Chapter 26

Protocols for Improvement of Black Pepper (*Piper nigrum* L.) Utilizing Biotechnological Tools

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Abstract

Black pepper, *Piper nigrum* L., the "King of spices" is the most widely used spice growing in the South-Western region of India. The humid tropical evergreen forest bordering the Malabar Coast (Western Ghats is one of the hot spot areas of plant bio-diversity on earth) is its center of origin and diversity. However, the crop faces constraints like rampant fungal and viral diseases, lack of disease free planting material, hence biotechnological tools can be utilized to address these problems and strides have been made successfully. The standardization of micropropagation, somatic embryogenesis, in vitro conservation, protoplast isolation, and genetic transformation protocols are described here. The protocols could be utilized to achieve similar goals in the related species of *Piper* too.

Key words Artificial/synthetic seeds, Cryopreservation, Embryo culture, Genetic transformation, In vitro conservation, Micropropagation, Plant regeneration, Somatic embryogenesis, Suspension cultures

Abbreviations

BA	Benzyl adenine
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog
NAA	α-naphthalene acetic acid
SH	Schenk and Hildebrandt
TDZ	Thiaduzuron
WPM	Woody plant medium

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1 Introduction

Black pepper, *Piper nigrum* L., is the most important spice of the world accounting for 40 % of export earnings from spices. India is the major producer and exporter of this spice and being the native home of black pepper, collection, cataloguing, and conservation of the precious genetic resources of this crop [1], development of resistant varieties are the major thrust areas in improvement of black pepper.

Molecular markers have been used in resolving complex phylogenetic problems in pepper and many reports on molecular characterization of Piper are available [2, 3]. Conserving and fingerprinting the genetic diversity in pepper and development of resistant varieties to "*Phytophthora* foot rot" are few areas that seek immediate solutions. In the past few years protocols utilizing biotechnological methods for commercial propagation, protoplast culture, genetic transformation and molecular profiling, conserving the genetic resources in in vitro and cryo banks have been developed [2, 4–6]. Genetic stability of micropropagated plants of *Piper longum* was validated using RAPD markers [3]. Similarly RAPD profiles for many cultivars of black pepper for identification of varieties have been studied [12]. The ability to manipulate and study plant tissues has led to the appreciation of role played by plant biotechnology in improving a crop species. Black pepper is no exception and the protocols developed for addressing crop-specific problems are reported.

2 Materials

2.1 Plant Material

Shoot tips, leaf segments, and nodal segments of *Piper nigrum* (Fig. 1) for all experiments and seeds for somatic embryogenesis.

Fig. 1 Black pepper vine

2.2 Surface Sterilization of Capsules

1. Diluted detergent, viz., two to three drops of Tween 20 in 50 ml distilled water.
2. Copper oxychloride as fungicide.
3. 0.1 % mercuric chloride.
4. 70 % ethanol.
5. Sterilized double distilled water.

2.3 Culture Vessels

1. Borosil test tubes and conical flasks.
2. Closures made of non-adsorbent cotton in gauze cloth.

2.4 Chemicals

Murashige and Skoog [7] medium (Table 1) for all in vitro culture methods.

2.5 Hardening of Plantlets

1. Potting mixture composed of sterilized vermiculite, garden soil, and sand (1:1:1) in equal proportions.
2. Micro-cups, polythene bags, and pots.

2.6 Molecular Profiling

2.6.1 Genomic DNA Isolation

1. 2× extraction buffer: (2 % cetyltrimethylammonium bromide (CTAB), 100 mM Tris–HCl, pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8, 1.4 M NaCl, 1 % polyvinylpolypyrrolidone (PVPP)).
2. Chloroform–isoamyl alcohol (24:1).
3. 100 % ethanol or isopropanol, 70 % alcohol.
4. TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8).
5. RNAse A (10 mg/ml).
6. 50× Tris–Acetate–EDTA (TAE) buffer (pH 8).
7. Agarose.
8. Ethidium bromide (10 mg/ml).
9. 6× loading dye (30 % glycerol, 5 mM EDTA, 0.15 % bromophenol blue, 0.15 % xylene cyanol).
10. 1000 bp DNA ladder.

2.6.2 For RAPD PCR

1. Taq DNA polymerase with 10× buffer.
2. 10 mM dNTPs: 10 mM each of dATP, dCTP, dGTP, and dTTP.
3. 25 mM $MgCl_2$.
4. 10 μM primers.

3 Methods

3.1 Micropropagation

Micropropagation protocol is used to multiply adequate planting material of black pepper (Fig. 2).

