Chapter 17

Scale-Up of *Agrobacterium rhizogenes*-Mediated Hairy Root Cultures of *Rauwolfia serpentina*: A Persuasive Approach for Stable Reserpine Production

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Abstract

Roots of *Rauwolfia serpentina*, also known as "Sarpagandha" possess high pharmaceutical value due to the presence of reserpine and other medicinally important terpene indole alkaloids. Ever increasing commercial demand of *R. serpentina* roots is the major reason behind the unsystematic harvesting and fast decline of the species from its natural environment. Considering *Agrobacterium rhizogenes*-mediated hairy root cultures as an alternative source for the production of plant-based secondary metabolites, the present optimized protocol offers a commercially feasible method for the production of reserpine, the most potent alkaloid from *R. serpentina* roots. This end-to-end protocol presents the establishment of hairy root culture from the leaf explants of *R. serpentina* through the infection of *A. rhizogenes* strain A4 in liquid B5 culture medium and its up-scaling in a 5 L bench top, mechanically agitated bioreactor. The transformed nature of roots was confirmed through PCR-based *rol* A gene amplification in genomic DNA of putative hairy roots. The extraction and quantification of reserpine in bioreactor grown roots has been done using monolithic reverse phase high-performance liquid chromatography (HPLC).

Key words Bioreactor, Hairy roots, HPLC, *Rauwolfia serpentina*, Reserpine, Up-scaling

1 Introduction

Rauwolfia serpentina, a distinguished member of family Apocynaceae, has drawn special attention all over the world because of its high medicinal importance. The plant is known to produce medicinally important terpenoid indole alkaloids (TIAs) such as reserpine, ajmalicine, ajmaline, serpentine, vomiline, and yohimbine. [1]. Among other TIAs, reserpine, a 3,4,5-trimethyl benzoic acid ester of reserpic acid, is the most potent alkaloid with remarkable antihypertensive properties. The alkaloids are concentrated mostly in the plant roots, reported to yield about 85–90 % of the total alkaloid content of the whole plant [2]. Presence of these

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TIAs contributes to the medicinal properties of *Rauwolfia* roots which are strong antihypertensive, laxative, anthelmintic, and diuretic in nature. The root extract is present as a major ingredient in a variety of commercially available drugs that are used for the treatment of hypertension, high blood pressure, mental illness, and problems related to central nervous system (CNS). The crude extract is also used in sedatives, aphrodisiac and antispasmodic medicines. Further, hypoglycemic and hypolipidemic activities are also reported from the root extract of other species of *Rauwolfia* [3]. The only source for the procurement of *Rauwolfia* roots for medicinal purposes is the natural reserves which due to unsystematic exploitation are fast declining. The International Union for the Conservation of Nature and Natural Resources (IUCN) enlisted this species among those endangered plants for which cultivation and conservation are prioritized through conventional and alternative methods in India (NMPB; www.nmpb.nic.in).

The *Agrobacterium rhizogenes*-mediated hairy root cultures are known as an alternative source for the production of plant-based secondary metabolites under laboratory conditions [4]. Regular demand of *R. serpentina* roots for commercial medicinal uses and limited supply from natural resource have laid the background for the establishment and use of *A. rhizogenes*-mediated hairy root cultures (HRCs) for TIA production [5, 6]. The hairy root cultures of various species of *Rauwolfia* represent a rich repository of a range of alkaloids as they produce noticeable amounts of these TIAs [6]. These easily manageable hairy root cultures can be a promising source for the production of reserpine and other valuable alkaloids. In the development and progress of techniques for commercial production of hairy root-based secondary metabolites, the up-scale culture of roots in bioreactors is the definitive step. Keeping the pharmaceutical importance of reserpine in mind, the present protocol was developed to establish *Agrobacterium rhizogenes*-mediated *R. serpentina* hairy root cultures for high reserpine biosynthesis and up-scaling of these hairy root cultures in a bench top, mechanically agitated 5 L capacity bioreactor. Before up-scaling in bioreactor, the transformed nature of hairy root line was confirmed through PCR based *rol* A gene amplification. The accumulation of reserpine was detected by high performance liquid chromatography.

2 Materials

2.1 Media Preparation

1. Stock solutions of Gamborg's B5 and Murashige and Skoog (MS) medium (*see* Table 1) (*see* **Notes 1** and **2**).
2. Agar.
3. Beakers, measuring cylinders, and glass rods (*see* **Note 3**).

