Protocols for In Vitro Propagation, Conservation, Synthetic Seed Production, Microrhizome Production, and Molecular Profiling in Turmeric (Curcuma longa L.)

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Chapter 27

Protocols for In Vitro Propagation, Conservation, Synthetic Seed Production, Microrhizome Production, and Molecular Profiling in Turmeric (*Curcuma longa* L.)

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Abstract

Turmeric is a rhizomatous herbaceous perennial but cultivated as annual, belonging to the family Zingiberaceae. It is a native of India and South East Asia. The tuberous rhizomes or underground stems of turmeric are used from antiquity as condiments, a dye and as an aromatic stimulant in several medicines. Turmeric is an important crop in India and it is used as a spice, food preservative, coloring agent, cosmetic as well as for its medicinal properties. Propagation is done vegetatively with rhizome bits as seed materials. It is plagued by rhizome rot diseases most of which are mainly spread through infected seed rhizomes. Micropropagation will help in production of disease-free seed. Sexual reproduction is rare in turmeric, making recombinant breeding very difficult. In vitro technology can thus become the preferred choice and it can be utilized for multiplication, conservation of genetic resources, generating variability, gene transfer, molecular tagging, and their utility in crop improvement.

Key words Conservation, Cryopreservation, Embryo rescue, In vitro conservation, Micropropagation, Microrhizome production, Molecular profiling, Plant regeneration, Somaclonal variation, Synthetic seeds, Turmeric

Abbreviations

BA	Benzyl adenine
IBA	Indole-3-butyric acid
Kin	Kinetin
NAA	α-naphthalene acetic acid
MS	Murashige and Skoog

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1 Introduction

Turmeric (*Curcuma longa* L.) is an important spice crop belonging to the family Zingiberaceae and known for its varied medicinal properties (Fig. 1). Turmeric is a native of India and South East Asia and is used since ancient times as a spice and a dye and cultivated extensively in China, India, Indonesia, and Thailand and throughout the tropics, including tropical regions of Africa and America. It is extensively used in Chinese, Ayurvedic, Unani, and Siddha systems of medicine and as a home remedy for various ailments. The coloring principle of turmeric "curcumin" is responsible for many medicinal properties [1] and used in cheese, spices, mustard, cereals, pickles, potato flakes, soups, ice creams, yogurts and also in pharmacy, confectionery, and food industries. Recent research is focused on turmeric's antioxidant, hepatoprotective, anti-inflammatory, anticarcinogenic, and antimicrobial properties, in addition to its use in cardiovascular disease and gastrointestinal disorders. India is the world's largest producer, consumer, and exporter of turmeric with an annual production of about 9,86,690 tonnes from an area of 1,94,330 ha during 2012–2013.

Turmeric rarely sets seeds, hampering recombination breeding. Conventionally propagated through rhizomes, its cultivated

Fig. 1 Turmeric plant

types exhibit much morphological variations probably due to accumulated vegetative mutations. The past few years have seen the solutions of many breeding bottlenecks being solved by the use of biotechnological tools. Somaclonal variation, anther and protoplast culture, rDNA techniques, etc. are successfully utilized for production of disease-free planting material, conservation and improvement of genetic resources. Protocols for various techniques have been standardized and are summarized below. Morphological and molecular variations among micropropagated and callus-regenerated plants were studied and variations were found in both but with higher percentage of variation in callus-regenerated somaclones [2, 3]. In vitro plants developed through microrhizome exhibited least amount of variations. The study inferred that this is due to the accumulated vegetative mutations (mosaic) in turmeric. The genetic fidelity studies of turmeric germplasm conserved in in vitro gene bank using RAPD profiling showed their genetic integrity [4].

2 Materials

2.1 Explant Materials Vegetative bud explants from rhizomes.

2.2 Glassware Borosilicate conical flasks (500 ml), glass bottles (250 ml), culture tubes (22 cm × 3.5 cm), and 250 ml conical flasks, culture vessels, tubes, bottles, and flasks. Cotton plugs (made of non-absorbent cotton covered with cheese cloth), aluminum foil, or polypropylene caps.

2.3 Growth Regulators Auxins—α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) 0–1.0 mg/l;

Cytokinins—6-benzylaminopurine (BA) and 6-furfurylamino purine (kinetin) 0–3.0 mg/l.

