Preface

Plants consistently synthesize, accumulate, and use a bewildering range of secondary metabolites as a part of their overall defense strategy. Many of these metabolites have been used around the world as medicines for various human health problems. In fact, more than 80% of the world's population relies on plants for principle health care. Nearly half of the medical prescriptions in the developed world are of plant origin.

In recent years the quest for quality of life and a common belief that plants are "natural and therefore safe" has paved the way for a wider acceptance of plant-based medicines worldwide. International trade in medicinal plants has become a major force in the global economy and the demand is increasing in both developed and developing countries. Thus, the continued rise in consumer demand for plant-based medicines and the expanding world population have resulted in indiscriminate harvest of wild species of medicinal plants. As well, a reduction of natural habitats for medicinal plants has placed many wild species in danger of extinction. The impact of rapid climate changes may also have an adverse effect on wild-plant species leading to the loss of useful genetic material. Most medicinal plants are harvested from the wild and the traditional agricultural and horticultural practices have not been developed even for most commonly used medicinal plant species.

The quality and consistency of the products are the most challenging issues facing the plant-based medicines. The production of medicinal metabolites in plants is affected by plant genotype, growth environment cultivation, harvesting, processing, and distribution. Medicinal plant preparations may also be contaminated with microbes and soil contaminants such as heavy metals, herbicides, pesticides, and other agricultural chemicals which can cause qualitative and quantitative changes in the levels of medicinal metabolites. The widespread occurrence of chemical variability and compromised quality of medicinal plants remain the major factors in inconsistent results of clinical trials of plant-based medicines. New regulations are currently being developed internationally to ensure consistency, safety and efficacy of plant-based medicines as well as how they are developed, manufactured, and marketed. Clearly, there is an imminent need for the development of new technologies and production approaches to improve the overall strategy of medicinal plant production to comply with up-coming legal regulations.

In vitro cell culture and controlled environment production systems offer an excellent opportunity for the selection and season-independent propagation of elite lines with specific, consistent levels of medicinal metabolites with minimum contamination. Additionally, the plant materials produced by in vitro techniques allow efficient application of the emerging analytical methods—such as metabonomics—for complete chemical profiling which has enormous potential for the discovery of new medicinal compounds.

Traditional breeding programs for medicinal plants are generally difficult to establish primarily due to lack of defined chemistry of medicinal components. Little knowledge of the genetic regulation of pathways of potential bioactive molecules further compounds this problem. In vitro techniques such as somaclonal variation, chemical mutagenesis, haploidy, protoplast fusion, and genetic transformation are applicable to create novel

germplasm. The impact of these techniques is perhaps greatest in the improvement of medicinal plants since the resulting genetic diversity may open avenues for the discovery of new medicinal metabolites and treatments. Furthermore, the genetic manipulation of plant cells and organs has great advantages for producing secondary metabolites and other bioactive natural products. Together with the tools of chemical and genomic analyses, the in vitro culture methods hold the key to fundamental research on the biochemical and molecular basis of the mode of action of plant-based medicines. This book provides a detailed step-by-step description of protocols for the establishment of in vitro cultures of important medicinal plants, their mass multiplication in a controlled environment, and step-wise secondary metabolite analysis, genetic transformation, large-scale metabolite production in a bioreactor, and molecular markers. The role of altered microgravity in plant metabolite production is also described. In addition, many of these protocols will provide a basis for much needed efforts of in vitro germplasm conservation or cryopreservation of medicinal plant species at the brink of extinction as well as efforts to protect them from the adverse impact of rapid climatic changes. This book will certainly appeal to graduate and post graduate students, researchers, biotechnologists, industry, government agencies, and could be used as a text book.

This book contains 31 book chapters, divided into five sections. Section I contains 16 chapters describing step-wise protocols for micropropagation and chemical analysis of secondary compounds of different medicinal plants. Section II contains five chapters which address the transgenic approach for producing secondary metabolites. Section III contains two chapters which cover molecular markers/microsatellites. Section IV has six chapters which address biotransformation, bioreactors, and metablomics. Finally, section V contains two review chapters describing plant secondary metabolites in altered microgravity and role of biotechnology in producing anti-cancer compounds. Each chapter has been peer reviewed and revised accordingly.

We appreciate the time and effort that all reviewers have put into the development of these chapters which aided in improving the quality of the material presented herein. We extend our most sincere thanks to the staff of Humana Press for giving us the opportunity to present this book to our audience.