Table 1
Composition of tissue culture media (mg/l) used in the study

	Murashige and Skoog	Schenk and Hildebrandt	Woody plant medium
NH_4NO_3	1650	–	400.00
$(NH_4)_2\ SO_4$	–	–	–
$NH_4H_2PO_4$	–	300	–
$CaCl_2{\cdot}2H_2O$	440	200	96.00
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	556.00
$MgSO_4{\cdot}7H_2O$	370	400	370.00
KCl	–	–	–
KNO_3	1900	2500	–
KH_2PO_4	170	–	170.00
K_2SO_4	–	–	990.00
$NaH_2PO_4H_2O$	–	–	–
Na_2SO_4	–	–	–
$CoCl_2{\cdot}6H_2O$	0.025	0.1	–
$CuSO_4{\cdot}5H_2O$	0.025	0.2	0.25
$FeSO_4{\cdot}7H_2O$	27.85	15.0	27.80
Na_2-EDTA	37.25	20.0	37.30
$MnSO_4{\cdot}4H_2O$	22.3	10.0	22.30
KI	0.83	1.0	–
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.1	0.25
$ZnSO_4{\cdot}7H_2O$	8.6	1.0	8.60
H_3BO_3	6.2	5.0	6.2
Inositol	100	1000	100
Nicotinic acid	0.5	5.0	0.50
Pyridoxine–HCl	0.5	0.5	0.50
Thiamine–HCl	0.1	5.0	1.0
Glycine	2.0	–	2.00

3.1.1 Preparation of Culture Media

Murashige and Skoog (MS) basal medium is used for culture. Prepare separate stocks for macronutrients, micronutrients, vitamins, amino acids, and plant growth regulators separately.

1. Mix the stocks as mentioned, add 30 g/l sucrose, dissolve well, and make up to 1 L.
2. Adjust the pH to 5.8 and add 6 g/l agar.

Fig. 2 Production of multiple shoots in vitro

3. Transfer it to culture tubes and flasks in the required quantity and autoclave at 121 °C for 20 min.

3.1.2 Establishment of In Vitro Culture

1. Explants (shoot tips) from hygienically maintained source plants are collected into conical flask containing a fungicide solution of copper oxychloride with a dilute detergent (*see* **Notes 1–3**).
2. Surface-sterilize the explants with 0.1 % mercuric chloride ($HgCl_2$) in laminar flow hood and wash with three changes of sterile double distilled water.
3. Dry them on sterile filter papers and cut to the required size.
4. Inoculate in the culture tubes containing nutrient media and incubate them in growth room at 25 ± 2 °C with 16 h photoperiod, light intensity 33.8 μmol/m^2 s.
5. MS medium fortified with BA (0.5 mg/l for bud break, and 3 mg/l for multiple shoots) and hormone free MS with or without charcoal (2 %) for rooting is standardized as the ideal medium (Fig. 3).
6. Transfer the well-developed plants to a potting mixture and maintain at high humidity (>85 %) either in the nursery or hardening facility for 15–30 days and slowly bring them to open conditions.

3.2 Somatic Embryogenesis from Mature Seeds

1. Mature and ripe seeds of black pepper are squeezed so as to remove the pericarp, followed by thorough washing under running tap water.
2. Surface-sterilize the seeds inside a laminar flow by treating with 0.1 % mercuric chloride for 10 min, followed by rinsing with three to four changes of sterile double distilled water.
3. Inoculate the seeds on solid SH [8] basal medium (Table 1) with 30 g/l sucrose, gelled with 0.8 % agar.

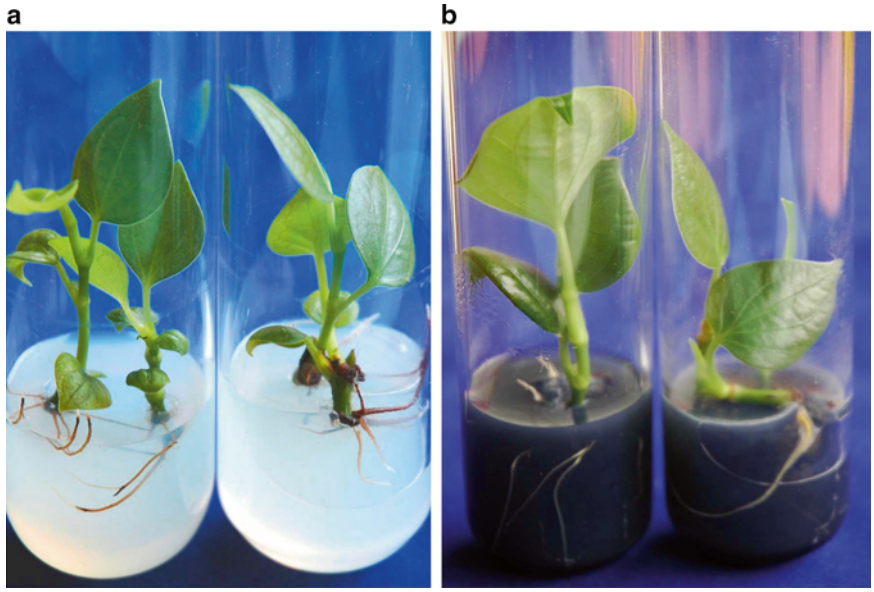

Fig. 3 (**a**, **b**) In vitro cultures of black pepper (activated charcoal is incorporated in the medium, whenever phenolics exudation into the medium is observed)