Table 1
Components of MS and B5 growth medium

Designated stock	Constituents of stock	Concentration (mg/L)	
		MS	B5
A	NH_4NO_3	1650	–
	$(NH_4)_2SO_4$	–	134
B	KNO_3	1900	2500
	$MgSO_4{\cdot}7H_2O$	370	250
	$MnSO_4{\cdot}4H_2O$	22.3	10
C	$ZnSO_4{\cdot}7H_2O$	8.6	2
	$CuSO_4{\cdot}5H_2O$	0.025	0.025
	$CaCl_2{\cdot}H_2O$	440	–
	KH_2PO_4	170	150
D	NaH_2PO_4	–	3
	H_3BO_3	6.2	0.75
	KI	0.83	0.25
	$Na_2MoO_4{\cdot}2H_2O$	0.25	0.025
	$CoCl_2{\cdot}6H_2O$	0.025	–
F	$FeSO_4{\cdot}7H_2O$	27.85	27.85
	Na_2EDTA	37.35	37.35
	Thiamine HCl	0.1	10
	Pyridoxine HCl	0.5	10
G	Nicotinic acid	0.5	1
	Glycine	2	–
	Folic acid	–	–
	Biotin	–	–

4. 0.1–10 ml pipettes and/or 0.5–100 µl micropipettes.
5. 1 N NaOH/1 N HCl.
6. 250 ml narrow-neck Erlenmeyer flasks.

2.2 Bacterial Stock Culture and Suspension

1. *Agrobacterium rhizogenes* strain A4 (pRA4).
2. Yeast Mannitol Broth (YMB) and Yeast Mannitol Agar (YMA) [7] (Table 2 *see* **Note 4**).
3. Disposable petri plates (90 mm).

Table 2
Composition of YMA/YMB

Constituents	Concentration (g/L)
KH_2PO_4	0.5
$MgSO_4 \cdot 7H_2O$	2
NaCl	0.1
Mannitol	10
Yeast extract	0.4
Agar-agar	1.50 %

4. Streaking loops.
5. Sample tubes with screw top (10 ml;) (*see* **Note 5**).

2.3 Infection, Co-cultivation, and Establishment of Hairy Root Cultures

1. Culture medium (semisolid MS in petri plates and liquid B5 in culture flasks).
2. In vitro maintained 6–8-week-old stock multiple shoot cultures of *R. serpentina* [8] (*see* **Note 6**).
3. Disposable syringes (1 ml).
4. Sterile distilled water.
5. Autoclaved scissors and 8–12″ rust-proof stainless steel forceps.
6. Sporidex antibiotic (Ranbaxy; India).

2.4 Confirmation of Transformed Nature of Hairy Roots

2.4.1 Isolation and Quantification of Genomic DNA of Normal and Putative Hairy Roots

1. Mortar and pestle.
2. Liquid nitrogen.
3. Autoclaved eppendorf tubes.
4. Buffers (Table 3, *see* **Notes 7** and **8**).
5. Chloroform–isoamyl alcohol (24:1; *v*/*v*).
6. 5 M NaCl in water.
7. Isopropanol (Propane-2-ol).
8. Desiccators with vacuum pump.
9. Milli-Q water.
10. Ethidium bromide stock (1 %) in water.
11. Agarose.
12. 6× loading dye (*see* **Note 9**).

2.4.2 Polymerase Chain Reaction (PCR) for Gene Amplification

1. PCR tubes (*see* **Note 10**).
2. Template DNA.
3. PCR master mixture (2×, Fermentas) (*see* **Note 11**).

Table 3
Composition of buffers

Extraction buffer	High salt TE	TAE buffer (50×; 1 L)
1.4 M NaCl	1 M NaCl	0.5 M EDTA (100 ml)
100 mM Tris–HCl	10 mM Tris–HCl	Tris base 242 g
20 mM EDTA	1 mM EDTA	57.1 ml Glacial acetic acid
2.5 % CTAB		
0.2 % β-mercaptoethanol		
1 % PVP		

4. Gene specific (*rol A*) primer(s). In the present study, to confirm the transformed nature of roots, *rol* A amplification is performed. However, for this purpose any of the *rol* genes (A, B, C) or even T_L sequence (left border sequence from bacterial plasmid which is essential for hairy root induction) from the bacterial origin can be amplified in host genome with their respective primers. Primer sequence for *rol* A:

   Forward 5′-GGAATTAGCCGGACTAAACG-3′ and

   Reverse 5′-CCGGCGTGGAAATGAATCG-3′
5. Marker DNA (Quick load 100 bp, NEB)
6. Milli-Q water.
7. Ice bath.
8. Thermal power PCR cycler (iCycler).

2.5 Up-Scaling of Hairy Roots in Bioreactor

1. Bioreactor setup (in the present protocol, model Bio flow 3000, M/s New Brunswick Scientific, USA; modified air lift with top driven mechanical agitation through marine blade impeller is used). The reactor setup is also fitted with probes for dissolved oxygen (DO), pH, and temperature.
2. 4–6-week-old stock hairy root cultures maintained in shake flasks [6] (*see* **Note 12**).
3. Gamborg's Liquid B5 culture medium.
4. Ethanol for surface sterilization.
5. Sterile forceps.