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Chapter 2

In Vitro Propagation of *Rauwolfia serpentina* Using Liquid Medium, Assessment of Genetic Fidelity of Micropropagated Plants, and Simultaneous Quantitation of Reserpine, Ajmaline, and Ajmalicine

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Summary

Rauwolfia serpentina holds an important position in the pharmaceutical world because of its immense anti-hypertensive properties resulting from the presence of reserpine in the oleoresin fraction of the roots. Poor seed viability, low seed germination rate, and enormous genetic variability are the major constraints for the commercial cultivation of *R. serpentina* through conventional mode. The present optimized protocol offers an impeccable end to end method from the establishment of aseptic cultures to in-vitro plantlet production employing semisolid as well liquid nutrient culture medium and assessment of their genetic fidelity using polymerase chain reaction based rapid amplification of polymorphic DNA analysis. In vitro shoots multiplied on Murashige and Skoog basal liquid nutrients supplemented with benzo[a]pyrene (1.0 mg/L) and NAA (0.1 mg/L) and in-vitro rhizogenesis was observed in modified MS basal nutrient containing NAA (1.0 mg/L) and 2% sucrose. In-vitro raised plants exhibited 90–95% survival under glass house/field condition and 85% similarity in the plants regenerated through this protocol. Field established plants were harvested and extraction of indole alkaloid particularly reserpine, ajmaline and ajmalicine and their simultaneous quantitation was performed using monolithic reverse phase high-performance liquid chromatography (HPLC).

Key words: Micropropagation, Liquid medium, *Rauwolfia serpentina*, Genetic fidelity, Reserpine, Ajmaline, Ajmalicine, HPLC, RAPD

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1. Introduction

Rauwolfia serpentina has drawn special attention all over the world in the pharmaceutical field for quite some time and still holds an important position because of its great medicinal value. The antihypertensive properties of *Rauwolfia* roots are attributed to presence of reserpine (3,4,5-trimethyl benzoic acid ester of reserpic acid, an indole derivative of 18-hydroxy yohimbine type) *(1)*. It is a relatively weak tertiary base occurring in the oleoresin fraction of the roots and has depressant action on central nervous system (CNS) and produces sedation and lowering of blood pressure. Administration of reserpine depletes the brain and peripheral vessels of serotonin (5-hydroxy tryptamine) and catechol amines. Besides, it is also reported to influence the concentration of glycogen, acetylcholine, γ-amino butyric acid, nucleic acid, and anti-diuretic hormones. Reserpine is now being used as a tool in physiologic studies of body functions and pharmacologic studies of other drugs.

Poor seed viability, low seed germination rate, and low vegetative propagation rate through root cuttings has hampered large scale commercial cultivation of *R. serpentina* through conventional mode and over exploitation of the natural resources has led to its inclusion in Red Data Book of India as an endangered plant species. The increasing demand for *Rauwolfia* roots in national and international markets and decreasing availability have encouraged many farmers to cultivate this pharmaceutically important plant. Large scale cultivation through in vitro clonal propagation may therefore be beneficial for the germplasm conservation and commercial cultivation of elite plants of *R. serpentina* for the production of reserpine at desirable level. Use of liquid medium has certain advantages over the use of nutrients in an agar based semisolid matrix because of better availability of oxygen by agitation and aeration *(2–4)*. Keeping in view these problems and overwhelming interest in the techniques of plant tissue culture, the present protocol was developed covering the following objectives; (a) production of quality planting material, (b) mass propagation of *Rauwolfia serpentina* plantlets employing liquid medium; (c) assessment of genetic fidelity of the in vitro raised somaclones, and (d) simultaneous analysis of reserpine, ajmaline and ajmalicine using reverse phase HPLC. This protocol would be of great help for commercial industrial units and research groups who are associated with the optimization of micropropagation of economically important plants particularly medicinal plants and secondary metabolite studies.

2. Materials

2.1. Media Preparation

1. Murashige and Skoog (MS) salts.
2. Phytohormone(s) (*see* **Tables 1** and **2**).
3. 4.0, 7.0, and 9.2 pH buffers.
4. 1 N NaOH.