Fig. 4 Somatic embryogenesis in black pepper

4. Incubate the cultures under complete darkness at 25 ± 1 °C, and observe the cultures for germination at weekly intervals.
5. After germination of majority of seeds (30–40 days), subculture them with micropylar region in contact with fresh medium.
6. Maintain the cultures and regularly observe for somatic embryogenesis from the micropylar ring tissue (usually occurs between 60 and 120 days after culture initiation in 10–25 % seeds).
7. Once somatic embryo induction is observed, allow five to ten embryos to form on the explants (Fig. 4).

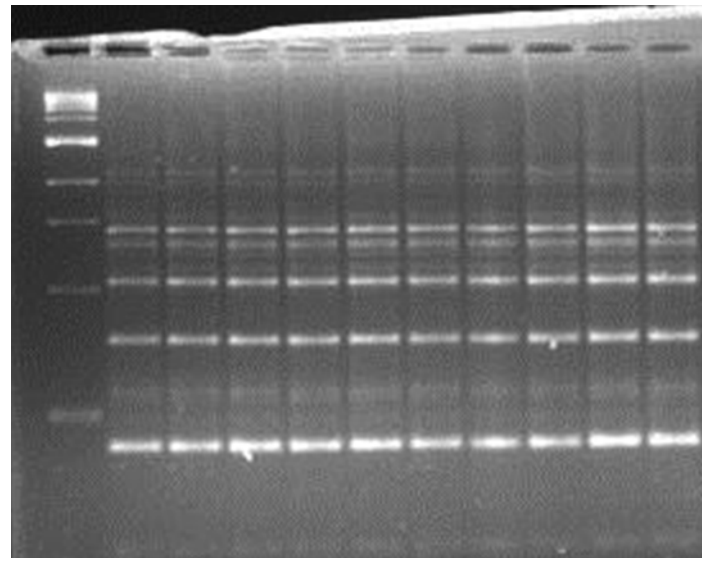

Fig. 5 RAPD profiles of plantlets developed through somatic embryogenesis

8. Transfer the explants with somatic embryos to the medium of same composition for proliferation combined with maturation or to SH medium supplemented with 15 g/l sucrose for more proliferation (Culture conditions remain the same).
9. Germinate the somatic embryos in agitating liquid SH medium containing 30 g/l sucrose at 110 rpm in the dark.
10. After complete formation of plants allow them to grow under light in the same flask (with medium replenishment once in 15 days) or transfer to individual tubes containing static liquid medium for better growth [9, 10].
11. After the formation of at least four leaves keep the cultures outside the culture room for 10 days
12. Transfer plants to 350 ml plastic cups filled with autoclaved river sand and cover with polythene bags having a few holes, till the emergence of fresh leaf growth.
13. Molecular profiles of these plants show absolute genetic uniformity (Fig. 5).

3.2.1 Somatic Embryogenesis from Leaf Explants

1. Prepare culture medium supplemented with BA (0.05–1 mg/l) and TDZ (0.05–1 mg/l).
2. Select very young leaves (preferably the freshly opened leaf of 1–2 cm size) from established aseptic cultures.
3. Culture the leaf explants in the medium prepared above and keep them in the dark for 15–20 days.
4. Keep the petiole of the leaf intact and in contact with the medium.
5. Cultures with callus growth are eliminated. Observe for direct somatic embryos from leaves, which occur only in 3–5 % cultures.

6. Transfer the leaf explants with somatic embryos very carefully to medium of same composition.
7. Observe for proliferation of somatic embryos by budding (*see* **Note 4**).
8. Culture bigger and mature embryos on solidified growth regulator free medium for development of plantlets.

3.3 Establishment of Cell Suspension Cultures

1. Supplement the basal MS medium with 2,4-D (0.5 mg/l) and BA.
2. Select leaf/stem/root explants from established aseptic cultures. Explants from aseptically germinated seedlings and embryos can also be used.
3. Culture the explants in the prepared medium and keep them in the dark for 15–20 days. Periodically observe for in vitro responses.
4. Cut explants into smaller pieces and callus growth from the cut ends would be observed. The majority of callus developed will be hard which can be transferred to regeneration medium for organogenesis and plant regeneration.
5. Separate friable and loose callus from the hard callus and put into the liquid medium of the same composition and agitate in orbital shakers at 100–150 rpm to establish suspension cultures.
6. Use these suspension cultures for radiation/mutation/in vitro selection studies.
7. Plate the selected calli on solidified regeneration medium for development of hard callus and plant regeneration. Addition of TDZ to the culture medium will enhance plant regeneration and sometimes somatic embryogenesis.
8. Transfer the hard callus into regeneration medium for plant development. Observe cultures periodically under the microscope for growth and differentiation and transfer to the suitable medium.