2.6 Chemical Analysis

2.6.1 Extraction of Alkaloids and High-Performance Liquid Chromatography for Reserpine

1. Dried root samples.
2. Chloroform–methanol, 3:1 (*v*/*v*).
3. Distilled water.
4. Hydrochloric acid.
5. Rotavapor R-144 (Buchi).
6. RP-18e Chromolith HPLC Columns (4.6×100 mm).

7. Glacial acetic acid.
8. Di-sodium-di-hydrogen orthophosphate.
9. HPLC-grade acetonitrile (Merck; Darmstadt, Germany)
10. HPLC-grade water (Sigma-Aldrich, USA).
11. Standard reserpine (Sigma-Aldrich, USA).

3 Methods

3.1 Preparation of Medium

1. Use MS and B5 stock solutions to prepare the culture medium (*see* **Notes 1** and **2**; Table 1)
2. For semisolid medium melt the calculated amount of agar separately. Normally 7.5–8 % agar is used in the protocol or unless otherwise stated.
3. Mix the required volume of stock solutions, Sucrose (30 g/l) and myoinositol (0.1 g/l) medium. In the case of semisolid medium add the aforementioned to the molten agar by stirring. In the case of liquid B5 medium do not add agar. Make the final volume of culture medium as per requirement.
4. Adjust the medium pH at 5.86 ± 0.02 with the help of 1 N NaCl/1 N HCl.
5. Dispense the medium in appropriate culture vessels. Use cotton plugs to seal the vessels.
6. Sterilize the medium at 121 °C at 15 lb pressure for 15–20 min.
7. Pour lukewarm semisolid MS medium in petri plates and allow it to solidify under laminar air flow.
8. Store the culture medium at 25 °C.

3.2 Maintenance of Stock Bacterial Culture and Preparation of Bacterial Suspension

1. Prepare the stock bacterial culture by streaking single cell bacterial colonies on semisolid YMA and incubate the cultures at 28 ± 2 °C. New bacterial colonies develop within 48 h incubation. Store the cultures at 4 °C and maintain the stock cultures by regular subculturing on to the same medium at every 6–8 week interval (*see* **Note 13**).
2. Take 5 ml liquid YMB medium in sterile sample tubes.
3. Inoculate the medium with single cell colonies from stock bacterial cultures with the help of sterile streaking loops.
4. Incubate the culture at 28 ± 2 °C; 75–80 rpm on a rotary shaker for 48 h (*see* Fig. 1a).

3.3 Infection and Co-cultivation of Leaf Explants

1. Excise juvenile (3–5-week-old) leaf explants from in vitro maintained shoot cultures of *R. serpentina* under the laminar flow.

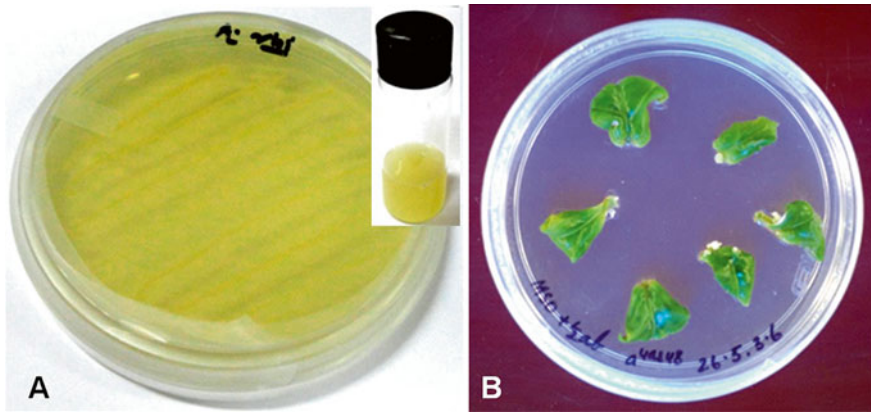

Fig. 1 Stock bacterial culture (**a**; *inset* 48-h-old suspension culture); Co-cultivation in leaf explants (**b**)

2. Prick the leaf explants with sterile needle dipped in 48 h-old bacterial suspension (*see* **Note 14**).
3. Leaf explants pricked with the sterile needle dipped in sterile water can be treated as control.
4. Place the infected explants on MS medium in petri plates for co-cultivation.
5. Incubate the petri plates in 40 μmol/m^2/s light intensity at 25 ± 2 °C.
6. Check the excessive growth of bacteria by transferring the explants on MS medium containing antibiotic (Sporidex, 1 mg/ml culture medium; *see* **Note 15**) after every 2–3 days till the vestige bacterial growth disappears (*see* Fig. 1b).