2.4 Gelling Agents Bacteriological grade agar, 7–8.0 g/l.

2.5 Culture Medium

- Murashige and Skoog (MS) basal medium (Table 1).
- Stock solutions for macronutrients, micronutrients, vitamins, vamino acids, and plant growth regulators (Table 2).
- Sucrose—30 g/l for multiplication and 90 g/l for microrhizome induction.

2.6 Culture Establishment Rhizomes from potted plants.

2.7 Culture Initiation MS medium supplemented with 3 % sucrose and 0.5 mg/l Kinetin.

Table 1
Composition of Murashige and Skoog[a] basal medium

Composition		Concentration (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2{\cdot}2H_2O$	440.00
Potassium orthophosphate	KH_2PO_4	170.00
Magnesium sulfate	$MgSO_4.7H_2O$	370.00
Micronutrients	Na_2EDTA	37.30
Sodium EDTA	$FeSO_4{\cdot}7H_2O$	27.80
Ferrous sulfate	H_3BO_3	6.20
Boric acid	$MnSO_4.4H_2O$	22.30
Manganese sulfate	KI	0.83
Potassium iodide	$ZnSO_4{\cdot}7H_2O$	8.60
Zinc sulfate	$Na_2MoO_4{\cdot}2H_2O$	0.25
Sodium molybdate	$CuSO_4{\cdot}5H_2O$	0.025
Copper sulfate	$CoCl_2{\cdot}6H_2O$	0.025
Cobalt chloride		
Vitamins		
Myo-inositol	$C_6H_{12}O_6$	100.00
Thiamine HCl	$C_{12}H_{17}CIN_4OS{\cdot}HCl$	0.10
Nicotinic acid	$C_6H_5NO_2$	0.50
Pyridoxine HCl	$C_8H_{11}NO_3{\cdot}HCl$	0.50
Amino acid		
Glycine	$C_2H_5NO_2$	2.00

[a]Murashige and Skoog [8]

2.8 In Vitro Conservation

In vitro regenerated shoot buds.

2.9 Production of Synthetic Seeds

In vitro regenerated shoot buds, protocorms, and callus; sodium alginate, and calcium chloride.

2.10 Long-Term Storage by Cryopreservation

Synseeds containing miniaturized shoot tips, pollen are used as explants, and are immersed in liquid nitrogen.

Table 2
Details of various stock solutions for MS medium

Stock	Composition	Stock strength	Quantity[a]
A	Macronutrients	×20	50 ml
	NH_4NO_3		
	KNO_3		
	$CaCl_2 \cdot 2H_2O$[b]		
	KH_2PO_4		
	$MgSO_4 \cdot 7H_2O$		
B	Micronutrients	×100	10 ml
	H_3BO_3		
	$MnSO_4 \cdot 4H_2O$		
	KI		
	$ZnSO_4 \cdot 7H_2O$		
	$Na_2MoO_4 \cdot 2H_2O$		
	$CuSO_4 \cdot 5H_2O$[a]		
	$CoCl_2 \cdot 6H_2O$[a]		
C	Micronutrients	×100	10 ml
	Na_2EDTA[a]		
	$FeSO_4 \cdot 7H_2O$[a]		
D	Vitamins	×100	10 ml
	Thiamine HCl		
	Nicotinic acid		
	Pyridoxine HCl		
E	Amino acid	×100	10 ml
	Glycine		
F	Myo-inositol	×100	10 ml
	Growth regulators		
	2,4-D	50 mg/200 ml	
	NAA	50 mg/200 ml	
	BA	50 mg/200 ml	
	Kinetin	50 mg/200 ml	

[a]For preparation of 1 L medium. Make up the remaining is double distilled water up to 900 ml adjust the pH and add the remaining water to 1000 ml
[b]Dissolved separately before mixing in the final stock

3 Methods

3.1 Micropropagation and Multiplication of True-to-Type Plants

3.1.1 Establishment of Cultures in Turmeric (Fig. 2)

1. Newly sprouting buds are used as a source of explants for the culture.
2. Wash the explants under running tap water for 15 min followed by detergent solution (Tween 20) for 10–15 min.
3. Surface-sterilize using copper oxychloride, Bavistin (10 min each), 70 % ethanol (30–40 s) and wash with sterile distilled water for 3–4 min to remove the remnants of the fungicide.
4. Before starting inoculation, clean/treat the laminar flow with ethyl alcohol (70 %) for 30–60 s, wash the explants again in sterile double distilled water followed by treatment with 0.1 % $HgCl_2$ for 3–5 min and rinse with sterile distilled water for 3–4 times.
5. Cut the sterilized buds into small pieces suitable for inoculation and dissect the sprouts and remove the outer leaf sheaths under aseptic condition.
6. Inoculate the explants in test tubes containing half-strength MS media supplemented with 3 mg/l BA and 1 mg/l NAA.