3.4 Production of Synthetic Seeds

1. 3 % sodium alginate is incorporated into MS medium as the encapsulation matrix.
2. The explants are mixed into the above medium, dropped into calcium chloride solution (1.036 g in 100 ml) and rotated gently for bead formation.
3. The solution is decanted and beads or Synseeds thus retrieved can be stored up to 9 months in sterile water with over 80 % viability (Fig. 6).

3.5 Protoplast Isolation

1. *Source tissue*: Leaves/callus is cut into small pieces to ensure proper enzymatic digestion, as it is difficult to peel off the epidermis.

Fig. 6 Recovery and growth of cryopreserved "synthetic seeds" of *Piper*

Table 2
Composition of enzyme solutions (ES) for isolation of protoplasts[a]

Code	Mannitol (%)	Macerozyme R-10 (%)	Hemicellulase (%)	Onozuka cellulase R-10 (%)
ES-1	10	0.5	–	1
ES-2	9	0.5	–	1
ES-3	8	0.5	–	1
ES-4	7	0.5	–	1
ES-5	6	0.5	–	1
ES-6	5	0.5	–	1
ES-7	10	0.5	0.5	2
ES-8	9	0.5	0.5	2
ES-9	8	0.5	0.5	2
ES-10	7	0.5	0.5	2
ES-11	6	0.5	0.5	2
ES-12	5	0.5	0.5	2
ES-13	10	1	–	3
ES-14	9	1	–	3
ES-15	8	1	–	3
ES-16	7	1	–	3
ES-17	6	1	–	3
ES-18	5	1	–	3

[a]Enzyme solutions are prepared in CPW medium

2. *Enzymatic digestion*: Enzyme digestion method is used to release the protoplasts by digesting the tissue with a mixture of macerozyme and cellulase (Table 2). Prepare enzyme mixture in Cell Protoplast Washing (CPW) medium (Table 3). The enzymes are filter-sterilized using Milli-Q filtration unit.

Table 3
Composition of media used for protoplast isolation and culture

Components	Cell protoplast washing (CPW) medium (mg/l)	Floating media (mg/l)	Protoplast culture media (mg/l)	
			I	II
NH_4NO_3	–	–	1650	1650
KNO3	101	101	1900	1900
$CaCl_2{\cdot}2H_2O$	1480	1480	440	440
$MgSO_4{\cdot}7H_2O$	246	246	370	370
KH_2PO_4	27.2	27.2	170	170
KI	0.16	0.16	0.83	0.83
H_3BO_3	–	–	6.2	6.2
$MnSO_4{\cdot}4H_2O$	–	–	22.3	22.3
$ZnSO_4{\cdot}7H_2O$	–	–	8.7	8.7
$Na_2MoO_4{\cdot}2H_2O$	–	–	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2{\cdot}6H_2O$	–	–	0.025	0.025
$FeSO_4{\cdot}7H_2O$	–	–	27.8	27.8
$Na_2EDTA{\cdot}2H_2O$	–	–	37.3	37.3
Myo-inositol	–	–	100	100
Nicotinic acid	–	–	0.5	0.5
Thiamine HCl	–	–	0.5	0.5
Pyridoxine HCl	–	–	0.5	0.5
Glycine	–	–	2	2
Sucrose	–	21 %	2 %	3 %
Mannitol	7–10 %	–	7 %	4 %
Gibberellic acid	–	–	0.5	0.5
BA	–	–	0.5	1
NAA	–	–	0.5	1
2,4-D	–	–	0.5	–

3. *Osmoticum*: Osmotic pressure of the medium is adjusted by addition of mannitol at different concentrations (8 %, 9 %, and 10 %) (*see* **Note 5**).
4. *Incubation conditions for protoplast isolation*: One gram mechanically macerated leaves from in vitro cultured plants is immersed in 10 ml isolation medium for pre-plasmolysis and incubated in the dark for 16 h.