3.4 Disinfection and Establishment of Root Cultures

1. Within 12 days of infection roots emerge at infection sites of leaf explants. Single root emerging from an infection site can be considered as an individual putative hairy root line (*see* Fig. 2a).
2. Excise the individual putative hairy roots (≥1 cm) emerging from the leaves.
3. Inoculate individual root lines separately on B5 semisolid medium containing antibiotic.
4. Incubate the petri plates in light at 25 ± 2 °C (*see* **Note 16**).
5. Upon growth, transfer the root lines individually (approximately 200 mg fresh tissue weight) in 250 ml Erlenmeyer flasks containing 30 ml liquid B5 growth medium. Incubate the liquid cultures on a rotary shaker at 60 rpm; 25 ± 2 °C in continuous light (*see* Fig. 2b).
6. Maintain the root cultures in shake flasks through regular subculturing at every 6-week interval in the same medium and similar culture conditions.

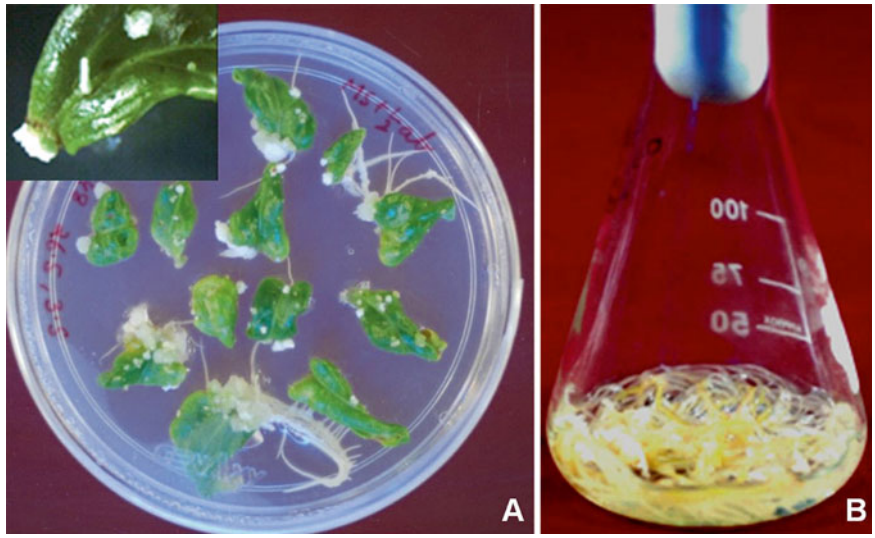

Fig. 2 Emergence of roots at infection sites of leaf explants (**a**; *inset* emergence of single root line); axenic root culture in liquid B5 medium (**b**)

3.5 Confirmation of Transformed Nature of Roots

3.5.1 Isolation of Genomic DNA from Different Root Lines

1. Isolation of genomic DNA is done by modified CTAB method [9].
2. Prepare the extraction buffer by mixing calculated amounts of cetyl trimethyl ammonium bromide (CTAB), 5 M NaCl, ethylene diamine tetraacetic acid (EDTA; pH 8.0), Tris–HCl (pH 8.0), and polyvinyl pyrrolidone (PVP) (*see* Table 3).
3. Make the final volume by adding sterile distilled water and warm the extraction buffer at 60 °C in water bath for 30 min.
4. Add β-mercaptoethanol to the extraction buffer prior to use (*see* **Note 17**).
5. Grind approximately 0.2–0.5 g fresh root tissue of putative root lines (six in present case) along with normal roots grown in vitro (control) to a fine powder in liquid nitrogen.
6. Immediately transfer the powdered tissue to sterile eppendorf tubes (1.5 ml micro centrifuge tubes) containing pre-warmed (55–60 °C) 1 ml extraction buffer (*see* **Note 18**). Inversely mix or gently shake the mixture to form slurry.
7. Incubate them at 60–65 °C in a water bath for 1 h for lysis of plant cell. Mix regularly at every 20 min interval.
8. Add equal volumes of chloroform–isoamyl alcohol (24:1) and gently mix by inversion for 10 min to form an emulsion.
9. Centrifuge the tubes for 10 min at 10,000×*g* at 25 °C.
10. Pipette out the upper aqueous layer (~800 μl) containing mostly nucleic acid.

11. Add 5 M NaCl (300 µl) solution and propanol-2-ol (0.6 volume of the total solution). Gently mix by inversion and allow this mixture to stand for 1–2 h at room temperature.
12. Centrifuge for 10 min at 10,000 rpm at 25 °C.
13. Discard the supernatant and wash the pellet with 80 % ethanol by centrifugation for 5 min at 10,000×*g* at 25 °C. Discard the supernatant and dry the pellet under vacuum for 1–2 min to remove the traces of alcohol.
14. Dissolve the pellet in high salt TE (400 µl) buffer (*see* Table 3). It may take some time to dissolve.
15. Add RNAse A (5 µl) and incubate at 37 °C in a water bath for 30 min.
16. Extract with equal volume chloroform–isoamyl alcohol (24:1) to remove the remaining proteins and other impurities by gentle inversion and centrifugation for 10 min at 10,000×*g* at 25 °C.
17. Transfer the upper aqueous layer to sterile eppendorf tube and add double volume of ice cold ethanol. Incubate at –20 °C for 1–2 h for the precipitation of DNA.
18. Centrifuge this mixture at 10,000×*g* for 10 min at 4 °C. Discard the supernatant and wash the pellet with 80 % ethanol at 10,000×*g* for 5 min at 25 °C.
19. After vacuum drying dissolve the pellet in 50 µl Milli-Q water and store at –20 °C for further use.

