

ED-XRF spectrometry-based trace element composition of genetically engineered rhizoclones vis-à-vis natural roots of a multi-medicinal plant, butterfly pea (*Clitoria ternatea* L.)

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Abstract The energy dispersive X-ray fluorescence setup incorporating a molybdenum secondary exciter was used for quantitative determination of major and minor elements in genetically transformed root somaclones (rhizoclones) of butterfly pea (*Clitoria ternatea* L.) which had been established via explant co-cultivation with *Agrobacterium rhizogenes*. The multi-elemental composition of these transformed rhizoclones was compared with that of the naturally grown in vivo donor plant. Trace elements namely Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Rb, Sr and Pb in addition to two macro-elements K and Ca were identified and quantified in root tissues of both sources. The elemental content of transformed root cultures was found to be *at par* with that of the natural roots of in vivo grown plants of the same species. These findings are implicated on the context of utilization of such *Agrobacterium*-mediated genetically transformed root cultures as a viable alternative to natural roots, the former being a fast-proliferating renewable resource of medicinally useful minerals essential for designing of effective drugs, besides providing an *ex situ* means for plant conservation.

Keywords Butterfly pea · ED-XRF · Transformed root cultures · Trace elements

Introduction

In consideration of the toxicity and carcinogenicity reportedly associated with modern synthetic medicines, plant-based drugs are gaining acceptance worldwide. Large-scale field cultivation of medicinal plants and subsequent extraction and purification of the active principle from them to be used in drug preparation is beset with certain serious drawbacks. These encompass low yield and/or poor quality of desired phytochemicals owing to environmental factors, non-availability of sufficient planting material because of territorial constraints or slow growth rate etc. In addition, bulk quantities of raw plant parts being needed for commercial-scale extractions, threat is consistently imposed on the natural biodiversity of medicinal plant species. This has necessitated development of sustainable alternatives in the form of rapidly multiplying in vitro culture systems such as callus cultures [1], cell suspension cultures [2] or root cultures [3] as a renewable source of pharmaceutically-relevant phytochemicals, derived from secondary metabolism, which include alkaloids, terpenoids, phenylpropanoids, quinones and steroids.

Pharmacological properties of potential medicinal plants are mostly attributed to bioactive metabolites and other constituents including organic compounds and trace elements [4, 5]. Presently the naturally occurring medicinal plants and tissue/cell cultures thereof are being exploited chiefly for their organic content, precisely secondary metabolites [6]. Unfortunately, little is known about the distribution or profiling of their inorganic elements. Mineral elements in form of macronutrients and micronutrients

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including trace elements are required for plant growth and metabolism. Interestingly, some of these elements are closely related to human health; hence knowledge of elemental content of potential medicinal plants and phytotherapies holds significance.

Inorganics are also known to play a key role in various metabolic functions in human body [7–10]. Trace element deficiency or abundance can result in many physiological disturbances including endocrine diseases in animals and humans [11, 12]. Inorganic elements such as Cu, Zn, Cr, and Se have been implicated in some metabolic disorders that include diabetes [13–15]. Zinc complex is the store house of insulin secreted from pancreas which plays an important role in glucose metabolism [16]. Chromium has important role along with zinc, calcium and manganese in glucose tolerance factor (GTF), which decreases the blood glucose level by utilizing insulin [17]. Potassium, calcium and trace elements especially chromium and zinc etc., are responsible for the secretion of insulin from β -cells of islets of Langerhans [18] and also important for the glucose metabolic regulation [19, 20]. Continuous intake of nutraceuticals that are excessively high in a particular trace element can influence changes in the functioning, forms, activities of some organs as concentrations of such element in the body tissue and fluid can rise above the permissible limit [9]. Interestingly, Rajukar and Damame [21] reported a higher concentration of inorganic micronutrients in selected medicinal plants compared to common non-medicinal plants; thus suggesting the former to have a greater tendency towards uptake of trace elements from soil. In addition to those which are essential for growth and productivity of plants, some relatively less essential trace elements are also absorbed by medicinal plants, the latter being known to enhance their curative properties [22]. A direct correlation between element profile and their therapeutic capabilities is yet to be elucidated; nevertheless quantitative data on major, minor and trace element contents in medicinal plants are of considerable significance in order to understand their pharmacological activity. With the renewal of worldwide interest in use for herbal medicines, the pressure is mounting on consistent maintenance of the standards, quality, safety, integrity and authenticity of the practices and products used in herbal drug development. Therefore, it is imperative that the elemental profile of the aforesaid *in vitro* culture systems should be accurately determined and compared with their *in vivo* counterparts to ensure effective drug design utilizing the former source.

Among the elementary analytical techniques, many have been developed to determine the trace elements, such as (i) neutron activation analysis (NAA), (ii) optical emission spectroscopy (OES), (iii) atomic absorption spectroscopy (AAS), (iv) mass spectroscopy (MS), (v) inductively coupled plasma mass spectroscopy (ICPMS) and (vi) total