Table 4
Composition of PEG solution[a] for protoplast fusion

Constituents	Molar conc.	g/l
NaCl	140 mM	8.18
KCl	5 mM	0.37
Na_2HPO_4	0.75 mM	0.11
Glucose	5 mM	0.90
$CaCl_2 \cdot 2H_2O$	125 mM	18.40
PEG (MW 4000)		400.00

[a]pH is adjusted to 5.8

5. *Protoplast purification*: A combination of filtration, centrifugation, washing, and floatation centrifugation is used to purify the protoplasts (*see* **Notes 6** and 7). Observation under inverted microscope (Leica) confirms enzymatic digestion and release of protoplasts.
6. *PEG-mediated hybridization/fusion*: Two droplets of protoplast suspension from the two species to be fused are added to another droplet of PEG solution (Table 4), and allowed to mix at room temperature for 30 min. They are maintained as droplet cultures on glass slides and observed periodically for fusion of protoplasts.
7. *Protoplast culture*: Culture protoplasts initially in the liquid medium in petri dishes and incubate in the dark at 25 °C. Fresh culture medium with 3 % sucrose and 7 % mannitol is added after every 7 days to replenish the nutrients. The samples are periodically observed for cell wall regeneration and cell division. After 40–60 days of culture they are plated on 0.25 % agarose medium and monitored for microcallus formation and further development.

3.6 Molecular Marker Profiling to Assess the Genetic Diversity

In this protocol, fresh and healthy leaves from in vitro conserved plants maintained in the in vitro gene bank are used for DNA isolation.

3.6.1 Isolation of DNA

3.6.2 DNA Extraction Procedure

CTAB method [11] is used for the isolation of genomic DNA. Stock solutions and buffers are prepared as in Tables 5 and 6.

1. Grind 2 g leaf tissue in the liquid nitrogen to a fine powder using prechilled mortar and pestle.
2. Transfer the powder to 50 ml oak ridge containing 10 ml preheated CTAB + β mercaptoethanol.

Table 5
Composition of various stock solutions for DNA isolation

Solutions	Method of preparation
1 M Tris–HCL (pH 8.0) 500 ml	60.55 g Tris base + 300 ml distilled water Adjust pH to 8, make up the volume to 500 ml. Dispense into reagent bottles and sterilize by autoclaving
0.5 M EDTA pH 8.0	93.05 g EDTA-disodium salt + 300 ml water Adjust pH to 8, make up volume to 500 ml. Dispense into reagent bottles and autoclave
5 M NaCl 500 ml	146.1 g NaCl + 200 ml water. Adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave
3 M sodium acetate (pH 5.2) 250 ml	61.523 g anhydrous sodium acetate + 200 ml water. Adjust the pH to 5.2 with glacial acetic acid (99–100 %). Autoclave
Ethidium bromide 10 mg/ml, 100 ml	1 g ethidium bromide + 100 ml distilled water. Dissolve on magnetic stirrer. Dispense into amber colored reagent bottle and store at 4 °C
70 % ethanol, 500 ml	360 ml ethanol + 140 ml distilled water. 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	Weigh 20.33 g $MgCl_2$, dissolve in double distilled water, make up to 100 ml, autoclave

Table 6
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 of Tris base; add 20 ml EDTA (0.5 M); 11.42 ml glacial acetic acid and around 150 ml distilled water. Dissolve the salt and adjust volume to 1 L. Autoclave
4	Gel loading buffer (6×): for 100 ml 0.25 % bromophenol blue 30 % glycerol	Dissolve 0.25 g 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

3. Incubate the sample at 65 °C for 30–60 min with occasional mixing by gentle shaking.
4. Add equal volume of chloroform–isoamyl alcohol (24:1). Spin at 38,638 × *g*, for 15 min, at 4 °C Transfer the aqueous phase to fresh tubes with cut tips.
5. Add 2/3 volume of ice-cold isopropanol and mix by gentle shakings.
6. Incubate at 4 °C for half an hour.
7. Centrifuge at 38,638 × *g*, for 15 min, at 4 °C.
8. Discard supernatant, add 70 % ethanol, and wash the precipitate by gentle swirling for 3–4 min.
9. Spin at 26,832 × *g*, for 10 min at 4 °C. Pour off supernatant, invert the tubes for 15 min to drain off excess alcohol, and vacuum-dry the pellet.
10. To the dried pellet, add 500 µl TE to redissolve the DNA and transfer the solution to 1 ml sterile microfuge tubes.

3.6.3 Purification Procedure

1. Add 2 µl DNase-free RNase (10 mg /ml) and incubate at 37 °C for 1 h.
2. Extract with equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), by spinning at 26,832 × *g* and transferring aqueous phase to fresh tubes.
3. Extract twice with equal volume of chloroform–isoamyl alcohol (24:1), by spinning at 26,832 × *g* and transferring aqueous phase to fresh tubes.
4. Add 1/10th volume of 3 M sodium acetate (pH 5.2) and 0.8 volumes of ice-cold isopropanol and mix by gentle shaking to precipitate the DNA.
5. Centrifuge at 26,832 × *g* for 5 min.
6. Decant supernatant carefully. Wash the pellet with 70 % cold ethanol.
7. Air-dry the pellet and dissolve in 250 µl TE (pH 8).