Table 1
Composition of Basal MS Medium

Designated Stock	Constituent of stock	Concentration (mg/L)	Stock (g/L)	Volume (mL) of the stock to be used for preparation of 1 L medium
A	NH_4NO_3	1,650	82.5	20
B	KNO_3	1,900	95	20
C	$CuSO_4{\cdot}5H_2O$	0.025	0.005	5
	$MgSO_4{\cdot}7H_2O$	370	74	
	$ZnSO_4{\cdot}7H_2O$	8.6	1.72	
	$MnSO_4{\cdot}4H_2O$	22.3	4.46	
D	$CaCl_2{\cdot}2H_2O$	440	88	5
E	$Na_2MoO_4{\cdot}2H_2O$	0.25	0.05	5
	H_3BO_3	6.2	1.24	
	$CoCl_2{\cdot}6H_2O$	0.025	0.005	
	KI	0.83	0.166	
	KH_2PO_4	170	34	
F	$FeSO_4{\cdot}7H_2O$	27.85	5.57	5
	Na_2EDTA	37.25	7.45	
G Vitamins	Thiamine–HCl	0.1	0.02	5
	Pyridoxine–HCl	0.5	0.1	
	Nicotinic acid	0.5	0.1	
	Glycine	2.0	0.4	
Other supplements	*myo*-inositol	100	To be added freshly	
	Sucrose	30,000		
	Agar	8,000		

Table 2
Details of Plant Growth Regulators

Plant growth regulators	Solubility	Sterilization	Storage
Auxin			
α-naphthelene acetic acid (NAA)	1 N NaOH	Co-autoclave	4°C
Cytokinin			
Benzyl- 6- amino purine (BAP)	1 N NaOH	Co-autoclave	4°C

5. 1 N HCl.
6. 250-mL wide-mouth Erlenmeyer flask.
7. 25 × 150 mm Culture tubes.
8. Beakers.
9. Measuring cylinders.
10. 0.1–10 mL pipets and/or 0.5-μL micropipets.
11. Glass rods (*see* **Note 1**).

2.2. Surface Sterilization and Establishment of In Vitro *Cultures*

1. 0.1% $HgCl_2$, Teepol.
2. 80% ethanol.
3. Savlon.
4. Sterile distilled water.
5. Culture medium.
6. 8”-12” Rust-proof stainless steel forceps.
7. Scissors.
8. Scalepl and/or razor blade.
9. Petri dishes.

2.3. Assessment of Clonal Fidelity of Micropropagated Plants

2.3.1. Isolation of Plant Genomic DNA

1. Eppendorf tubes.
2. Oakridge tubes.
3. Dessicator with vacuum pump.
4. Mortar and pestle.
5. Liquid nitrogen.
6. Buffers (*see* **Table 3**).
7. 5 mg/mL Ethidium bromide (EB) stock in water.
8. Agarose.
9. 6X Loading dye.

Table 3
Details of Buffers and Dyes

Buffers (pH 8.0)					
Extraction buffer		**High salt TE buffer**		**TAE buffer (50X)**	**Loading dye (6X)**
NaCl	1.4 M	NaCl	1 M	Tris base 242 g	30% glycerol in water
CTAB	2.5%	Tris–HCl	10 mM	EDTA (0.5 M) 100 mL	0.25% bromo phenol blue
Tris–HCl	100 mM	EDTA	1 mM	Glacial 57.1 mL acetic acid	0.25% xylene cyanol
EDTA	20 mM				Store this at –20°C
β-mercapto-ethanol	0.2%			Add water to make 1 L	
PVP	1%				

2.3.2. PCR for DNA Amplification

1. Template DNA.
2. Taq DNA polymerase.
3. Deoxyribonucleoside triphosphates (dNTPs) mix (dATP; dTTP; dCTP and dGTP).
4. 10X polymerase buffer.
5. Primer(s).
6. λ marker DNA double (HindIII + EcoRI) digested (Genei, Bangalore).
7. Polymerase chain reaction (PCR) tubes.
8. Milli-Q water.
9. Ice.

In this protocol MAP, OPA, and OPB series of primers were used (*see* **Table 4**). MAP primers were designed at CIMAP *(5)* (custom synthesized by M/s Genei; Bangalore, India). OPA and OPB primers were designed at Operon Technologies Inc. (Germany).

2.4. Quantitative Analysis of Indole Alkaloids

2.4.1. Extraction of Indole Alkaloids

1. Chloroform.
2. Methanol.
3. Distilled water.
4. Hydrochloric acid.
5. Vacuum-rotavapor.
6. Filtration assembly.
7. Micropipets.
8. Rotavapor R-144 grinding device (Buchi, Switerzerland).