3.1.2 Multiplication of Contamination-Free Cultures in Turmeric (Fig. 3)

1. Use 1-week-old, contamination-free cultures for multiplication. Explants are taken out carefully under sterile conditions and transferred into multiplication medium MS medium containing 5 mg/l BA and 1.0 mg/l NAA.
2. Use 7- to 8-week-old cultures for subculturing and for further multiplication. 20–25 plants can be harvested from 8-week-old culture.

3.2 Direct Regeneration of Plantlets from Immature Inflorescence

1. Collect immature, 1- to 10-day-old inflorescence (during flowering season) and sterilize.
2. Remove the outer bracts under aseptic condition and transfer remaining inflorescence to culture medium.

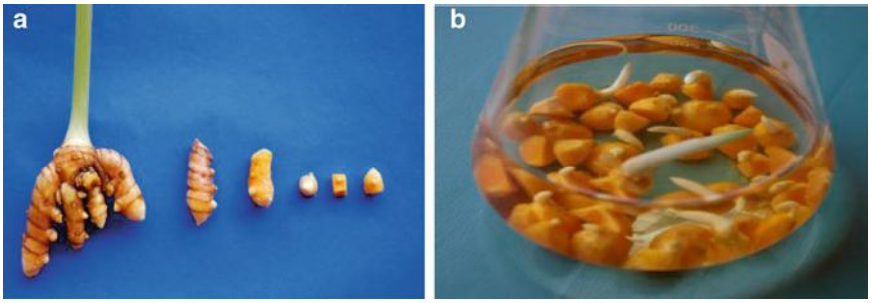

Fig. 2 (**a**, **b**) Explant sources of turmeric

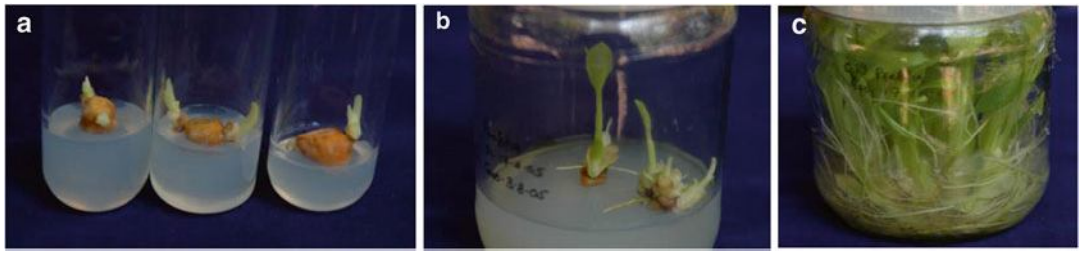

Fig. 3 (**a**–**c**) Stages of culture establishment and in vitro multiplication in turmeric

Fig. 4 (**a**–**d**) Stages in microrhizome induction and in vitro rhizome formation in turmeric

3. Use MS medium amended with 10 mg/l BA and 0.2 mg/l 2,4-D.
4. MS liquid medium having 1 mg/l NAA enhances rooting.

3.3 In Vitro Rooting and Production of Plantlets

1. Turmeric cultures do not require separate culture medium for rooting. Once the shoots become around 3–4 cm they automatically root in the media used.
2. MS liquid medium with 1 mg/l NAA can be used to improve rooting before transplanting to the soil.

3.4 Microrhizome Induction (Fig. 4)

Technology for microrhizome development in turmeric was standardized [5].

1. Microrhizome formation will be noticed in MS medium supplemented with 90 g sucrose after 90–100 days of inoculation and reaches maturity by 120 days.
2. Plant out microrhizomes directly in micro-cups or in the field for normal rhizome development.