3.6.4 Quality Analysis of DNA

1. Prepare 0.8 % agarose gel in 1× TAE buffer, add ethidium bromide (10 mg/ml), and cast the gel.
2. Load 3 µl DNA sample mixed with 2 µl gel loading buffer (6×).
3. The gel is run at 60 V, for 2 h.
4. Visualize the image under UV Transilluminator in Bio-Rad Gel Doc 1000 system and take a printout.

3.6.5 PCR Amplification

1. Amplify 20–50 ng of genomic DNA in a reaction mix containing 1.0 U *Taq* DNA polymerase, 1 µM primer, 1.5–2.0 mM $MgCl_2$, and 0.125 mM each of dNTPs, and 1× *Taq* DNA polymerase buffer.

2. The amplification profile consists of an initial denaturation of 3 min at 94 °C followed by 35–40 cycles of denaturation for 1 min at 94 °C, annealing for 37 °C for 1 min, and extension at 72 °C for 2 min and final extension for 6 min at 72 °C.

3.6.6 Gel Electrophoresis

1. The amplified RAPD products are separated by horizontal electrophoresis in 1.5 % (w/v) agarose gel, with 1× TAE buffer, stained with ethidium bromide (0.5 μg/ml), and analyzed under ultraviolet (UV) light.
2. The length of the DNA fragments is estimated by comparison with DNA ladder.
3. Variability is then scored as the presence or absence of a specific amplification product. Each gel is analyzed by scoring the present (1) or absent (0) polymorphic bands in individual lanes.
4. The binary matrix is transformed into similarity matrix using Dice similarity (NTSYS-PC 2.01; Numerical Taxonomy System of Multivariate Programs).

3.7 Genetic Transformation

1. Cut off the healthy young leaves from a sterile plant in the laminar flow hood and place them in a petri dish with sterile water. Place 3 Whatman 1 filter papers cut in circles (sterile) in a petri dish. Wet them with sterile water and place two leaves over the filter paper. Cut out disks (about 5 mm diameter) using a cork borer. Using a bacterial loop, transfer each disk from water to a piece of dry Whatman 1 paper. Blot the disk dry and then place it inverted on MS medium. Incubate for two days in a tissue culture room.
   (a) 2 ml culture of the *Agrobacterium tumefaciens* strain (pBZ 100) (Kan 50/μml, 28 °C) (*see* **Notes 8** and **9**).
2. Day 2: Inoculate 20 ml YEP culture with 0.1, 0.2, 0.4, and 0.6 ml of the starting culture containing *A. tumefaciens*, in separate flasks.
3. Monitor the cultures by measuring the O.D. at 600 nm. Use a culture that has an optical density value of 1 at 600 nm. Transfer 5 ml culture to a sterile petri dish. Using a bacterial loop, transfer the leaf disks to *Agrobacterium* suspension. Leave for two minutes and then blot dry on a Whatman 1 paper. Place the leaf disks on MS medium (Table 1) and incubate for 2 more days.
4. Transfer the leaf disks on to the selection medium containing kanamycin in which only plant cells transformed with neomycin phosphotransferase would grow. This medium also contains carbenicillin, which would kill *Agrobacterium* (Fig. 7). Among the antibiotics concentrations tried, tolerance and survival is better in 25–100 mg/ml [12].
5. Callus appears around the edges of the leaf disks. The leaf disk can be selected on the antibiotic-amended MS medium with

Fig. 7 Regeneration from ***Agrobacterium*** treated tissues of black pepper in selection medium

2,4-D 1 mg/l, NAA 1 mg/l, sucrose 30 g/l, 8 g/l agar, kanamycin (<100 mg/l), and carbenicillin (<100 mg/l). Regeneration of the transformed cells can be carried out subsequently in the shoot multiplication (WPM supplemented with 3 mg/l BA and 1 mg/l Kin) and rooting in basal WPM media (*see* **Note 10**). After the development of roots, the plants are transferred to moist vermiculite in pots and transferred to growth chambers and subsequently to the greenhouse.

3.8 In Vitro Conservation [13]

3.8.1 In Vitro Storage by Minimal Growth

1. Shoot tips (2–3 cm long) are collected from 3-year-old plants, thoroughly washed in running tap water and then wiped with 70 % ethanol.
2. The explants are surface-sterilized in 0.1 % mercuric chloride for 3 min, followed by rinsing in sterile distilled water three times.
3. Shoot tips are cultured on the WPM supplemented with 3 mg/l BA + 1 mg/l Kin for the induction of multiple shoots [6, 8, 10]. This is used as the base material for further studies.
4. The cultures could be stored up to 360 days with 80 % survival in half WPM (Woody Plant Medium) medium supplemented with 15 g/l each of sucrose and mannitol in sealed culture vessels and the growth rate was also reduced.
5. For encapsulation of axenic shoot tips (1–2 mm) different concentrations of sodium alginate are tried and 4 % is the optimum concentration. Encapsulated shoot tips are cryopreserved using vitrification technique.
6. In encapsulation-vitrification method, the encapsulated shoot tips are pre-cultured on MS medium supplemented with 0.3 M, 0.5 M, and 0.7 M sucrose (pH 5.8) for three days followed by dehydration with PVS2 (containing 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, and 15 % (w/v) DMSO mixed in MS medium containing 0.4 M sucrose) solution (100 %) at pH 5.8, and 0 °C for 3 h. After dehydration, the