reflection X-ray fluorescence (TXRF), etc. Energy dispersive X-ray fluorescence (ED-XRF) has been of special interest to biologists as this technique is rapid, non-destructive, sensitive and relatively cheap while at the same time it enables simultaneous qualitative, semi-quantitative and quantitative analysis of samples of any size or number and without chemical pre-treatment [23]. Quantitative and qualitative analyses by XRF techniques are performed without chemical digestion and a great number of elements can be determined simultaneously in a short time [24–26]. Major advantages of ED-XRF over inductively coupled plasma atomic emission spectrometry (ICP-AES) and atomic absorption spectrometry (AAS) include the ease of analysis of solid samples without need for rather tedious and potentially hazardous procedures, the ability to analyze matrix components such as silica, and the avoidance of poor recovery for some metals during the digestion required for ICP-AES and AAS [27]. In an effort to save money, space, sample preparation time, or simply to add an analytical instrument to their process many companies will decide to evaluate ED-XRF analyzers as a substitute for their standard wavelength dispersive X-ray fluorescence (WD-XRF) analysis. The ED-XRF analyzer uses an X-ray source to excite the sample but it may be configured in one of two ways. The first way is direct excitation where the X-ray beam is pointed directly at the sample. Filter made of various elements may be placed between the source and sample to increase the excitation of the element of interest or reduce the background in the region of interest. The second way uses a secondary target, where the source points at the target, the target element is excited and fluoresces, and then the target fluorescence is used to excite the sample. A detector is positioned to measure the fluorescent and scattered X-rays from the sample and a multichannel analyzer and software assigns each detector pulse an energy value thus producing a spectrum [28, 29]. In case of ED-XRF the “excitation efficiency” is usually expressed in ppm per-count-per-second (cps) or similar units; this being the other main factor for determining detection limits, repeatability, and reproducibility. The relative excitation efficiency is improved by having more source X-rays closer to but above the absorption edge energy for the element of interest. The advent of commercially available ED-XRF measurements has provided an economical and powerful analytical tool for environmental, clinical, geological, pharmacological, biological and biochemical research and quality control.

This ED-XRF technique has been successfully applied to a number of plant materials. Obiajunwa et al. [9] used this technique to analyze essential and trace element contents of 20 Nigerian medicinal plants. Ekinci et al. [10] quantified trace elements of 15 different medicinal plants of Turkey. Queralt et al. [30] did quantitative determination of

macro- and microelement contents of five medicinal plants, *Taraxum officinale*, *Eucalyptus globules*, *Plantago lanceolata*, *Matricaria chamomilla* and *Mentha piperita* and their infusions using XRF and ICP methods. Choudhury and Garg [31] analyzed the variation in essential, trace and toxic element contents of the curry leaf tree (*Murraya koenigii*) collected from different states of India. Elemental composition of several vegetable samples such as *Brassica oleracea* var. acephala [32], *Utricea dioico* and *Spunacia oleracea* [33] were also determined using ED-XRF methods. Recently, this technique has been used for comparing inorganic elemental content of in vitro root and leaf callus culture vis-à-vis their in vivo counterparts of *Andrographis paniculata* [34] and *Phyllanthus amarus* [35] respectively.

Butterfly pea (*Clitoria ternatea* L.; Family: Fabaceae) is a perennial twinning legume that has been adopted in the Indian traditional system of medicine, the *Ayurveda*, as a memory enhancer, nootropic, antistress, anxiolytic, antidepressant, anticonvulsant, tranquilizing and sedative agent [36]. The root contains a wide range of secondary metabolites, including pentacyclic triterpenoids, taraxerol & taraxerone [37, 38], and flavonol glycoside 3,5,4'-trihydroxy-7-methoxyflavonol-3-O- β -D-xylopyranosyl-(1,3)-O- β -D-galactopyranosyl(1,6)-O- β -D-glucopyranoside [39]. These exhibit pharmacological activities, including antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, local anaesthetic, antidiabetic, insecticidal, blood platelet aggregation-inhibiting, and vascular smooth muscle relaxing properties [40].

In view of this, modern biotechnological intervention could be an effective means to exploit the plant species for its medicinal value. As most of the active principles of pharmaceutical relevance are present in the roots of this plant species, in vitro root cultures could be the most appropriate source from which the useful secondary metabolites could be extracted in order to cater to the requirement of drug design and manufacture. In this context, the neoplastic 'hairy root' disease caused naturally in plants due to infection by the soil-dwelling Gram-negative phytopathogenic *Agrobacterium rhizogenes* is, indeed, interesting. The disease develops consequent upon integration into the host plant genome of oncogenic coding sequences and their over-expression, resulting in uncontrolled auxin biosynthesis following transfer of T-DNA from the large Ri plasmids of infecting *Agrobacterium* via a well-characterized signal transduction [41]. *Agrobacterium*-transformed hairy root cultures of *Clitoria ternatea* L. could serve as an effective and sustainable source of medicinally important phytochemicals needed as raw ingredients for manufacturing the selective drug types so as to target specific diseases. At present, naturally occurring plants and their in vitro cultures are being exploited mostly for their organic content (secondary metabolites; [6]); however, little is known about their inorganic element distribution.

Therefore, it is imperative that the elemental composition of *Agrobacterium*-mediated genetically transformed in vitro root culture systems should be determined and compared *vis-a-vis* their in vivo root counterparts to ensure effective drug development using the former source. Additionally, these cultures thus emerging as an alternative source of taraxerol, an important secondary metabolite with anti-cancer activity [42], can serve as an efficient means for *ex situ* conservation of this plant species of pharmaceutical significance.

Materials and methods

Induction and proliferation of transformed root cultures

Plant material

Nodal segments, internodes and leaf explants were collected from a butterfly pea (*Clitoria ternatea* L.) plant grown in the experimental pots in the Department of Botany, Utkal University, Bhubaneswar (India) (Fig. 3a). Explants were washed under running tap water (15 min) followed by treatment with 7.5 % (v/v) lizol (Reckitt Benckiser, India) for 30 min and rinsed 5–6 times using autoclaved tap water. These were surface-disinfected by treatment with 0.1 % (w/v) mercuric chloride (8 min) followed by rinsing with autoclaved distilled water (5–6 changes). Nodal segments were inoculated into full-strength Murashige and Skoog [43] medium (MS) augmented with 1 mg l⁻¹ BA (⁶N-benzyladenine). Multiple shoots developed in vitro were cut into single node pieces (0.8–1.0 cm) and cultured in 300 ml screw-capped glass jars (Excel Corporation, Alleppey, Kerala, India), containing MS medium augmented with 1 mg l⁻¹ BA (20 ml per jar) for further multiplication and renewable establishment of axenic shoot cultures. Cultures were maintained in a culture room (25 ± 1 °C, 35–40 μ mol m⁻² s⁻¹ photon flux density [PFD], 60 % RH).