3.5 Callus Culture, Plant Regeneration from Callus, and Inducing Variability (Fig. 5)

Technology for plant regeneration from various tissues in turmeric is available, and used to exploit somaclonal variation for increasing variability in this crop [6].

1. Wash new buds under running tap water followed by washing with 0.1 % $HgCl_2$ for 5–10 min. Rinse the explants 3–4 times to wash away the traces of $HgCl_2$.

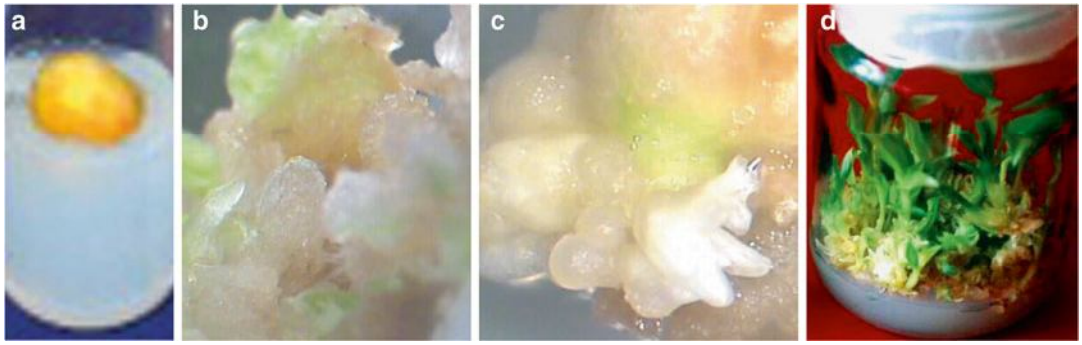

Fig. 5 Different stages of plant regeneration in turmeric, (**a**) Explant, (**b**) organogenesis from callus, (**c**) embryogenesis, (**d**) well-developed plantlets

2. Remove the outer leaf primordial under aseptic condition and place a small segment of bud on MS media supplemented with 13.6 μm 2,4-D and 16 μm NAA for callus induction.
3. Subculture 2-month-old callus on MS medium containing 0.2 mg/l 2,4-D and 10 mg/l BAP for organogenesis and plant regeneration.

3.6 Hardening and Planting Out

1. Take out healthy and well-rooted in vitro plantlets from the culture media and wash carefully to remove the traces of culture medium.
2. Transfer to polythene bags containing garden soil, sand, and farmyard manure in equal proportions and keep in humid chamber.
3. Maintain 90–100 % relative humidity for 20–30 days for hardening and establishment.

3.7 Encapsulation and Synthetic Seed Production

1. Suspend in vitro grown shoots/somatic embryos in MS basal medium supplemented with 4 % (w/v) Na alginate, 2 M glycerol, and 0.4 M sucrose.
2. Drop the mixture containing micro-shoots, with a sterile pipette into 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose and leave for 20 min to form beads 4 mm in diameter, each bead containing at least one shoot.
3. Store the beads in sterile containers for 6–8 months [7].
4. Transfer to MS medium amended with 1 mg/l BAP + 0.5 mg/l NAA for regrowth.

3.8 Conservation of Genetic Resources

3.8.1 Medium Term In Vitro Conservation by Slow Growth

In Vitro Storage by Minimal Growth (Fig. 6)

1. Use vegetative bud explants and establish cultures in MS [8] basal medium containing 0.5 mg/l kinetin.
2. Multiply established shoot cultures on MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP with an average of 8 shoots per culture in 90 days of culture.
3. The in vitro developed shoots are maintained on half-strength MS medium amended with 15 g/l each of sucrose and mannitol in sealed culture tubes up to 12 months with 80 % survival.
4. Maintain cultures the same medium with yearly subculture up to 7 years.
5. The miniaturized in vitro stored cultures can be recovered to normal size by transferring to the multiplication medium (MS + 1 mg/l BAP + 0.5 mg/l NAA), thereby resuming growth and multiplying normally in 3 weeks of culture. Multiple shoots and roots are produced in the same medium.
6. Transfer the rooted plantlets to nursery cups and maintain at 90–100 % relative humidity for 20–30 days for hardening and establishment with 80–90 % survival [9, 10].