Table 4
Nucleotide Sequences of Primers Used for RAPD Analysis

Code	5′ to 3′	Code	5′ to 3′
OPA-2	TGCCGAGCTG	OPB-1	GTTTCGCTCC
OPA-3	AGTCAGCCAC	MAP-4	TGCGCGATCG
OPA-7	GAAACGGGTG	MAP-6	GCACGCCGGA
OPA-9	GGGTAACGCC	MAP-9	CGGGATCCGC
OPA-11	CAATCGCCGT	MAP-10	GCGAATTCCG
OPA-13	CAGCACCCAC	MAP-13	GTGCAATGAG
OPA-20	GTTGCGATCC		

2.4.2. High-Performance Liquid Chromatography Analysis of Indole Alkaloids

1. 100 × 4.6 ID RP-18e () Chromolith HPLC Column (Merck; Darmstadt, Germany).
2. HPLC-grade acetonitrile (Merck; Darmstadt, Germany).
3. HPLC-grade water.
4. Di-sodium-di-hydrogen orthophosphate.
5. GR-grade glacial acetic acid.
6. 0.45-μm nylon HPLC filter paper (Millipore).
7. Ajmaline (Sigma).
8. Reserpine (Sigma).
9. Ajmalicine (Hi-media).

3. Methods

3.1. Media Preparation

1. Prepare the MS *(6)* stock solutions as categorized (**Table 1**) (*see* **Notes 2** and **3**).
2. Use the stock solutions as stated in the Table 1 (*see* **Note 4**).
3. Melt agar separately.
4. Properly mix the required volume of stock solutions, sucrose, and *myo*-inositol.Add to the molten agar by stirring. Make the final volume of culture medium as per requirement.
5. Shoot multiplication semisolid medium (RS): Add BAP 1.0 mg/L and NAA 0.1 mg/L and adjust to pH 5.68 using 1 N NaOH and 1 N HCl.

6. Shoot multiplication liquid medium (RL): Prepare as RS medium but do not add agar (*see* **Note 5**).
7. Root induction medium (RI): Prepare semisolid half strength MS medium with 2% sucrose. Add 1.0 mg/L NAA and adjust the pH as above.
8. Dispense this medium in appropriate culture vessels (*see* **Note 6**). Sterilize at 121°C at 15 lb pressure for 15–20 min. Make the slant by tilting the tubes 45 and allow the medium to solidify.
9. Store the culture medium at 25°C.

3.2. Explant Preparation

1. Prepare the explants by cutting 1.5–2.5 cm long nodal segments containing 2 axillary buds (*see* **Fig. 1**) with the help of a sharp blade/razor.
2. Thoroughly wash the explants in continuous flow of tap water for 1 h and then with 1% Teepol solution followed by Savlon.

3.3. Surface Sterilization of Explants and Establishment of In Vitro *Cultures*

1. Sterilize the explant surface with 80% ethanol for 30 s and 0.1% $HgCl_2$ for 3 min under a laminar air flow hood (*see* **Notes 7** and **8**) followed by rinsing (3–4×) with sterilized distilled water (*see* **Notes 9** and **10**).
2. Slice off the exposed end slightly with the help of sterilized sharp blade and transfer the explants to a sterile Petri plate.
3. Inoculate single nodal explant in the culture tube (*see* **Fig. 2a**) containing RS media and incubate in the culture room under 14-h photoperiod, light intensity 54 μmol/m^2 s provided by cool white fluorescent light, 25 ± 2°C temperature and 60% relative humidity.
4. After in vitro establishment, these cultures can be multiplied and maintained indefinitely in 250-mL flasks (*see* **Fig. 2b**) by inoculating 6–8 explants per culture vessel. The cultures may be used as a source of inoculum for further routine multiplication practice.

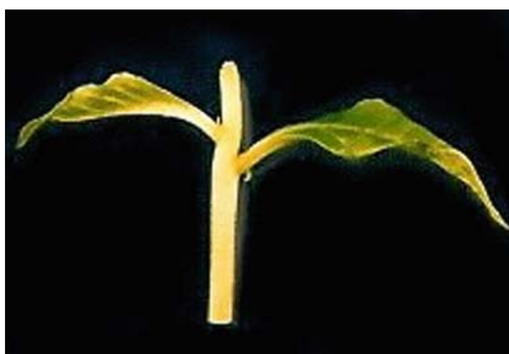

Fig. 1. Single nodal explant.

Fig. 2. In vitro establishment and multiplication of aseptic cultures of *R. serpentina* (**a–c**); semisolid medium (**a–b**) and liquid medium (**c**).

3.4. Multiplication and Maintenance of In Vitro *Cultures Using Liquid Medium*

1. Transfer 8–10 nodal explants from pre-existing aseptic stock cultures in the RL liquid medium in 250-mL flask (*see* **Fig. 2c**) containing 30 mL medium.
2. Place the culture flasks on an orbital shaker at 75 rpm in culture room.