3.5.2 Quantification of DNA and PCR Amplification

1. The DNA yields can be measured either by spectrophotometer (at 260 nm) or by agarose gel (0.8 %) electrophoresis and its visualization under transilluminator (Preparation of agarose gel is described below. For loading of gel *see* **Note 19**; Fig. 3a).
2. To use appropriate amount of sample DNA and ease of pipetting, the DNA dilutions are required. Normally, single PCR reaction mix requires 20–25 ng of DNA. The 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.
3. Polymerase chain reaction: Prepare the PCR reaction mixture by adding sample DNA (1 µl), forward and reverse *rol* A gene primers (1 µl each from 10 pM stock), PCR master mix (12.5 µl). Make up the volume to 25 µl by adding Milli-Q water (*see* **Note 20**).
4. Prepare the PCR reaction mixture on ice bath.
5. Spin the PCR tube containing PCR mix for few seconds for proper mixing.
6. Place the tubes in thermal cycler for amplification. Set the conditions for amplification as:

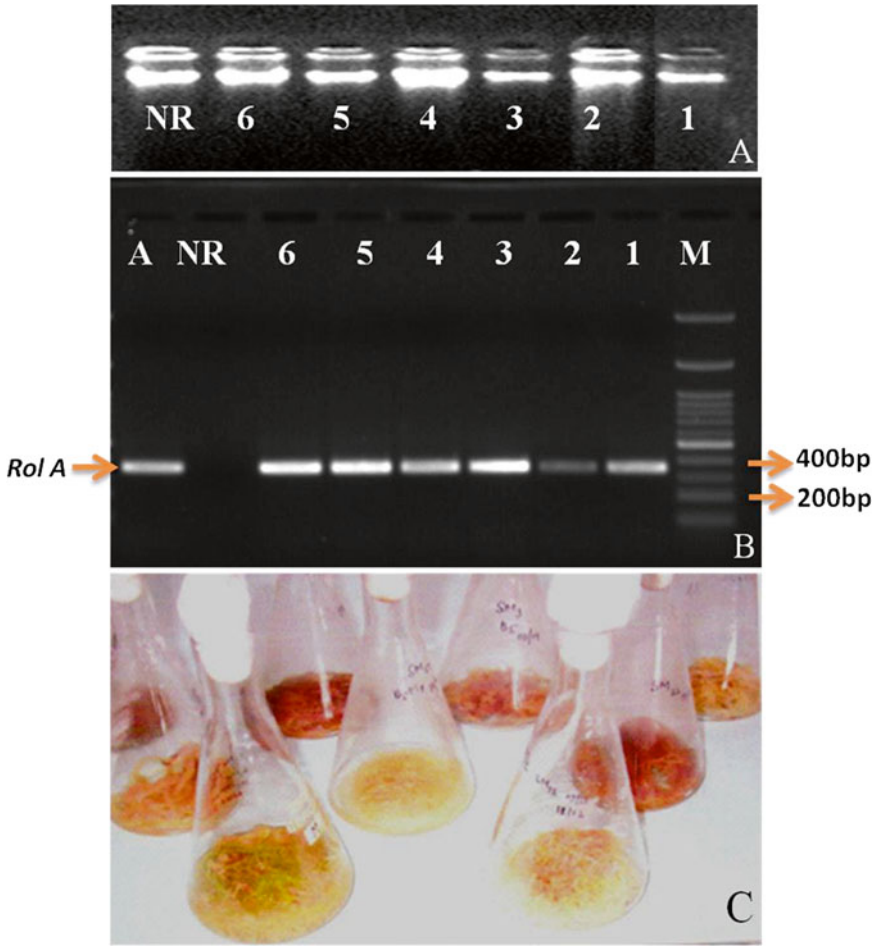

Fig. 3 Quantification of DNA obtained from normal roots and putative hairy root lines 1–6 (**a**); amplification of *Rol* A gene in genomic DNA of *A. rhizogenes* strain A4 (**b**), normal roots (NR) and hairy root lines 1–6. M = marker DNA; hairy root cultures of *R. serpentina* (**c**)

(a) Initial denaturation at 94 °C for 5 min;

(b) 35 cycles each consisting of a denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, amplification at 72 °C for 2 min;

(c) Final extension at 72 °C for 5 min followed by storage of reaction at 4 °C for infinite period.

7. Gel electrophoresis: To prepare agarose gel (0.8–1.2 %), add measured quantity of agarose powder to the required volume of 1× TAE buffer. Boil and gently swirl the solution till agarose get completely melts. Allow it to cool to 45–50 °C. Add EtBr when the temperature of gel remains lukewarm (*see* **Notes 21** and **22**).