3.8.2 Long-Term Storage of Encapsulated Shoot Tips by Cryopreservation

Cryopreservation (Fig. 7)

1. Initiate the rhizomes excised from field-grown plants on MS medium supplemented with 0.5 mg/l kinetin, 20 g/l sucrose, 0.7 % agar in tissue culture tubes at 22 ± 20 °C under 278 cd for a period of 2 weeks. Adjust pH 5.8 of the medium prior to autoclaving at 121 °C for 15 min. Multiply the plantlets obtained through initiation culture in MS medium containing BAP (1.0 mg/l) and NAA (0.5 mg/l). Excise apical shoot tips with two leaf primordial (about 1–2 mm in size) for cryopreservation.
2. For osmoprotection suspend the excised shoot tips in 0.3 M sucrose for a period of 3 days in the dark.
3. Dehydrate the osmoprotected shoot tips with ice-cold PVS2 solution at 0 °C for 3 h. PVS2 solution contains 30 % (w/v)

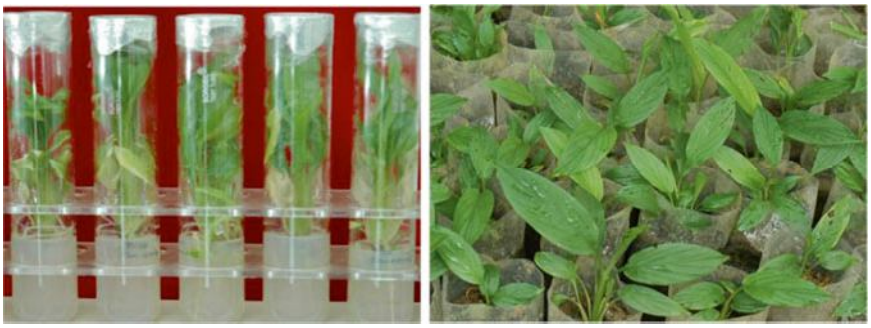

Fig. 6 In vitro storage by minimal growth in turmeric

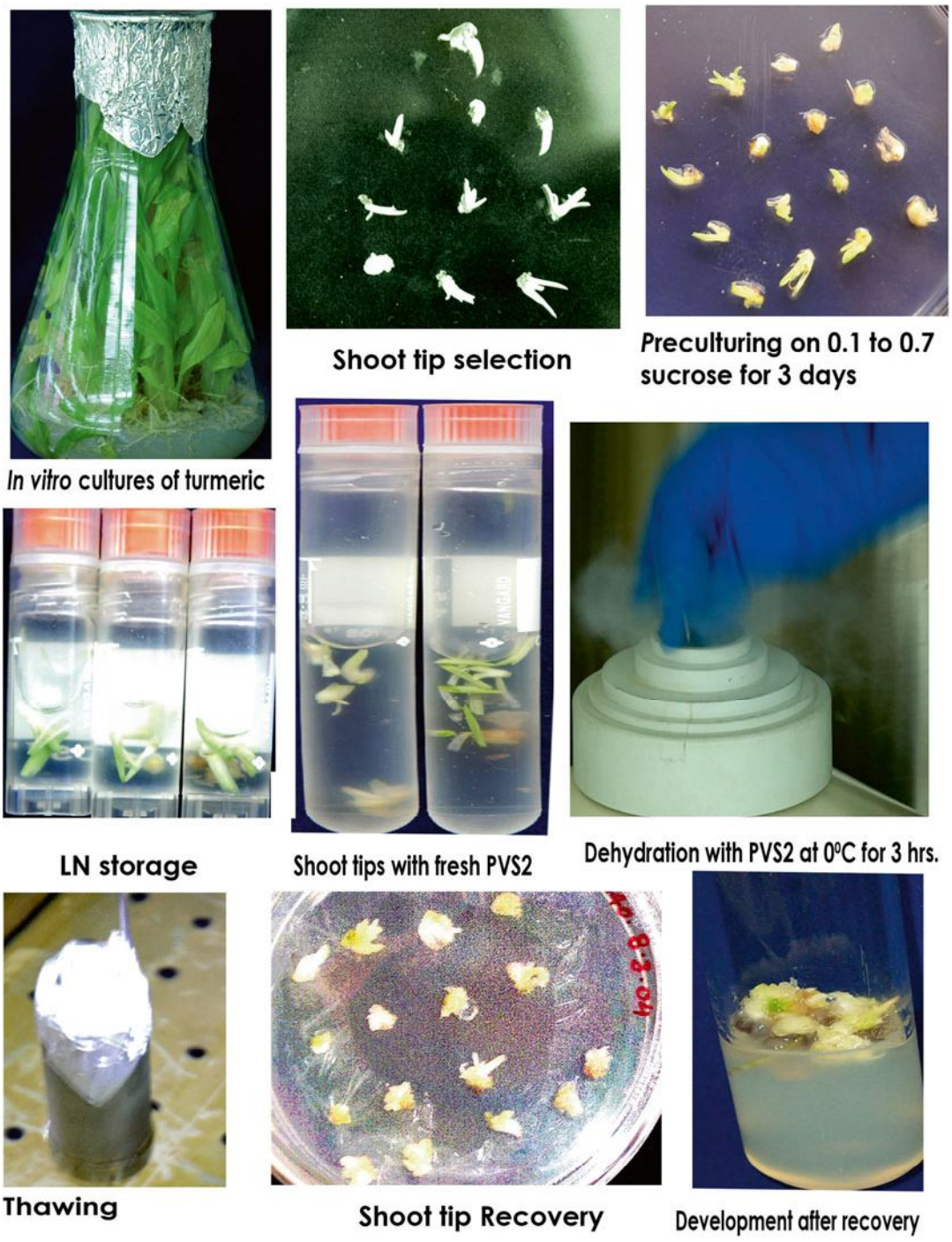

Fig. 7 Various stages in cryopreservation of turmeric

glycerol, 15 % (w/v) ethylene glycol, and 15 %(w/v) DMSO in MS medium supplemented with 0.4 M sucrose (pH 5.8). After dehydration, suspend 10 shoot tips in 1 ml PVS2 solution in a 1.8 ml cryo-tube and then plunge into liquid nitrogen for 1 h.

Thawing and Regrowth

1. Thaw by rapidly immersing the cryotubes in water bath at 40 °C for 1 min
2. For encapsulation-vitrification method, PVS2 is drained off from the vitrified samples, and replaced with 1.2 M sucrose solution twice at 10 min intervals.