beads (ten beads containing shoot tips), are immersed in 0.8 ml PVS2 solution in a 1.5 ml cryo-tube, and are frozen rapidly by direct immersion into liquid nitrogen (−196 °C) and kept for 1 hour.

7. After 1 hour the samples are thawed at 40 °C for 90 s in a water bath. PVS2 solution is decanted and the beads are washed thrice with 1.2 M sucrose (2 min/washing).
8. For recovery, the encapsulated shoot tips are re-cultured on medium containing 0.5 mgl^{-1} BAP and kept in the dark for 2 weeks under a 16 h light–8 h dark photoperiod.
9. Subsequent subculture is done every 20 days. Six-month-old plants are hardened.

3.8.2 Cryopreservation of Zygotic Embryos

1. Embryos are extracted from the surface-sterilized seeds and placed on Murashige and Skoog medium with 2 M glycerol + 0.4 M sucrose in the dark at 22 ± 2 °C for 3 days before being used for freezing experiments.
2. Zygotic embryos are cryopreserved using the vitrification technique. Zygotic embryos are incubated for 3 h at 0 °C in a loading solution containing ice cold PVS2 vitrification solution.
3. Zygotic embryos are frozen rapidly by direct immersion in liquid nitrogen after direct treatment with full-strength vitrification solution. About ten embryos can be immersed in 1 ml PVS2 solution/1.8 ml cryotube.
4. After storage at −196 °C the embryos could be retrieved by thawing. This is done by plunging the cryotubes for 90 s in a water bath at 40 °C.
5. The PVS2 solution is eliminated by washing the embryos twice with medium containing 1.2 M sucrose.
6. Embryos are then transferred on MS basal media with 0.1 M sucrose. Survival is measured by assessing the percentage of embryos having germinated [3]. Around 80 % recovery of cryopreserved embryos can be obtained (*see* **Note 11**).

4 Notes

1. Endogenous bacterial contamination is a major problem affecting black pepper cultures. Initial inoculation of explant containing antibiotics will help in reducing the bacterial contamination. But these cultures could not be retained in antibiotic containing medium as the antibiotics will affect further growth and multiplication, and hence they must be shifted to fresh culture medium without antibiotics within one week.
2. Initial reduction of sucrose to 15 g/l will help to reduce the growth of bacterial contamination. These initial aseptic

cultures will be the source explants (leaves, shoot tips, nodal segments, and roots) for callus induction, plant regeneration, establishment of embryogenic suspension cultures, and genetic transformation experiments.

3. The micro shoots, embryos derived from aseptic cultures will be the starting materials for all cryopreservation experiments. This will increase the efficiency of cryopreservation and recovery of cryopreserved propagules.
4. Transfer the somatic embryos to plant growth regulator free liquid medium and agitate on orbital shaker at 100 rpm for production of large number of somatic embryos. These operations in the dark enhance adventive embryogenesis and maturation of embryos.
5. During protoplast isolation osmotic strengths of cytoplasm and the isolation medium need to be balanced, to prevent plasmolysis or bursting of the protoplasts.
6. After digestion, the enzyme solution containing protoplasts is filtered through a stainless steel mesh (60 mesh size from Sigma) to remove larger particles of undigested tissues and cell clumps.
7. The filtrate is distributed into sterilized screw capped centrifuge tubes and centrifuged in Beckman tabletop centrifuge for 10 min at 700 rpm. The protoplasts form a pellet at the bottom of the tube. The supernatant enzyme solution is removed using a Pasteur pipette without disturbing the pellet. The pellet is suspended in Cell Protoplast Washing (CPW) medium. Centrifugation and resuspension in fresh medium is repeated three times so as to wash the protoplasts and remove traces of enzyme solution. After washing, the pellet of protoplasts is resuspended in 1 ml CPW medium and layered on top of 9 ml floatation medium (Table 4) and centrifuged at 700 rpm for 10 min. The live protoplasts form a band at the interphase, which is collected with a Pasteur pipette and transferred to culture medium.
8. Black pepper leaf tissues could be transformed using *Agrobacterium tumefaciens*. The construct used contained gene for osmotin, a PR protein known to induce *Phytophthora* resistance, and kanamycin as antibiotic selection marker [10]. Leaf explants from both juvenile as well as mature tissues and zygotic embryos are used as explants. Among the different variables tested, while performing transformation, viz., co-cultivation period, pre-culture of explants, temperature, and pH, co-cultivation of 48 h is efficient. Use leaf explants from both juvenile as well as mature tissues and zygotic embryos as explants for transformation.
9. The treated tissues are transferred in the selection medium (WPM with BAP 1 mg/l + Kinetin 1 mg/l + Cefotaxime 250 mg/l + Kanamycin 50 mg/l). Putative transgenics shoots are regenerated from leaf disks treated with *Agrobacterium* containing osmotin.