8. In a pre-prepared gel casting tray fitted with desired comb, pour the molten agarose gently by avoiding any bubble formation. Allow the gel to solidify. As the gel solidifies carefully remove the comb and submerge the gel tray in the gel reservoir containing 1× TAE buffer.
9. Load the amplified DNA on 1.2 % agarose gel stained with 0.5 μg/ml EtBr in 1× TAE buffer (*see* **Note 23**).
10. Turn on the power supply. Take note of polarities (red-to-red, black-to-black) as the DNA in gel runs from negative to positive end (*see* **Note 24**). Run the loaded gel for 2 h and visualize the bands on a gel documentation system (*see* Fig. 3b).

3.6 Up-Scaling of Hairy Roots in Bioreactor

3.6.1 Bioreactor Setup and Culture

1. The bioreactor used for the present study is of 5 L capacity, modified air lift type. The thick glass culture vessel consists of top driven mechanical agitation through marine blade impeller.
2. The culture vessel is fitted with probes for dissolved oxygen (DO), pH, and temperature to control and optimize the respective culture conditions.
3. In this protocol, an autoclavable nylon mesh (pore size 200 μm) is manually fabricated in the center of the vessel that divides the culture vessel into two halves. In upper half of the vessel, mesh provides anchorage surface to the inoculated and growing tissue and rescues them from getting sunk in the medium during growth phase. The lower half of the vessel consists of a single sparger and a marine blade impeller provided for aeration and agitation respectively (*see* Fig. 4).
4. A hydrophobic membrane filter (Whatman, USA; 0.22 μm) is used to pass the influent air from sparger into the medium.
5. All the parts of the reactors are thoroughly washed and then surface-sterilized with ethyl alcohol prior to assembling the unit and lubricated with silicon grease to make the unit airtight.
6. Pour the liquid B5 medium supplemented with 3 % *w/v* sucrose into the assembled reactor culture vessel.
7. In present protocol, the complete air tight unit filled with growth medium (2.5 L; pH adjusted to 5.86 ± 0.02) is sterilized by autoclaving at 120 °C and 15 lbs pressure for 15 min.
8. The sterile culture vessel is inoculated on a clean laminar bench provided with HEPA filters with 5–6-week-old hairy root cultures (*see* **Note 25**). A fast growing hairy root line (whose transformed nature has been confirmed through *rol* gene amplification) is selected for the cultivation in reactor.
9. The impeller speed is maintained at 75 rpm throughout the culture duration. The experiment is conducted at 25 ± 2 °C in continuous light provided externally by compact fluorescent lamps.

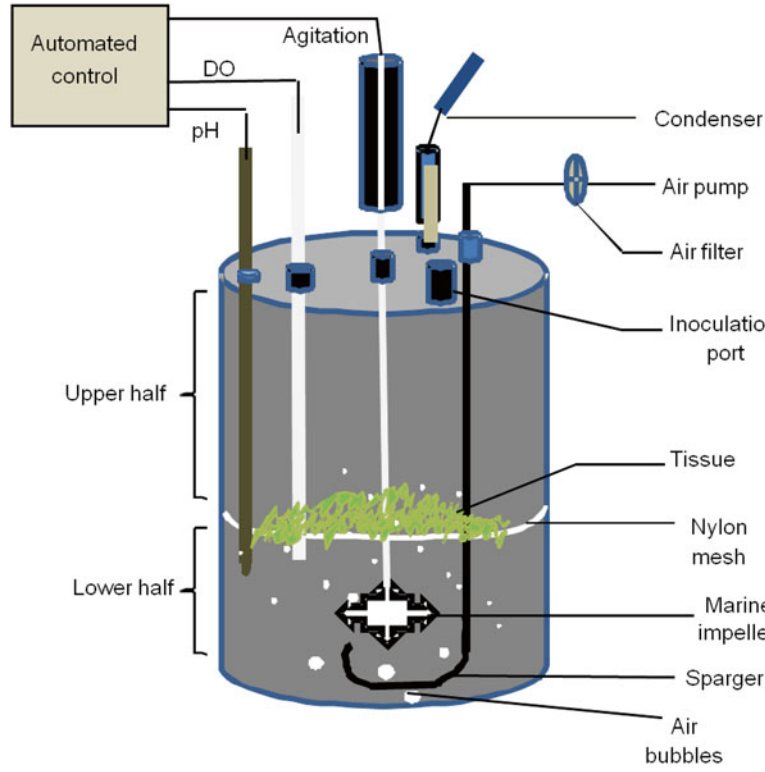

Fig. 4 Diagrammatic presentation of reactor configuration

10. The bioreactor is operated for 11 weeks. During this culture period rapid growth of roots resulted in the formation of root clumps (*see* Fig. 5a; [10]). This has led to the depletion of dissolved oxygen in the medium and an increased oxygen demand. Therefore, the air supply is increased from 0.25 to 1.0 L/min.