3. Propagules after thawing are placed on MS + BA (1 mg/l) and NAA (0.5 mg/l) medium in petri dishes and maintained at the same culture conditions as stock explants.
4. Around 80 % recovery of cryopreserved shoots can be obtained [11].

3.9 Molecular Profiling [11, 12]

3.9.1 Isolation of DNA

There are various protocols for isolation of DNA from plant tissues. For turmeric CTAB method was used [13]. The protocol used for extraction of DNA from turmeric leaf tissues is as follows (Tables 3 and 4)

1. Grind 5 g young leaves in the liquid nitrogen with a mortar and pestle and add 25 ml preheated (65 °C) CTAB buffer. Add 0.2 % β-mercaptoethanol prior to use.
2. Incubate at 60 °C for 30 min.
3. Extract with equal volume of chloroform–isoamyl alcohol (24:1) at 26,832 × *g* for 10 min at room temperature.
4. Take the aqueous phase and add 2/3rd volume of ice-cold isopropanol.
5. Incubate at −20 °C for 2 h and centrifuge at 26,832 × *g* for 15 min at 4 °C.
6. Discard the supernatant and invert the tube on paper towel for few minutes.
7. Dissolve the pellet and add 1.5 ml TE buffer. Store at room temperature.
8. Add 10 μg/ml RNase A and incubate at 37 °C for 30 min.
9. Add equal volume of Tris saturated phenol, mix it well and centrifuge at 26,832 × *g* for 10 min.
10. To the aqueous phase, add equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), shake and centrifuge at 26,832 × *g* for 10 min.
11. Take the aqueous phase and add equal volume of chloroform–isoamyl alcohol (24:1), shake and centrifuge at 26,832 × *g* for 10 min.