Bacterial strains and culture media

The wild type A4, and engineered A4T strain of *Agrobacterium rhizogenes* (kind gift from Dr. Tepfer, Laboratoire de Biologie de la Rhizosphere, Institut National de la Recherche Agronomique, Versailles, Cedex, France), harboured an agropine-type pRiA4 while 8196 had mannopine-type pRi. LBA 9402 was a rifampicin resistant strain possessing an agropine-type Ri plasmid pRi1855. A4, A4T and 8196 strains were 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 (NH₄)₂SO₄, 5 g l⁻¹ NaCl; pH 6.6). LBA 9402 strains were grown at 26–28 °C in modified YEB medium

(5 g l⁻¹ nutrient broth, 1 g l⁻¹ yeast extract, 5 g l⁻¹ peptone, 5 g l⁻¹ sucrose, 15 g l⁻¹ agar; pH 7.4). The pH of the medium was adjusted prior to autoclaving and the medium cooled to 40 °C in a water bath. Thereafter, 2 ml of 1 M MgSO₄·7H₂O and 50 mg l⁻¹ rifampicin from respective filter-sterilized stock solutions were added. For explant infection, a loop-full 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. A 100 µl aliquot of the overnight suspension was re-inoculated into fresh medium (10 ml/25 ml Erlenmeyer flask). Prior to bacterial inoculation, acetosyringone (Sigma, USA; 100 mM stock solution in DMSO) was added to the culture medium. The cultures were re-grown for 16–18 h on a reciprocal shaker (120 rpm) at 28 °C to obtain an inoculum for infecting explants.

Transformation

Stem internodal segments and leaf explants from the outdoor-grown plant after surface-disinfection, as well as those from in vitro grown axenic shoot cultures (3–4 week-old), were used for transformation. Apical portions of the cut internodal surfaces and leaf midribs were inoculated with 10–30 µl of an overnight culture of *Agrobacterium* (10⁹ bacteria ml⁻¹) by means of a sterile hypodermic needle. *Agrobacterium*-treated explants were plunged into 300 ml screw-capped jars containing 0.6 % (w/v) agar-solidified MS basal medium without growth regulator supplements (MS0; 20 ml per jar) and the jars were kept inside the culture room (25 ± 1 °C) under diffused light with a low PFD (10–15 µmol m⁻² s⁻¹). Following co-cultivation (2–8 days), explants were transferred to 0.6 % (w/v) agar-solidified MS0 supplemented with a bactericidal antibiotic. Control cultures, containing similar explants but wounded with the hypodermic needle without bacteria, were maintained under similar light and temperature conditions as for inoculated cultures.

Establishment of transformed root cultures

For initiating transformed root clones (rhizoclones), individual roots (1.0–1.5 cm) that developed along the inoculated surface were excised and each transferred to 5 cm diam Petri dish (Tarsons, India) and eventually to 300 ml screw-capped glass jars (Excel Corporation, Alleppey, Kerala, India), each containing 10–20 ml of MS0 agar medium. A set of transformed root clones were also inoculated on MS medium supplemented with different concentrations of auxins (0.1–1.0 mg l⁻¹ indole-3-acetic acid [IAA] or indole-3-butyric acid [IBA]). The media were supplemented with the bactericidal antibiotic cefotaxime (250–1000 µg ml⁻¹; Sigma, USA). Root cultures were incubated at 25 ± 1 °C and 10–15 µmol m⁻² s⁻¹ PFD and sub-cultured

every 2–4 week—intervals onto fresh medium, the antibiotic concentrations being gradually reduced to 250 µg ml⁻¹ and 100 µg ml⁻¹ during the second and third passages, respectively. Subsequently, roots were maintained in MS0 medium free from the antibiotic. Root cultures in liquid medium were grown on a rotary shaker (80 rpm) in the culture room (25 ± 1 °C; 10–15 µmol m⁻² s⁻¹ PFD).

Polymerase chain reaction

Template DNA was isolated from different fast-growing hairy root lines (rhizoclones) according to a modified CTAB extraction protocol [44]. Root DNA was isolated from the non-transformed donor plant so as to serve as a negative control. A4T was used as a source of plasmid DNA to serve as a positive control. The oligonucleotide primers (Bangalore Genei, Bangalore, India), were based on the known nucleotide sequence of the *T_L*-DNA of *Agrobacterium rhizogenes* A4 [45] and designed for amplification of *rolB* ORFs according to Soudek et al. [46]. The forward (21-mer) and reverse (20-mer) primer combination used for amplification of *rolB* gene of A4T were 5'-GCA CTT TCT GCA TCT TCT TCG-3' and 5'-CCT GCA TTT CCA GAA ACG AT-3' respectively. The optimum PCR mixture (25 µl) contained 1U *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8), 50 mM KCl, 1.5 mM MgCl₂, 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 one min and extension (copy-synthesis) at 72 °C for three min for the first cycle followed by 33 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1.5 min. The final cycle was culminated with an additional step of extension at 72 °C for 5 min prior to hold at 14 °C. The amplified product was separated by 1 % (w/v) agarose gel electrophoresis in 1X 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 rocker, followed by destaining in double-distilled water (30 min). The gel was then photographed using a Gel Documentation unit (Bio-Rad, USA).