3.6.2 Harvesting of Roots

1. Harvest the cultured roots from the reactor vessel (*see* Fig. 5b). Carefully separate the thick clumps of roots from the baffle assembly. Wash the roots in running tap water to remove the traces of medium.
2. Dry the roots on filter paper towel to remove excess water.
3. Dry the harvested roots in hot air oven at 50 °C.

3.7 Chemical Analysis

3.7.1 Extraction of Indole Alkaloids and HPLC Analysis

1. Grind 1.0 g oven-dried *R. serpentina* hairy roots into fine powder.
2. Extract the root powder thrice with (3 × 10 ml) chloroform and methanol (3:1) over night at room temperature.
3. Pool the extracts and dry under vacuum, 417 bars at 40 °C in a Rotavapor (R-144) (Buchi).

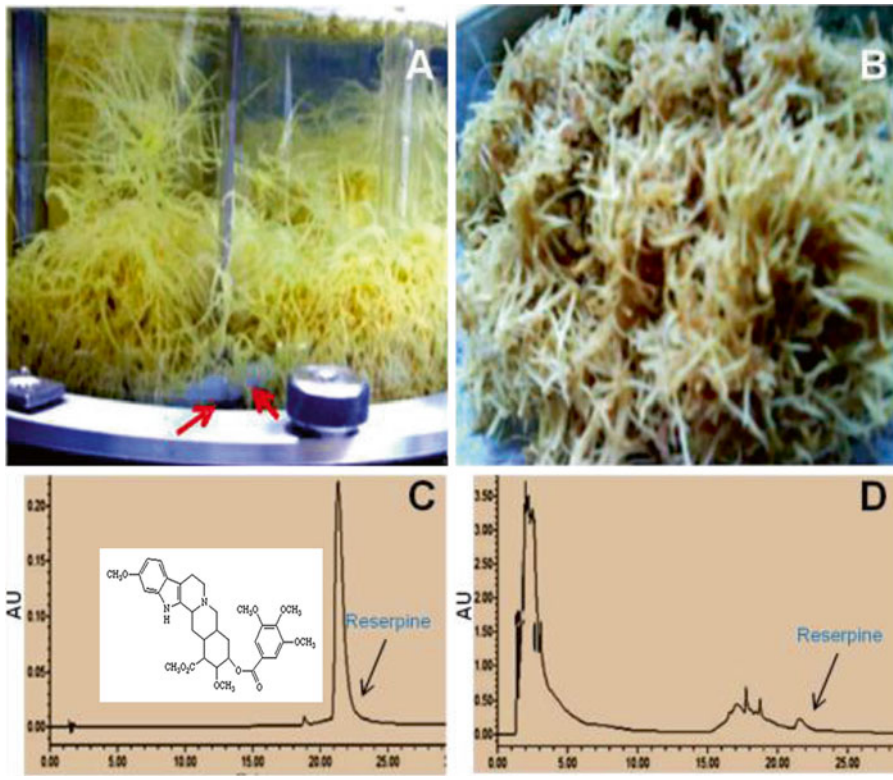

Fig. 5 Growth performance of *R. serpentina* hairy root culture in bioreactor. Dense root growth during culture (**a**), *arrows* indicate the position of nylon mesh; Hairy root biomass harvested after 11 weeks of culture (**b**); HPLC chromatogram of standard reserpine (**c**) and in hairy roots grown in bioreactor (**d**)

4. Redissolve 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 reserpine and other indole alkaloids through HPLC.
5. Before analysis redissolve the dried extract in acidic methanol i.e., methanol:HCl—98:2 (*v*/*v*) through ultrasonication. Centrifuge the dissolved extract (equivalent to 1 g/ml on tissue dry weight basis) at 10,000 rpm for 30 min.
6. Stock solution of standard reserpine: dissolve 1 mg standard reserpine in 1 ml of methanol.
7. Filter the sample extract and standard reserpine solutions through HPLC millipore filter paper (0.45 mm).
8. Analyze the samples through an analytical HPLC system. In present method, the HPLC system consists of a LC-20 AD solvent delivery pump, a DGU-20A 5 degasser, a CTO-20A column oven, a 10AF auto sampler, and a SPD-M 20A photodiode array detector.

9. Quantitative estimation of reserpine is done using reversed-phase HPLC gradient method with photodiode array (PDA) detection method.
10. A gradient program (pump A acetonitrile and pump B 0.01 M phosphate buffer containing 0.5 % glacial acetic acid; pH 3.5) is used. The samples are run at a flow rate of 1.0 ml/min at 26 ± 2 °C. A chromolith RP-18e HPLC column, 4.6 × 100 mm, is used for all the analyses.
11. Data acquisition is performed on Lab Solution 3.21 at a wavelength of 254 nm.
12. The reserpine identity in the sample run is confirmed by R_f comparison. The area under respective peak is recorded and used for percent content of the alkaloid in the *R. serpentina* hairy root sample (*see* Fig. 5c, d).