3.5. Root Induction

1. For in vitro rhizogenesis, transfer 6 to 8 cm long microshoots to the semisolid RI medium filled in tubes or flasks. All steps should be carried in laminar air flow.
2. Remove the in vitro developed plantlets (*see* **Fig. 3a**) after 8 wk and properly clean them gently removing the agar under the tap water (*see* **Note 11**).

3.6. Acclimatization and Field Transfer

1. Transfer the plantlets to the glass culture tubes containing tap water for in vitro hardening in such a way that only their root portion should remain inside the water (*see* **Fig. 3b**). Keep these plantlets in culture room for 10 d.
2. Transfer the in vitro hardened plantlets to glass house in earthen pots (*see* **Fig. 3c**) containing sand:soil in 2:1 ratio under 70–80% humidity by covering with glass beakers. The pots may alternatively be placed in a temporary poly house.
3. After 45–60 d as new leaves starts appearing transfer the plants to the field conditions.

Fig. 3. In vitro rhizogenesis and hardening in *R. serpentina* (**a**–**c**); rooted plantlets (**a**); in vitro hydroponic hardening (**b**) and in vivo hardening.

3.7. Assessment of Clonal Fidelity of Micropropagated Plants

3.7.1. Preparation of Extraction Buffers

1. Add calculated amount of Cetyl Trimethyl Ammonium Bromide (CTAB), NaCl, Ethylene diamine tetra acetic acid (EDTA), Tris–HCl and Polyvinyl pyrolidone (PVP), make the final volume by distilled water and warm at 55°C in water bath for 30 min.
2. Right before the use, add β-mercaptoethanol to the extraction buffer under fume hood (*see* **Note 8**).
3. Aliquot the desired amount of extraction buffer in to the Oakridge.

3.7.2. Isolation of Plant Genomic DNA

This is an adoption to the CTAB method modified by the Khanuja group *(7)*

1. Grind 1.0 g fresh young leaves of the test samples to a fine powder in liquid nitrogen.
2. Transfer the powdered leaf tissue to sterile oakridge tubes containing 4 mL freshly prepared extraction buffer, pre-warmed to 56°C. Shake the mixture to form a slurry (*see* **Note 12**).
3. Incubate the tubes at 60–65°C in a water bath for 1–2 h for cell wall lysis.
4. Add equal volumes of 4 mL chloroform: isoamyl alcohol (24:1) and gently mix by inversion for 10 min to form an emulsion.

5. Centrifuge the tubes for 10 min at 10,000 rpm and 25°C. Three layers will be formed: (a) a lower layer containing chloroform with dissolved proteins, lipids and chlorophylls; (b) a middle layer containing cell debris and dissolved green cell wall; and (c) an upper aqueous layer containing nucleic acid along with dissolved polysaccharides.
6. Separate the upper aqueous layer (approximately 4 mL) and transfer in to a fresh sterile oakridge tube (*see* **Note 13**) and add 1.5 mL 5 M NaCl solution and 3.3 mL (i.e., 0.6 volume of the total solution) of isopropanol. Gently mix by inversion and allow this mixture to stand for 1–2 h at room temperature. At this stage nucleic acid floating in the solution could be observed. This can either be scooped or alternatively could be centrifuged for 10 min at 10,000 rpm and 25°C.
7. Discard the supernatant and wash the pellet, containing the DNA, with 1 mL 80% ethanol by centrifugation for 5 min at 10,000 rpm and 25°C.
8. Discard the supernatant and dry the pellet under vacuum for 1–2 min to remove the traces of alcohol.
9. Dissolve the pellet into 1 mL high salt tris-ethylene diamine tatraacetic acid (TE) buffer. It may take some time to dissolve. Add 1 μL RNAse and incubate at 37°C in a water bath for 30 min.
10. Extract with equal volume 1 mL chloroform to remove the remaining proteins and other impurities by gently inversion and centrifugation for 10 min at 10,000 rpm and 25°C.
11. Transfer the upper aqueous layer approximately 1 mL to two fresh sterile Eppendorf tubes and add double volume of absolute ice cold ethanol and incubate at –20°C for 1–2 h for precipitation of DNA.
12. Centrifuge this mixture at 10,000 rpm for 10 min at 4°C. Discard the supernatant and wash the pallet with 80% ethanol at 10,000 rpm for 5 min at 4°C.
13. After vacuum drying dissolve the pellet in 100 μL sterile triple distilled/Milli-Q water and store at –20°C for further use.