ED-XRF analysis of multi-elemental content

Instrumentation

The elemental analysis was performed using Mo K X-rays generated from a secondary molybdenum target of an ED-XRF system available at the Ion Beam Laboratory, Institute of Physics, Bhubaneswar, India [34]. The system

incorporates a low power (50 W) air-cooled tungsten anode X-ray tube as an excitation source with triaxial geometry (Fig. 1). The X-ray tube was operated at 30 kV and 0.6 mA current. The X-rays from the tube were exposed on a molybdenum secondary exciter and the characteristic K X-rays of molybdenum (17.8 keV, the weighted average energy of the Mo $K\alpha$ and $K\beta$ lines) were used to excite the characteristic X-rays of elements present in all the samples. The advantage of employing secondary exciter is to prevent high background and hence lower detection limit in the fluorescence spectra [47]. The fluorescent X-rays were collected using a peltier cooled silicon drift detector (SDD), RÖNTEC XFLASH® 1000 X-ray detector having a thin Be window (0.3 mm thickness) with an active area of 5 mm² and processed by a high resolution (180 eV at 5.9 keV) amplifier. The detector attained $-15\text{ }^{\circ}\text{C}$ using this peltier cool mechanism. The spectra were recorded by using a PC-based multi-channel analyzer. The spectral data were analyzed using the computer program AXIL [48]. Prior to irradiation of the samples pellets of NIST CRMs were irradiated for calibration.

Sample preparation

Fresh roots from in vivo donor plant and in vitro transformed roots of *Clitoria ternatea* L. were collected and thoroughly washed with distilled water and kept for 24 h in deep freezer at $-20\text{ }^{\circ}\text{C}$ to solidify the moisture content in the material. Then these plant materials were dried in a lyophilizer at $40\text{ }^{\circ}\text{C}$ for 8 h and subsequently powdered by using the agate mortar. To 100 mg of the fine powdered samples, a binder such as cellulose (Qualigen, India) was added in 1:1 ratio. Then they were thoroughly mixed and homogenized and

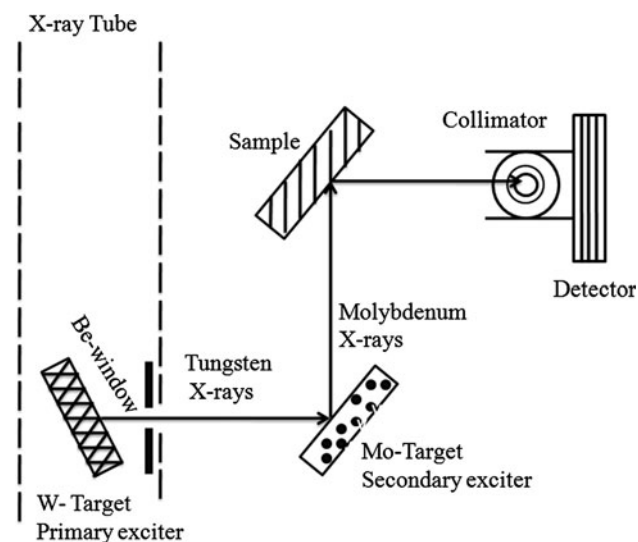


Fig. 1 Schematic representation of the triple axis geometry of ED-XRF set-up

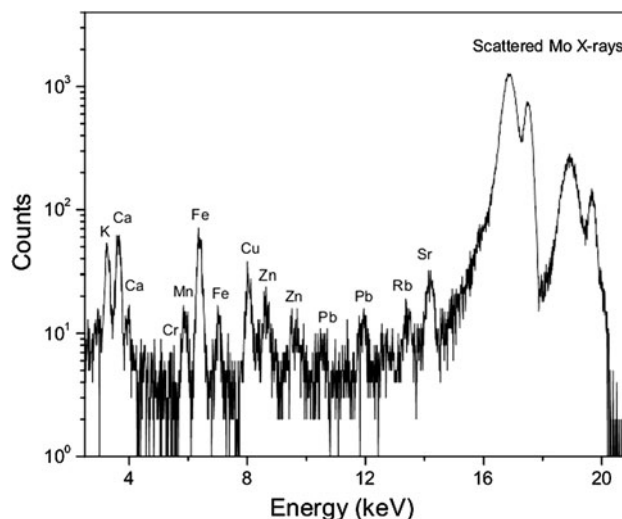


Fig. 2 The XRF spectrum of NIST-Tomato leaves (1573)

pressed (6 tons per pellet area) into pellets of 2.54 cm diameter and 2 mm thickness in a KBr hydraulic press, frequently used to prepare samples for FTIR spectroscopy using KBr (potassium bromide) as binding material. In a similar manner pellets were also prepared from the certified reference materials (CRM) viz. apple leaves (NIST-1515), orchid leaves (NIST-1571) and tomato leaves (NIST-1573) obtained from the National Institute of Standards and Technology (NIST), USA. Prior to irradiation of the sample pellets of NIST CRMs were irradiated as standards for calibration, quantification and verification of results.

