Co-A. Nickel (Ni) is an important micronutrient which helps in the absorption of Fe, in adrenaline and glucose metabolism, and improving bone strength. Along with other trace elements and vitamins it facilitates smooth functioning of the human body. Nevertheless, long-term ecological or anthropogenic exposure of humans to Ni via food chain or work place might cause chronic disorders or mutagenic/carcinogenic changes [66].

Strontium (Sr), albeit considered a non-essential trace element, has been shown by recent studies to have influenced bone turnover [67] and has been applied in the form of Sr ranelate in successful therapeutic treatment of osteoporosis. Sr is chemically very similar to Ca, and can replace Ca, however, little is known about the role of Sr in normal bone metabolism as well as in bone disorders [68]. Rubidium (Rb) is believed to possess the capacity to act as a nutritional substitute for potassium due to their metabolic interchangeability [69]; while to date, bromine (Br) has no known essential role in human health.

Conclusion

This is the first report on using PIXE technique to quantify the major and minor inorganic elements in a genetically transformed rhizoclone of *Sida acuta* Burm.f., induced by *A. rhizogenes*. The contents of bioactive elements in human healthcare were higher in the selected rhizoclone in comparison with natural roots and in vitro non-transformed roots. Interestingly, it is estimated that genetically transformed "hairy roots" of *S. acuta*, in quantities less than 160 g d.wt., can suffice to provide nearly all tested essential elements in amounts catering to *per diem* requirements of the human body. The ideal multi-elemental composition in the root samples support their integration in modern phytotherapy ("rhizotherapy") and is, thus, important for the development of root-based drugs in the form of natural metallo-organic compounds which are more effective and relatively safe with minimal side effects. Thus, these specialized root cultures could be a fast-proliferating

renewable resource of medicinally useful inorganic nutrients as complementary medicine to confer protection against various human diseases.

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