3.7.3. Quantification of DNA

1. The DNA can be quantified by running on 0.8% agarose gel checking the absorbance at 260 nm (*see* **Note 14**).
2. Agarose Gel: Mix 2 mL 50X TAE buffer to a final volume of 100 mL. Add 800 mg agarsoe, boil, and cool to 50–60°C. Carefully add EB. Seal the free ends of gel tray, fix the combs, and dispense the molten gel. Allow it to solidify. Remove the comb and put the gel tray in the gel reservoir containing 1X TAE buffer. Make sure the gel is fully submerged (*see* **Notes 15** and **16**).

3. Mix the DNA sample, loading 6X dye and Milli-Q water (1 + 2 + 9 μL) by repeated pipeting. Load in the wells of gel carefully (*see* **Note 17**).
4. Close the lid of the gel reservoir and turn on the power supply. The gel runs from the – pole (black) towards the + pole (red). Check after few minutes if the gel is running. Check the gel regularly to prevent the samples from running off the gel.
5. For PCR, 20 ng amount of DNA is sufficient per reaction therefore dilution of DNA should be made with sterile Milli-Q water in such a way that 1 μL should contain approximately 20–25 ng of DNA (*see* **Note 18**).

3.7.4. PCR for DNA Amplification (8)

1. Amplification reaction was performed using Bio-Rad i-cycler version 4.006.
2. PCR is carried out in a total volume of 25 μL for each reaction in 0.2 mL PCR tube.
3. Set up the PCR reaction mixture (*see* **Table 5**) (*see* **Note 19**).
4. Taq polymerase should be added at the end.
5. All this should be carried out in ice.
6. Transfer this reaction mixture in PCR tube and spin it for few seconds for uniform mixing.

Table 5
Details of Setting up the PCR Reaction Mixture

Stock	Reagents	Final concentration	Volume (μL)/reaction
	Water		19.3
10X	Polymerase buffer	1X	2.5
10 mM	dATP	100 μM	0.25
10 mM	dGTP	100 μM	0.25
10 mM	dCTP	100 μM	0.25
10 mM	dTTP	100 μM	0.25
3 U/μL	Taq DNA polymerase	0.6 U	0.2
	Primer	5 pmole	1.0
	Genomic DNA	20–25 ng	1.0

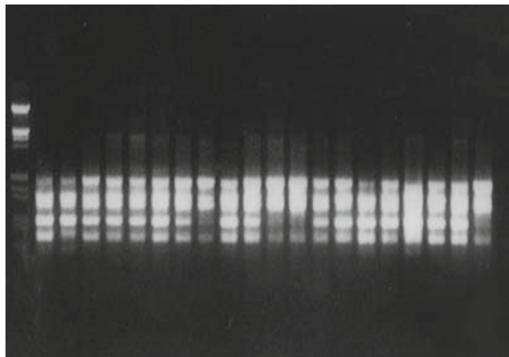

Fig. 4. RAPD profile of different samples of *R. serpentina* with primer OPA 13.

7. Carry out the PCR in the thermal cycler using the following conditions: (a) initial denaturation at 94°C for 5 min; (b) 45 cycles each consisting of a denaturation step at 94°C for 1 min, primer annealing step at 35°C for 1 min, amplification at 72°C for 2 min step; (c) final extension at 72°C for 5 min followed by arresting the reaction at 4°C for infinite period.
8. Load the amplified DNA on 1.4% agarose gel in 1X TAE buffer stained with 0.5 μg/mL EB. Photograph (*see* **Fig. 4**) on a gel documentation polaroid system.
9. Run the amplified products on the gel with molecular weight marker, λ Eco RI-Hind III digest.
10. The polymorphic bands were analyzed using image master 1-D elite software.
11. The similarity Index (SI) between two individuals = (2 × No. of common bands)/(Total No. of bands).
12. NTSys PC version 2.02j was used for analysis of the complete data set.
13. Similarity estimates were calculated by using Nei and Li coefficients *(9)* and cluster analysis was carried out by UPGMA method, Unweighted Pair Group Method Arithmetic Mean Averages.

3.8. Indole Alkaloids Analysis

3.8.1. Harvesting

1. Roots grow deeper inside the soil therefore plant is uprooted by digging out.
2. Wash the roots with tap water and allow drying under shade.

3.8.2. Extraction of Indole Alkaloids

1. Grind 1.0 g shade-dried roots of *R. serpentina* to powdered form.
2. Extract the powdered material 3× (3 × 10 mL) for 8 h with chloroform and methanol in 3:1 ratio at room temperature.

3. Pool the extracts and dry under vacuum, 417 bars at 40°C. in Rotavapor. R-144 (Buchi) to obtain a solid mass.
4. Re-dissolve the dried extract in small amount of chloroform and methanol (3:1) and transfer to small glass tube and allow the solvent to evaporate and dry in desiccators and store in refrigerator at 4°C. This extract can be used for quantitative analysis of the indole alkaloids through HPLC.