Data analysis

The elemental concentrations were determined using calibration-curve method by comparing the peak areas and heights of the sample with that of certified reference material (standard). The XRF spectrum and the certified and measured values of elemental concentrations of NIST-Tomato leaves (1573) are presented (Fig. 2; Table 1). The elemental concentrations were determined using the equation [49]

$$m_j = N_{ij} / I_0 G \varepsilon \sigma_{ij} \beta_i$$

where m_j is the concentration of the j th element present in the sample, N_{ij} is the net counts per unit time for the i th group of X-rays of j th element, $I_0 G$ is the intensity of the exciting radiation incident on the sample visible to the detector, ε is the detector efficiency for the j th element (provided by the manufacturer of the detector), σ_{ij} is the theoretical X-ray fluorescence cross section [50, 51] at 17.8 keV excitation energy and β_i is the self absorption correction factor that accounts for absorption of incident and emitted X-rays in the sample.

Table 1 Certified and measured values of elemental concentrations of NIST-Tomato leaves (1573)

Elements	NIST-Tomato leaves (1573)	
	Certified value	Measured value
K (%)	2.70 ± 0.05	2.69 ± 0.05
Ca (%)	5.05 ± 0.09	5.06 ± 0.08
V	0.835 ± 0.01	0.810 ± 0.01
Cr	1.99 ± 0.06	1.86 ± 0.04
Mn	246 ± 8.0	252 ± 8.0
Fe	368 ± 7.0	376 ± 7.2
Co	0.57 ± 0.02	0.57 ± 0.03
Ni	1.59 ± 0.07	1.67 ± 0.07
Cu	4.7 ± 0.14	4.6 ± 0.14
Zn	30.9 ± 0.70	29.8 ± 0.66
As	0.112 ± 0.004	0.089 ± 0.003
Se	0.054 ± 0.003	0.066 ± 0.004
Rb	14.89 ± 0.27	14.43 ± 0.25

Data represented mean values with SE

Statistical analysis

All transformation experiments were set up in a completely randomized design (CRD). Each treatment consisted of 10 replicate jars, each containing four internodes. Each experiment was repeated three times. Data were analysed using 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 [52].

Results

Genetic transformation and root development

Localized swellings first appeared at the inoculated sites of the intermodal explants and these developed into compact nodular outgrowth in 10–15 days. Such outgrowths were initially soft and fragile, but later (in 12–23 days) from a majority of them (up to 85 %) small hairy protuberances became evident (Fig. 3b; Table 2). These aerial roots (ca. 4–7 per explants) were different from normal subterranean roots in exhibiting a characteristic lack of geotropism. They were ageotropic, most being plagiotropic (growing parallel to the culture medium) while some displayed negative geotropism with a tendency to grow towards light and away from the culture medium. Roots resulted from inoculation were further characterized by their prolific growth on MS basal medium (semisolid/liquid) lacking growth regulators and a high incidence of lateral branching. They grew as closely interwoven masses over the surface of the culture medium and up to the sides of the culture

dish (Fig. 3c–e). In vivo explants collected from naturally occurring plants were more responsive than those obtained from axenic shoot cultures; internodes performed better than leaf explants (Table 2). Uninfected control explants, lacking bacterial treatment, did not produce roots at the inoculation sites. These, especially the stem internodes, developed 1 or 2 roots from the basal portion immersed inside the culture medium, but they failed to grow when excised and transferred to basal medium lacking growth regulator (MS0).

PCR amplification for detection of *rolB* genes in the transformed rhizoclones

Genomic DNA isolated from several independent transformed hairy root clones, capable of showing auxin-independent growth, revealed the expected amplification product of 206 bp specifying A4 *rolB* gene (Fig. 4). This indicated the integration of the *Agrobacterium* ORF as part of the pRi T_L -DNA into the recipient plant genome via genetic transformation. Amplification product was not detected in DNA from untransformed plants when subjected to PCR amplification with the gene primers specific to the *rolB* gene sequences.

Multi-elemental content

The elemental composition of genetically transformed hairy roots of *Clitoria ternatea* L. was identified and quantified using ED-XRF technique and this was compared with the roots from naturally grown donor plant (Table 3). The representative spectra of in vivo root sample are shown in Fig. 5a and that of 30 day-old transformed hairy roots are shown in Fig. 5b. Fourteen different elements were detected in the processed pellets in concentrations (mg kg^{-1}) which exhibited a retrogressive trend as K, Ca, Sr, Fe, Rb, Zn, Mn, Cu, Ni, Pb, V, Cr, Co and Se. The concentrations of all the elements tested were found to be at par in transformed roots compared to the non-transformed root sample. Two macronutrients namely K and Ca were detected in markedly high quantities whilst Fe was available in quantity just above the trace level ($>100 \text{ mg kg}^{-1}$). Under natural growth condition, the roots had two major elements, i.e. potassium ($86265.5 \text{ mg kg}^{-1}$) and calcium ($52122.04 \text{ mg kg}^{-1}$). Elements like Rb, Zn, Mn, Cu and Ni were detected but only in trace amounts whereas V (0.18 mg kg^{-1}), Cr (0.11 mg kg^{-1}), Co (0.13 mg kg^{-1}), Se (0.05 mg kg^{-1}), As (0.04 mg kg^{-1}) and Pb (0.22 mg kg^{-1}) were either in negligible quantities or at a level too-low-to-be detected. ED-XRF analysis revealed a reducing trend in contents of trace elements (mg kg^{-1}) as Sr (154.33), Fe (98.65), Rb (75.8), Zn (50.72), Mn (38.82), Cu (7.55) and Ni (2.08).