Table 3
Composition of various stock solutions for DNA isolation

Solutions	Method of preparation
1 M Tris–HCl (pH 8.0) 500 ml	Dissolve 60.55 g Tris base (Sigma) in 300 ml distilled water. Adjust pH to 8 by adding concentrated HCl. Adjust volume to 500 ml. Dispense into reagent bottles and sterilize by autoclaving
0.5 M EDTA pH 8.0	Dissolve 93.05 g f EDTA-disodium salt (sigma) in 300 ml water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml. Dispense into reagent bottles and autoclave
5 M NaCl 500 ml	Weigh 146.1 g NaCl (Merck) add 200 ml water and mix well. When the salts get completely dissolved, adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave
3 M Sodium acetate (pH 5.2) 250 ml	Dissolve 61.523 g anhydrous sodium acetate (Qualigens) in 200 ml water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99–100 %). Autoclave
Ethidium bromide 10 mg/ml, 100 ml	Add 1 g ethidium bromide to 100 ml distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. Dispense into amber colored reagent bottle and store at 4 °C
70 % ethanol, 500 ml	Take 360 ml ethanol; mix with 140 ml distilled water. Dispense into reagent bottle and store at 4 °C
Chloroform–isoamyl alcohol (24:1), 500 ml	Measure 450 ml chloroform and 20 ml isoamyl alcohol. Mixed and stored in room temperature
1 M $MgCl_2$, 100 ml RNase A (10 mg/ml)	Weigh 20.33 g $MgCl_2$, dissolve in double distilled water, make up to 100 ml, autoclave. Make up 10 mg/ml RNase in distilled water. Boil for 10 min to destroy DNase. Divide into 1 ml aliquots and store at −20 °C

12. To the aqueous phase add one-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and incubate at −20 °C for 1 h or at −70 °C for 30 min
13. Centrifuge at 26832 × *g* for 10 min and wash the pellet in 70 % ethanol (26,832 × *g* for 5 min).
14. Air-dry the pellet and dissolve in 1.5 ml TE and estimate the yield.
15. Estimate DNA amount using Scanning Shimadzu Spectrophotometer. DNA shows a clear absorbance peak at 260 nm and value of 1.0 OD_{260} is calculated equivalent to 50 μg/ml. DNA solution is considered to be pure if the value of OD_{260}:OD_{280} is 1.8. The DNA is visualized on (0.8 %) agarose gel for its quality and stored at −20 °C.

3.9.2 Molecular Profiling Using ISSR (Fig. 8) and SSR Primers

1. Perform amplification in a total volume of 25 μl including 2.5 μl 10× Taq DNA polymerase Buffer, 50 ng DNA, 0.15 mM dNTP, 1.5 mM $MgCl_2$, 0.4 μM primers, and 1U Taq DNA polymerase primers.

Table 4
Composition of various buffers used for DNA isolation

	Buffer	Method of preparation
1	CTAB Extraction Buffer: for 1 L 100 mM Tris–HCl (pH 8.0) 20 mM EDTA (pH 8.0) 1.4 M NaCl 2 % CTAB (w/v) Merck 0.2 % β-mercaptoethanol (v/v)-Merck	Measure 100 ml Tris (1 M), 280 ml NaCl, 40 ml EDTA (0.5 M). Mix with about 400 ml of hot distilled water, add 20 g CTAB to this. Adjust final volume to 1 L. Dispense into reagent bottles and autoclave. Just before use, add 0.2 % β-mercaptoethanol
2	TE (0.1 mM) buffer…for 100 ml 100 mM Tris–HCl (pH 8.0) 0.1 mM EDTA (pH 8.0)	Take 1 ml Tris–HCl (1 M), 20 ml EDTA (0.5 M). Mix with 99 ml sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave
3	TAE buffer 10×: for 1 L	Weigh 48.4 g Tris base; add 20 ml EDTA (0.5 M); 11.42 ml glacial acetic acid and around 150 ml double distilled water. Dissolve the salt and adjust volume to 1 L. Autoclave
4	10× DNA loading dye	Ficoll (Type 400) 25 % (w/v), bromophenol blue 0.4 % (w/v) and xylene cyanol FF 0.4 % (w/v). Dissolve 0.25 g of BPB in 99 ml 30 % glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense into reagent bottles and keep in 4 °C