3.8.3. HPLC Analysis of Indole Alkaloids

1. Re-dissolve the dried extract in acidic methanol, methanol: HCl – 98: 2 *(v/v)* using ultra-sonication. The extract is equivalent to 1 g/mL on tissue dry weight basis.
2. Centrifuge the dissolved extract at 10,000 rpm for 30 min.
3. Prepare stock solutions of reference alkaloids i.e., ajmaline, ajmalicine and reserpine by adding 1 mg/mL of methanol separately.
4. Prepare a mixture of three alkaloids by adding the equal volume of each standard. The concentration of working solution of the standard solution is now 0.33 mg/mL.
5. Prior to injection, filter the samples and standards through 0.45 μm nylon HPLC millipore filter paper.
6. Quantitative estimation of ajmaline, ajmalicine and reserpine was carried out by reversed-phase HPLC gradient method using photodiode array (PDA) detection method *(10)*.
7. In, this protocol an analytical HPLC system consisted of a LC-20AD solvent delivery pumps, a DGU-20A_5 degasser, a CTO-20A column oven, 10 AF auto-sampler and a SPD-M 20A photodiode array detector was used. Data acquisition was performed on Lab Solution 3.21. The separation was achieved with a binary gradient program for pump A – acetonitrile, and pump B – 0.01 M phosphate buffer (NaH_2PO_4) containing 0.5% glacial acetic acid; pH 3.5.
8. A chromolith RP-18e HPLC column, 4.6 × 100 mm ID, is used for all the analysis.
9. Column temperature was maintained at 26 ± 2°C.
10. Analysis was performed at a flow rate of 1.0 mL/min through out the gradient run (*see* **Table 6**) and the data acquisition was performed at a wavelength of 254 nm.
11. Equilibrate the HPLC with mobile phase till the base line is established (*see* **Note 20**).
12. The identity of individual alkaloid in the sample run was confirmed by R_f comparison as well as UV-spectra match with the library of the standards maintained in the LC solution software. The area under respective peak were recorded (*see* **Fig. 5**) and used for percent content of reserpine, ajmaline and ajmalicine in the *R. serpentina* root sample.

Table 6
Gradient Program for the Separation of Test Indole Alkaloids

Time (min)	Acetonitrile concentration (%) (pump A)	Phosphate buffer concentration (%) (pump B)	Flow rate (mL/min)
0.01	15	85	1.0
9.00	15	85	1.0
9.01	25	75	1.0
10.00	25	75	1.0
10.01	30	70	1.0
12.00	30	70	1.0
12.01	35	65	1.0
30.00	35	65	1.0
50.00	15	85	1.0