Fig. 3 Induction and establishment of hairy root cultures of *Clitoria ternatea* transformed by *Agrobacterium rhizogenes* A4T strain. **a** A potted plant of butterfly pea (*Clitoria ternatea* L.). **b** Formation of nodular outgrowth and root emergence at the inoculated sites of an internode in MS0 15 days following infection with overnight-grown bacterial suspension. **c** A well proliferated rhizoclone HRA4B5 in agar-solidified MS0 after 30 days of culture. **d** A well proliferated rhizoclone HRA5E7 in agar-solidified MS0 after 30 days of culture. **e** A well proliferated rhizoclone HRA7C3 in agar-solidified MS0 after 30 days of culture

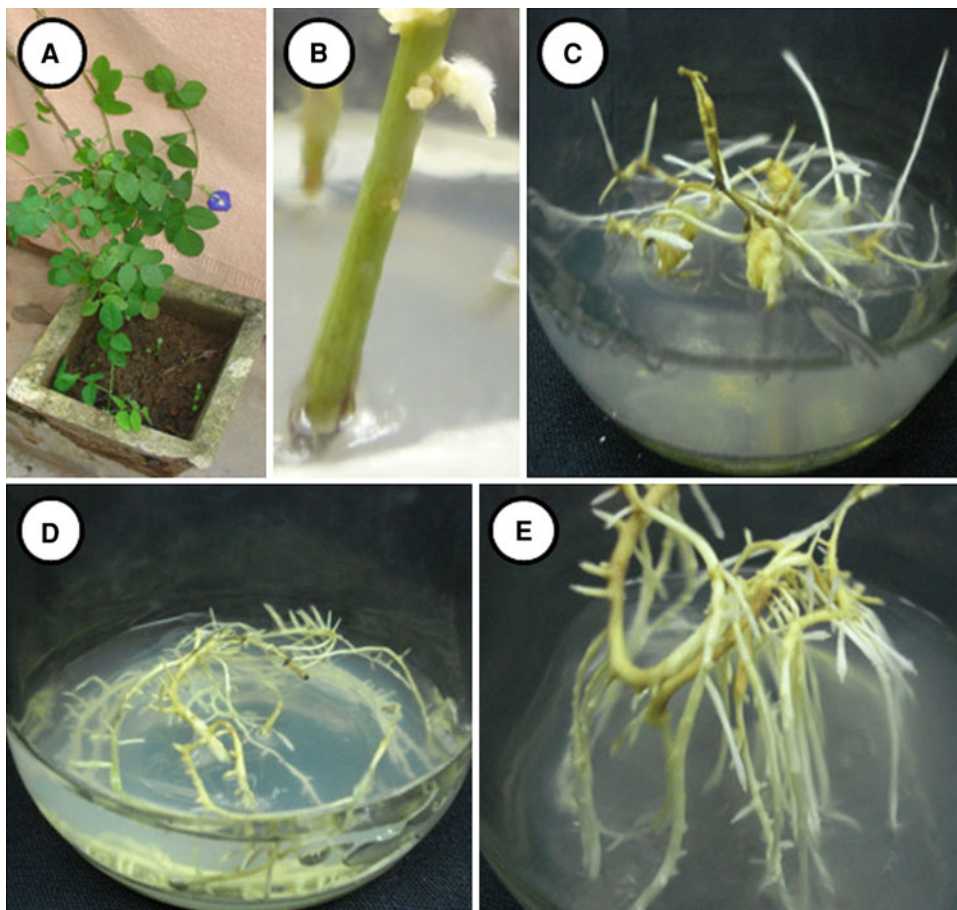


Table 2 Hairy root induction in genetically transformed *Clitoria ternatea* following explant co-cultivation with *Agrobacterium rhizogenes* strains

Strains	Days for root emergence		% Root emergence		Roots per explant	
	Internode	Leaf	Internode	Leaf	Internode	Leaf
A4 (pRi A4)	15–18	18–20	78.33 ^b	68.33 ^b	4.3 ^b	1.5 ^c
A4T (pRi A4)	12–15	15–18	85.83 ^a	73.26 ^a	7.5 ^a	3.8 ^a
LBA 9402 (pRi 1855)	20–23	22–25	72.40 ^c	56.66 ^c	3.8 ^c	1.7 ^b

Data pooled from a total of 3 separate co-cultivation experiments each comprising of 10 replicate jars containing 1–4 explants per jar. Mean values within column with *different alphabets* (in superscript) are significantly different ($P < 0.05$; Duncan's new multiple range test)

Elemental composition of transformed root cultures was comparable with that of roots from in vivo grown donor plants. Potassium and calcium had values of 88024.6 and 53474.2 mg kg⁻¹ respectively which were very close to that of in vivo roots. With respect to micronutrients the results were also closely comparable. The estimated value (mg kg⁻¹) of V (0.12), Cr (0.15), Co (0.09), Se (0.08) and Pb (0.16) were too as low as those of roots from naturally grown plants. On the other hand, the remaining trace elements were arranged in a decreasing order based on the estimated values (mg kg⁻¹) as strontium (Sr, 158.64), iron (Fe, 100.52), rubidium (Rb, 78.1), zinc (Zn, 54.36),

manganese (Mn, 42.77), copper (Cu, 8.03) and nickel (Ni, 1.42). No arsenic was detected in transformed root samples.

Between the elemental composition of transformed root and natural root, there were some variations in mean values which were more pronounced (>30 %) with regards to Se (37.5 %), V (33.3 %), Co (30.77 %), and Ni (31.73 %) and relatively less pronounced as for Pb (27.27 %), Cr (26.7 %), Mn (14.17 %), Zn (7.63 %), Rb (6.43 %) and Cu (4.22 %). In this study, transformed roots had a general trend of having accumulated higher levels of microelements barring V, Co, Ni and Pb.