10. MS [7] and WPM [14] medium can be used for in vitro cultures in *Piper* species.
11. Black pepper cultures could be stored under minimal growth conditions up to 360 days in half WPM or MS medium supplemented with 15 g/l each of sucrose and mannitol in sealed culture vessels. The cultures could be retrieved with 80 % survival. The conservation of genetic resources in in vitro gene bank utilizing medium and long -term cryopreservation methods has led to development of a conservation strategy (Fig. 8), which can be used as a safe alternative to germplasm conservation.

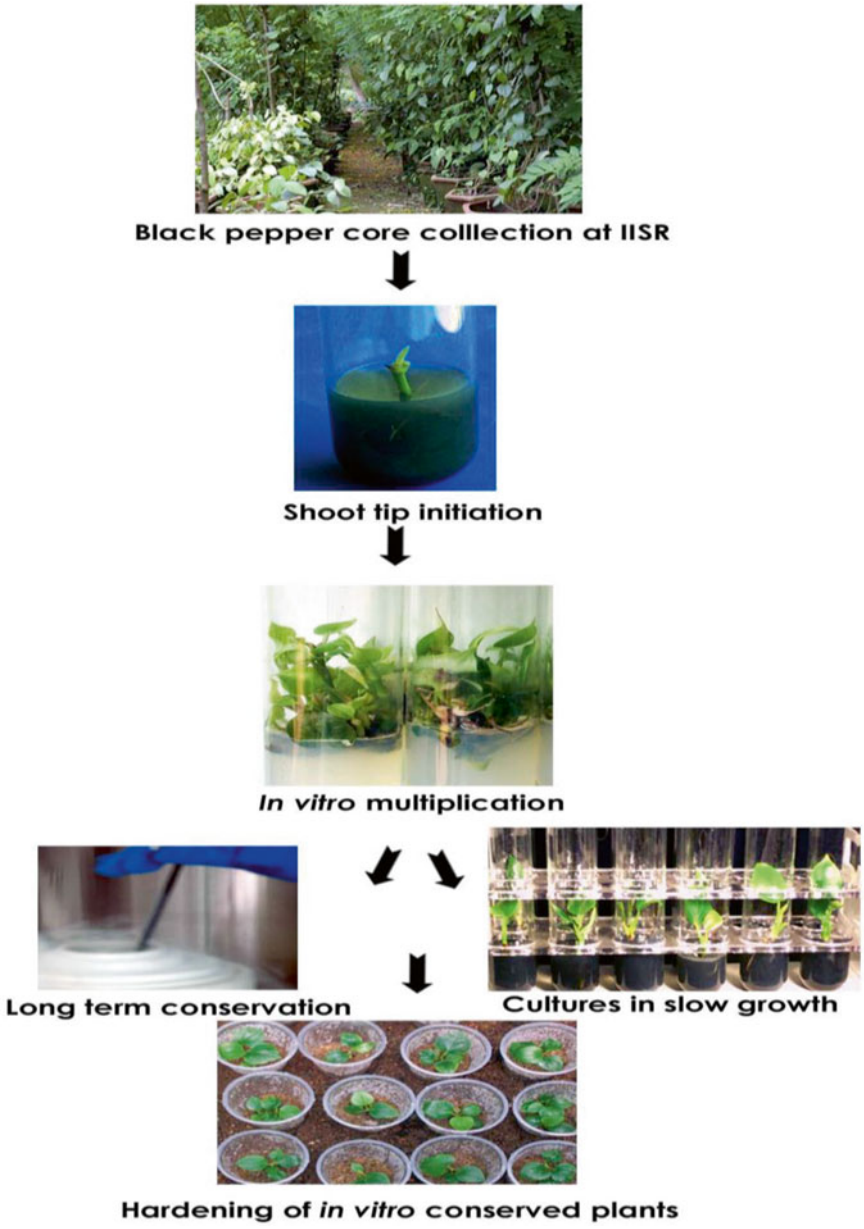

Fig. 8 Schematic representation of the in vitro conservation strategy adopted in black pepper

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