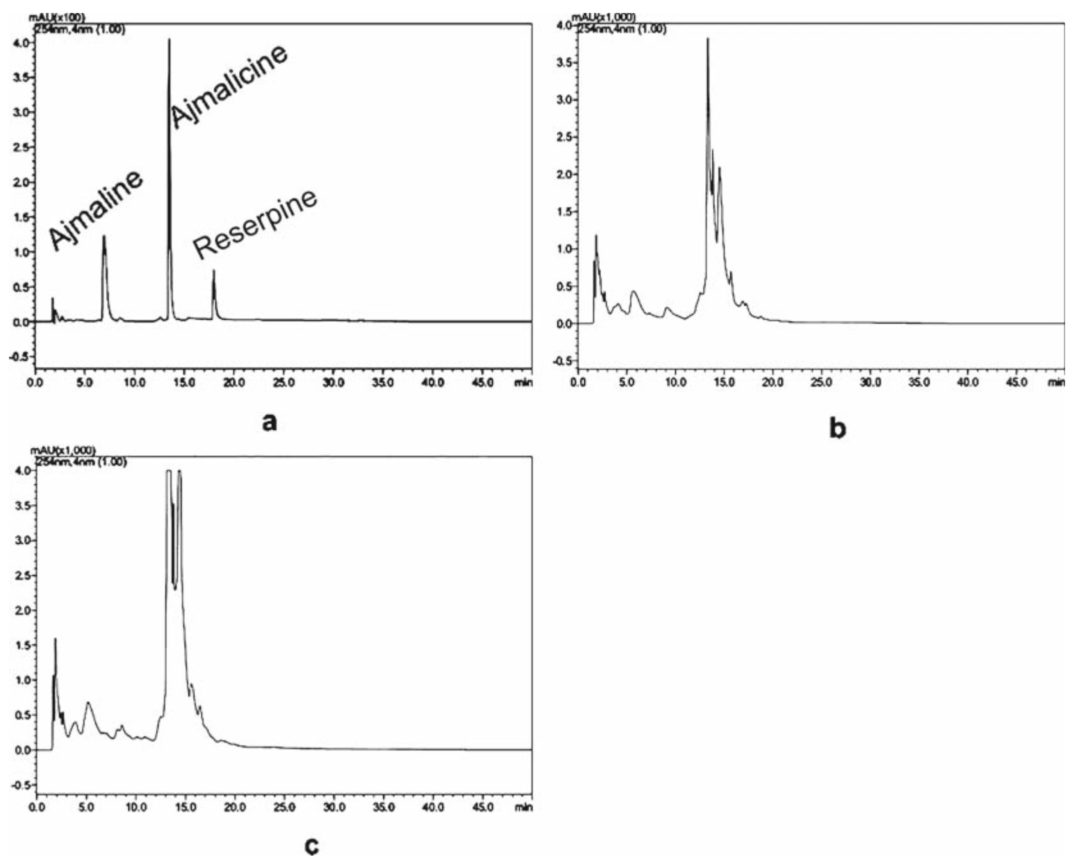

Fig. 5. Monolithic reverse phase HPLC separation of reserpine, ajmaline and ajmalicine in mixture of reference compounds (**a**) *R. serpentina* root sample of micropropagated plants showing ajmaline (**b**) and reserpine **(c)**.

13. The percent content on the basis of dry weight was calculated by using the area under respective peak in sample run and standard run.
14. The results were calculated using the following formula:

$$\%\text{content} = \frac{\text{Peak area (sample)}}{\text{Peak area (standard)}} \times \frac{\text{Concentration (standard)}}{\text{Concentration (sample)}} \times 100$$

4. Notes

1. Before using, soak all the glass wares overnight in chromic acid solution. Then clean with a liquid detergent and thoroughly wash with tap water to remove the last trace of detergent. Finally, rinse glass ware with double distilled demineralized water and dry in hot air oven at 150°C for 2 h.
2. An iron stock solution is prepared separately because of the problem of iron solubility. Usually the iron stock is prepared in a chelated form as Na Fe–ethylene-diamine tetraacetic acid (EDTA).
3. Dissolve $FeSO_4$ and Na EDTA separately in warm water, mix both solutions and make the final volume.
4. The MS basal salt mixture is also commercially available in powdered form.
5. Method for preparing liquid and solid medium is almost same; the only difference is presence or absence of agar.
6. For initial establishment culture tubes are preferred to avoid cross contamination.
7. Regularly check the air flow gauge of laminar air flow chamber. After regular intervals clean the pre-filters of the laminar air flow by washing followed by vacuum cleaning. Switch on the UV light for 30 min. Before starting any activity in the laminar air flow bench, wipe the surface of the laminar air bench with alcohol frequently during any aseptic operation. Fumigate the entire transfer room.
8. Avoid contact of body parts with $HgCl_2$, β-merceptoethanol, and EB as all these chemicals are highly mutagenic, carcinogenic and hazardous chemicals.
9. Possible source of danger exists if a person, after flaming an instrument reinserts the hot instrument into the alcohol dip. **CAUTION:** Ethanol is inflammable! One should be very careful.
10. UV irradiation may also cause some serious health risks.
11. In vitro roots are delicate do not let them break.

12. Mix extraction buffer and samples quickly; do not let the samples thaw.
13. Cut the tip to widening the orifice of pipette enables the easy suction and separation of upper fraction without causing any disturbance to the lower layer.
14. Alternatively DNA yield may also be also checked spectrophotometrically.
15. While preparing the agarose gel, take care that final volume should never be reduced due to evaporation during boiling. Take care do not entrap any air bubble.
16. Clean the gel tray, gel reservoir, combs and other materials with ethanol properly before and after use.
17. While loading the sample, do not let the sample spill out. Wear gloves during the entire operation and prevent contamination.
18. Pay attention to the quality of DNA-bad when there are many small fragments; and loading the amount of RNA – high when there is much smear above sample on the gel.
19. To minimize the error and for convenience mix all the dNTPs in equal amount and make a stock in advance, and then take 1 μL for each PCR reaction.
20. The method used for the analysis is precise, accurate, robust and validated. The general steps of chromatography should be followed. First of all the loop of the auto-sampler should be cleaned with at least 100 μL methanol three times and the system should be allowed to run at least for 30 min to achieve minimum noise signals.

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