4 Notes

1. Store the stock solutions of basal growth medium in refrigerator. Avoid contamination in stock solutions while using. Always prepare iron stock by dissolving $FeSO_4$ and Na EDTA separately to overcome problem of iron insolubility. Na EDTA is used to get iron in chelated form (Na Fe–ethylene-diamine tetraacetic acid).
2. Prepare the stock solutions of the growth medium using the following equation:

   $N_1V_1 = N_2V_2$

   Where: N_1 = concentration of stock solution

   N_2 = required concentration of the solution in the medium

   V_1 = volume of the stock solution needed

   V_2 = final volume of the medium
3. All glassware should be cleaned with a liquid detergent and thoroughly washed with tap water. Rinse the glassware with double distilled water and dry in hot air oven at 150 °C for 2 h before use.
4. Alternatively, this can be prepared by the Yeast Mannitol Agar/Broth (YMA/YMB) salt mixture which is also commercially available. Adjust the medium's pH at 7.0.
5. As an alternative to sample tubes, test tubes can also be used to prepare bacterial suspension.
6. Multiple shoot cultures of *R. serpentina* can be established and maintained easily in hormone-supplemented MS medium under standard in vitro conditions [8].
7. Add water to make the required volume of 1 × TAE buffer. Maintain the buffer at pH 8.0.

8. Use autoclaved bottles to store the TE and TAE buffers to avoid any contamination during DNA isolation and gel electrophoresis.
9. Prepare 6× loading dye by mixing glycerol (30 % in water), 0.25 % bromophenol blue, and 0.25 % xylene cyanol. Store the dye at –20 °C.
10. Autoclave the PCR tubes prior to use to avoid any contamination during the procedure as contamination at this stage may affect the results.
11. Commercially available PCR master mix is a 2× concentrated mixture of *Taq* DNA polymerase, dNTPs, and all other components required for PCR, except DNA template and primers. Use of readymade PCR master mix reduces contamination and handling errors during pipetting. Individual components can also be mixed separately to prepare PCR reaction mix with Milli-Q water.
12. Morphological variations can be seen in different hairy root clones of *R. serpentina* [10]. Any visibly healthy (free from callus and browning) and fast growing hairy root line can be selected for up-scaling.
13. Careful subculturing and maintenance of bacterial stock culture is required as these cultures get easily contaminated which ultimately lead to the loss of original culture. Ensure the maintenance of original bacterial culture by cross-checking the presence of *rol* gene through PCR.
14. While pricking the leaf explants care should be taken. A slight break in epidermal layer of leaf surface would be sufficient for bacterial cells to infect and transform wounded host cells. Little extra pressure during pricking can result in the death of leaf cells which ultimately leads to an unsuccessful exercise.
15. Dissolve the powder content of a 500 mg Sporidex capsule in 10 ml distilled water (50 mg antibiotic in 1 ml water). Dissolve it and filter through Whatman filter paper. Filter-sterilize the antibiotic solution under laminar flow with the help of syringe filters before mixing in the medium. Store the sterile antibiotic solution in sterile tubes in refrigerator.
16. After the establishment of axenic root cultures, gradually remove the antibiotic from the culture medium.
17. β-mercaptoethanol is added to the extraction buffer just before use. Care should be taken while using the chemical as it is considered toxic.
18. Avoid delay in transferring the powdered tissue to the extraction buffer. Do not let thawing of tissue.
19. Loading of gel for DNA quantification: Mix well the DNA sample (2 μl), loading 6× dye (2 μl), and Milli-Q water (8 μl). Load the 12 μl mixture in the wells of gel carefully.

20. Alternatively, the PCR reaction mix can be prepared by adding individual components. For this, add sample DNA (1 μl), 10× Reaction buffer (2.5 μl), 10 mM dNTPs (1 μl, i.e., 0.25 μl each dNTP), reverse and forward primers (1 μl of 10 pM stock), and *Taq* DNA polymerase (0.2–0.3 μl). Make up the volume to 25 μl by adding Milli-Q water.
21. There can be a significant loss of solution volume due to evaporation of water while melting the agarose. This can cause change in the concentration of agarose as well as will increase the concentration of buffer. The simplest way to get rid of this problem is to replenish the amount of water lost by evaporation.
22. The final concentration of ethidium bromide in gel should be 0.5 μg/ml.
23. Avoid contact of body parts with β-mercaptoethanol and EtBr. These chemicals are highly mutagenic and carcinogenic.
24. Check the gel regularly to prevent the samples from running off the gel.
25. Before inoculating the reactor vessel switch on the UV light for 30 min. and surface-sterilize the laminar bench with alcohol. Wipe the head plate of reactor with alcohol and inoculation port should also be heated to avoid contamination while inoculation. Care should also be taken while aseptic transfer of root tissue as any contact with inoculation port can damage the tissue due to heat.

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