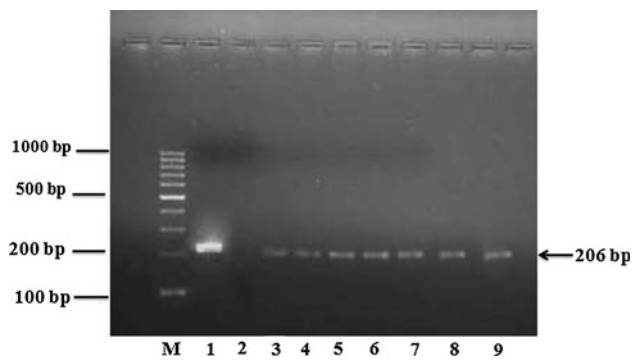


Fig. 4 PCR amplification of *rolB* gene in rhizoclonal lines resulted from transformation with A4T. *M* 100 bp DNA ladder, *Lane 1* Plasmid DNA from A4T (Positive control), *Lane 2* Root DNA from a non-transformed plant (negative control), *Lane 3–9* DNA from seven randomly selected rhizoclonal lines HRA3A7, HRA3B4, HRA4B5, HRA5D10, HRA5E7, HRA7B5, and HRA7C3

Table 3 ED-XRF based estimation of elemental concentration in transformed hairy roots of *Clitoria ternatea* L. vis-à-vis roots of the naturally grown donor plant

Elements	Natural roots (mg kg ⁻¹)	Transformed roots (mg kg ⁻¹)
Potassium (K)	86265.5 ^b	88024.6 ^a
Calcium (Ca)	52122.04 ^b	53474.2 ^a
Vanadium (V)	0.18 ^a	0.12 ^b
Chromium (Cr)	0.11 ^b	0.15 ^a
Manganese (Mn)	38.82 ^a	42.77 ^a
Iron (Fe)	98.65 ^b	100.52 ^a
Cobalt (Co)	0.13 ^a	0.09 ^b
Nickel (Ni)	2.08 ^a	1.42 ^b
Copper (Cu)	7.55 ^b	8.03 ^a
Zinc (Zn)	50.72 ^b	54.36 ^a
Arsenic (As)	0.04 ^a	Nd
Selenium (Se)	0.05 ^a	0.08 ^a
Rubidium (Rb)	75.8 ^b	78.1 ^a
Strontium (Sr)	154.33 ^b	158.64 ^a
Lead (Pb)	0.22 ^a	0.16 ^b

Data pooled from 3 replicates for each sample

Mean values within row followed by the *same alphabet* (in superscript) are not significantly different ($P \leq 0.05$; Duncan's new multiple range test)

Nd not detected

Discussion

The ED-XRF technique used in this investigation provided a highly sensitive device permitting detection and estimation of the multi-elemental composition of *Agrobacterium*-mediated transformed *in vitro* hairy root cultures vis-à-vis roots of naturally occurring plants. The trivial difference in elemental composition between *in vitro* transformed roots

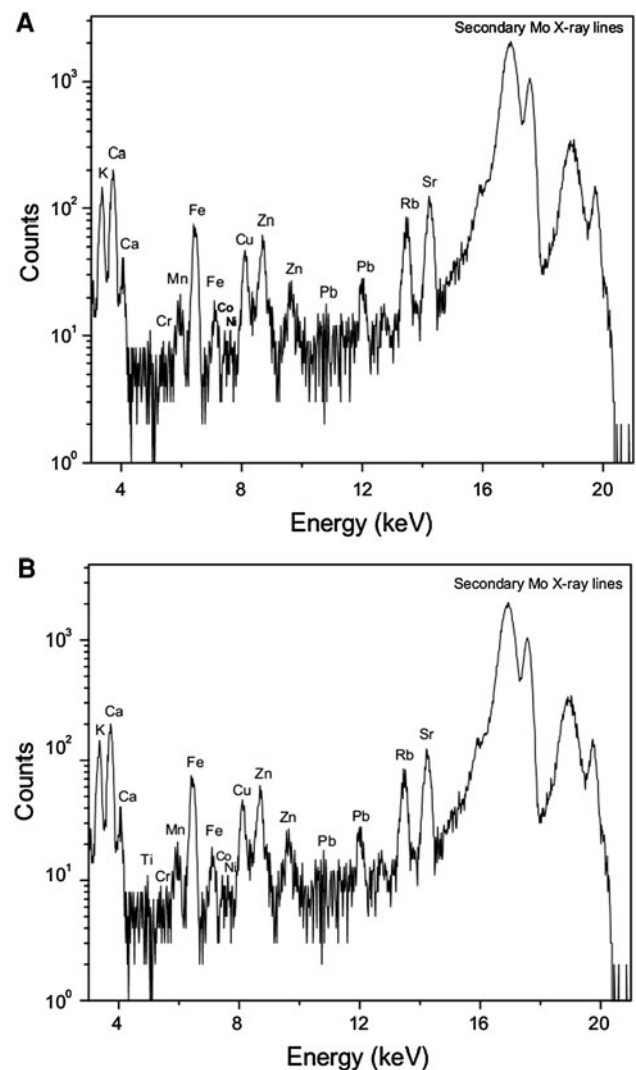


Fig. 5 **a** Representative ED-XRF spectra of roots of naturally grown plant *Clitoria ternatea* L. **b** Representative ED-XRF spectra of genetically transformed rhizoclone (HRA7C3) of *Clitoria ternatea* L. after 30 days

and that of roots from *in vivo* plants could be attributed to various factors like the 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 preferential absorbability of a particular plant or *in vitro* culture for the corresponding element from the respective planting substrate. The concentrations of major elements such as K and Ca were reasonably high in both the transformed root and *in vivo* root samples. There are a number of reports on high K and Ca levels in different medicinal plants [9, 24, 26]. The ionic movement of K along with Na is crucial to life and is most important during excitation and transmission of the action potential in nerve cells. K is an activator of some enzymes, in particular a

co-enzyme for normal growth and muscle function [53]. Normal concentrations of K are required for optimal secretion of insulin [54] and its concentration in serum below optimal level can cause insulin resistance [55]. Ca is known to enhance the quality of bone and teeth. Besides, it acts in the process of coagulation, regulation of heartbeat, cellular permeability, muscular contraction, transmission of nerve impulses and enzymatic activity, in addition to its neuromuscular, systemic and cardiac function [10]. Sufficient levels of Ca are required for release of insulin [56]. It is also reported to play an important role in glucose tolerant factor (GTF), which decreases the blood glucose level by utilizing insulin [17].