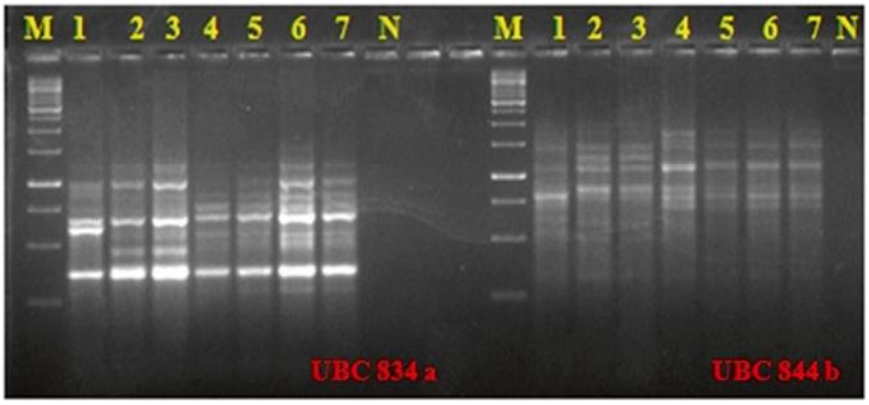

Fig. 8 ISSR profiling of released varieties of turmeric (Lanes 1–7 represent turmeric varieties Suvarna, Suguna, Sudharsana, Prabha, Prathiba, Alleppey Supreme, and Kedaram) (**a**) Using the primer UBC 834a 5′-AGAGAGAG AGAGAGAGCT-3′ and (**b**) Using primer 815 (5′-CTC TCT CTC TCT CTC TG-3′)

2. employ Amplification in a programmable thermal cycler with cycling regimes of an initial denaturation at 94 °C for 5 min, followed by a 33 repeats of a PCR core cycle of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, followed by a final extension cycle of 72 °C for 10 min.

Table 5
ISSR primers for DNA profiling of turmeric varieties

Sl no	Primer name	Sequence
1	UBC 810	5′-GAG AGA GAG AGA GAG AT-3′
2	UBC 811	5′-GAG AGA GAG AGA GAG AC-3′
3	UBC 812	5′-GAG AGA GAG AGA GAG AA-3′
4	UBC 815	5′-CTC TCT CTC TCT CTC TG-3′
5	UBC 834a	5′-AGAGAGAGAGAGAGAGCT-3′
6	UBC 835a	5′-AGAGAGAGAGAGAGAGCC-3′
7	UBC 844b	5′-CTCTCTCTCTCTCTCTGC-3′
8	UBC 850a	5′-GTGTGTGTGTGTGTGTCC-3′
9	UBC 864	5′-ATGATGATGATGATGATG-3′

3. Use the ISSR Primers listed in Table 5.
4. Resolve amplicons electrophoretically alongside a 1 kb ladder, on a 1.5–2 % agarose gel stained with 0.5 mg/ml ethidium bromide in a 1× TAE buffer (pH 8.0), and run at 90 V for 3 h in an electrophoresis unit.
5. Carry out duplicate amplification to confirm reliability of the bands. Amplified products are listed as discrete character states in a present (1) or absent (0) matrix.
6. Relationship among genotypes is evaluated using the Unweight Pair Grouping Mathematical Average (UPGMA) analysis and plotted to produce a dendrogram using NTSyS software.

4 Notes

1. The explant source and stage are the most important criteria for establishing contamination-free cultures with good in vitro responses.
2. Disease- and virus-free source material must be used and the individual plants are also checked to be free from symptoms.
3. Plant the rhizome bits in the greenhouse under protected conditions with 4–5 in. of sand on top and periodically (once in 20 days) spray/drench 0.3 % copper oxychloride/Bavistin/Dithane M-45 to minimize contamination.
4. Since explants from underground are used, there will be a high chance of contamination. To minimize the costs only small amounts (5 ml) of half-strength MS media are used.

5. Fungal and bacterial contamination appears within 5–10 days and the contaminated cultures are discarded.
6. An initial period of 25–30 days is required for the explants to accustom to the nutrient medium before exhibiting the first signs of growth and bud break.
7. The shoot tips show signs of growth in 3–4 weeks and within 5–6 weeks axillary shoot buds also emerge.
8. After activation of rhizome buds, the newly sprouted buds are excised and transferred to fresh media.
9. MS medium [8] is best suited for turmeric. Adjust the pH to 5.8 before adding agar. Agar is melted to ensure uniform distribution in the medium, which is autoclaved at 121 °C at 16 psi for 20 min.
10. For microrhizome induction, use sufficiently grown plants under in vitro conditions. Under sterile conditions separate the well-grown plants and use them for the induction of microrhizome. Trim the roots and shoot before transferring into microrhizome induction media.

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