ED-XRF analysis revealed the presence of several micro-nutrients and trace elements such as V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Rb and Sr in transformed root and in vivo root samples of the medicinal plant species *C. ternatea* L. Some of these inorganic elements have been implicated in metabolic disorders such as diabetes [13–16]. In addition to the macronutrient K and Ca, appropriate levels of Cr, Mn, Cu and Zn are essential for secretion and effective action of insulin. Cr is a critical co-factor in insulin action [20]. Reportedly, it is an active component of GTF [57]; deficiency of Cr causes impaired action of GTF, hyperglycemia, glycosuria [58], hyperinsulinemia, decreased insulin receptor number, decreased insulin binding, diabetes mellitus-associated neuropathy and vascular pathologies [59]. A naturally occurring biologically active form of chromium called chromodulin has been described that apparently has a role in carbohydrate and lipid metabolism as part of a novel insulin-amplification mechanism. Chromodulin is an oligopeptide that binds four chromic ions and facilitates insulin action in converting glucose into lipids and CO₂. Mn with glutamic acid and vitamin C intensively lowers the blood glucose level in diabetes and shows diuretic action. Cu possesses insulin like activity and its deficiency leads to glucose intolerance, decreased insulin response and increased glucose response [56]. Besides, Cu plays a major 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 a number of oxidase enzymes involved in the stabilization of matrices of connective tissue, oxidation of ferrous ion, synthesis of neurotransmitters, bestowal of pigment to hair and skin, assurance of immune system competence, generation of oxidative energy and protection from free radicals [9]. Zn plays an important role in production, storage and regulation of insulin [12]. Insulin is stored in pancreatic β -cells as insulin-Zn complex and deficiency of Zn can lead to increased insulin resistance and hyperglycemia [60]. Low levels of Zn were detected in people suffering from diabetes [61]. Zn is an essential component of enzymes and functions as a component of transcription factors known as

zinc fingers that binds to DNA and activates the transcription of a message and imparts stability to cell membranes. Its deficiency is associated in impairment in healing, taste and growth leading to dwarfism. It has also been suggested that low Zn status increases the susceptibility to osteoporosis and to pathological changes caused by the presence of excessive reactive oxygen species or free radicals. Fe plays role in oxygen and electron transport. Co is an integral component of vitamin B₁₂, which is an essential nutrient for non-ruminant animals and humans. It is known that 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 CoA, formed by the oxidation of odd-chain fatty acids, to succinyl CoA. Reportedly, there is a connection between Se deficiency and protein malnutrition disease (Kwashiorkor), multiple sclerosis, cancer and heart diseases. It has been reported that in cancer, as Se level reached or exceeded the mean value for the carcinoma group, the tumour was usually confined to the origin, distant metastasis occurred less frequently, and multiple primary lesions and recurrences seldom appeared. Also, it has been shown that the doses of Se required for maximum inhibitory effects in cancer prevention are considerably higher than those required for nutrition adequacy [62]. V has also been suggested to play a role in treatment of cancer [7].

Additional to the essential inorganic nutrients, both the in vivo and transformed root samples were found to accumulate the toxic element Pb in negligible amount, though arsenic was not detected in any root samples from genetically transformed rhizoclonal. Lead was detected at a lower level (0.16 mg kg⁻¹) in transformed roots compared to that in in vivo roots (at 0.22 mg kg⁻¹). The accumulation of Pb in root cultures could perhaps owe to the inherent contaminants of the gelling agent (culture medium) extracted from marine macro algae. Nevertheless, such in vitro transformed root cultures can be safely recommended as a source of herbal medicines in view of the recommendation by World Health Organisation (WHO, Geneva) citing maximum permissive levels for arsenic and lead as 1 and 10 mg kg⁻¹ respectively in raw plant materials [63].

Conclusion

For the first time, the investigation embodied in this report demonstrates that the genetically transformed roots induced by *Agrobacterium rhizogenes* can accumulate certain major and minor inorganic elements at a comparable level to that in the in vivo roots of the naturally occurring plants which was used as the tissue donor.

Scaling-up of such fast-growing in vitro root cultures using bioreactors can be exploited further as a renewable source of minerals essential for designing effective nutrient-conjugated drugs. This will be in addition to providing a remarkable means for conserving the natural stock of the selected plant species *Clitoria ternatea* L., bestowed with proven multi-medicinal properties. Genetically transformed root cultures, being capable of rapid proliferative growth without necessitating exogenous growth regulator supplements, offer a sustainable alternative to the native rooted plants. This will automatically preclude the need for massive uprooting of rare and threatened medicinal plants for their root-products. In essence, the optimized protocol for induction and establishment of in vitro root cultures via *Agrobacterium*-mediated genetic transformation holds a paramount potential as an efficient ex situ conservation strategy for elite plant species especially in which the active principle of pharmaceutical significance is confined to the root system.

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